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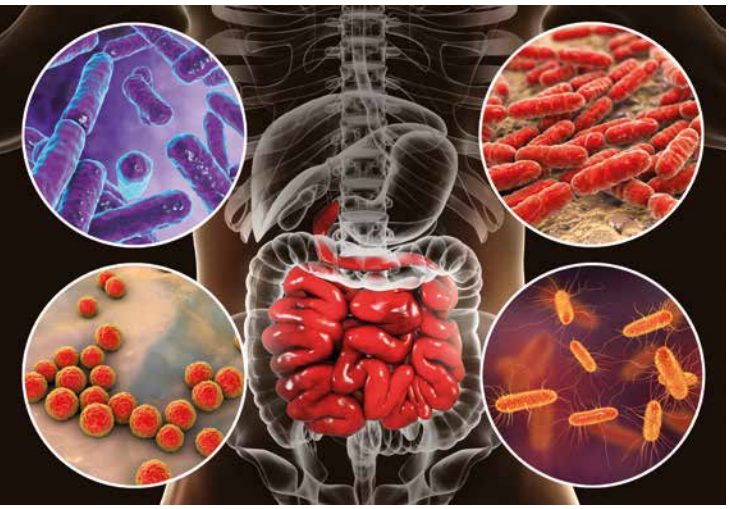

STATE OF RESEARCH ON THE INTERACTIONS BETWEEN FOOD ADDITIVES, THE GUT MICROBIOME AND THE HOST

A FOOD SAFETY PERSPECTIVE

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
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ABBREVIATIONS

ADI	Acceptable Daily Intake
ADME	absorption, distribution, metabolism and excretion
AMR	antimicrobial resistance
ARfD	Acute reference dose
ASB	artificially sweetened beverage
ASF	Altered Schaedler Flora
ASVs	amplicon sequence variants
BCFA	branched-chain fatty acids
bw	body weight
CAD	Codex Alimentarius Commission
CEN	European Committee for Standardization
CMC	Carboxymethyl cellulose
DNA	Deoxyribonucleic acid
DSS	dextran sulfate sodium
EFSA	European Food Safety Authority
EHC	Environmental health criteria
FAO	Food and Agriculture Organization of the United Nations
FDA	Food and Drug Administration (United States)
FMT	faecal microbiota transplant
GF	germ-free
GFSA	Codex General Standard for Food Additives
GI or GIT	gastrointestinal tract
GMC	glycerol monocaprylate
GML	glycerol monolaurate
GPR	G-protein coupled receptor
HFD	high-fat diet
IBD	inflammatory bowel disease
INS	International Numbering System
ISO	International Organization for Standardization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
Lcn2	lipocalin-2
LCS	low-caloric sweeteners
LFD	low-fat diet

LOAEL	lowest observed adverse effect level
LPS	lipopolysaccharide
mADI	Microbiological acceptable daily intake
MIC	Minimal inhibitory concentration
MS	mass spectrometry
MUC2	Mucin 2, oligomeric mucus/gel forming protein
NAS, NCAS	Non-caloric artificial sweeteners
NCD	non-communicable disease
NMR	nuclear magnetic resonance
NNS	non-nutritional sweeteners
NOAEC	no-observed adverse effect concentration
NOAEL	no-observed adverse effect level
OECD	Organization for Economic Cooperation and Development
OGTT	oral glucose tolerance tests
OTU	operational taxonomic unit
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SCFA	short-chain fatty acids
SDO	Standard development organizations
SPF	specific pathogen-free
SSL	sodium stearoyl lactylate
STAN	Standard
T2D	Type-2 diabetes
TiO_2	titanium dioxide
TLR	toll-like receptors
VICH	Veterinary International Conference on Harmonization
WHO	World Health Organization

EXECUTIVE SUMMARY

The gut microbiome refers to the microbial community composed of bacteria, viruses, fungi, and archaea that live in the gastrointestinal tract of animals. These microorganisms interact with the host in physiological activities, including digestion and immune response. The gut microbiome is highly dynamic and responsive to physico-chemical factors, such as pH, oxygen pressure, and diet composition. These factors can influence the stability of the microbial community (i.e. diversity, composition, and function of the microbiome) and influence how it interacts with the host. While there is no universally agreed-upon definition for the terms "healthy microbiome" and "dysbiosis," they are frequently employed to describe the possible influence of the gut microbiome on overall health and disease.

Food additives are added to foods for a variety of technological reasons (e.g. emulsifiers, preservatives), to improve appearance (e.g. colours), or to enhance the organoleptic properties of the product (e.g. sweeteners). Only food additives that have undergone thorough risk assessments are considered safe for consumption and can be used in food production. Such evaluations, typically of a toxicological nature, pay limited consideration to the potential impact of the additive–gut microbiome interactions. However, given the potential of the microbiome to biotransform dietary components, including food additives, and possibly influence health, it is logical to investigate if microbiome data should be incorporated into the risk assessment processes for food additives. Given the challenges of the current state of microbiome science, the consideration of microbiome data in risk assessments needs to be conducted carefully because regulatory science requires robust and reliable scientific evidence.

The main objective of this review was to critically **assess the current state of the research** evaluating the impact of select food additives and gut microbiome interactions and the consequential implications for host health. This involved: (1) gathering and assessing the amount, quality and reliability of scientific information; (2) identifying limitations, gaps, and research needs; and (3) exploring the applicability of microbiome data in food safety risk assessments. By conducting this exercise, it was possible to make recommendations to guide and improve microbiome science for risk assessment. This review was not intended to provide an opinion on whether the evaluated substances are beneficial or harmful to the gut microbiome or human health.

This work features original research manuscripts published between January 2010 and June 2022 selected through a literature search conducted between September 2021 and June 2022.

Research conducted to evaluate the impact of select food additives on the gut/faecal microbiome and potential subsequent effects in the host has been based on a broad diversity of study designs and methodological approaches of variable quality, which has limited the ability to compare results and make definite conclusions. One of the limitations hindering the understanding of study outcomes was the tendency to exclude null results from the interpretation of findings and the unclear meaning of the magnitude and biological relevance of the effects in both the gut microbiome and host. Additive categories most frequently studied were sweeteners (acesulfame K, aspartame, saccharin, sucralose, steviol glycosides, xylitol), emulsifiers/stabilizer/thickeners (several additives belong to multiple classes: carboxymethyl cellulose, polysorbate 80, carrageenans) and colours (mainly titanium dioxide).

The exposure of animal and *in vitro* models to the different additives included in this review often led to microbial changes of unclear biological relevance, although some researchers suggested their potential influence in observed host effects. Such host effects were related mainly to alterations of intestinal homeostasis, metabolic dysfunctions (often focused on glucose intolerance) or inflammatory responses. The evaluations were conducted to bring insights into the potential contribution of food additives to the increased prevalence of chronic disorders such as metabolic syndrome, inflammatory bowel disease or colon cancer. The effects of additives seemed dose-dependent and were often reported to affect predisposed or sensitive individuals or result in aggravation of the condition in disease models.

Some additives appeared to have no or limited effects on the gut microbiome and host (e.g. aspartame, acesulfame K, silver nanoparticles). In contrast, some others led to contradictory results, such as saccharin, sucralose or carrageenans. Despite most effects being observed in surrogate models, the limited number of human interventional trials conducted primarily using non-nutritional sweeteners (saccharin, sucralose, aspartame, steviol glycosides) and one study evaluating carboxymethyl cellulose led to a limited number of effects. These investigations were typically shorter and used lower additive doses than animal studies. Moreover, investigations to evaluate the causal role of the gut microbiome in observed host effects were limited, and the protocols used to conduct faecal microbiota transplants were diverse.

In general, to ensure that scientific data is robust enough to be suitable for risk assessment, several aspects need to improve: (1) gut microbiome research and the peer-review process, which best be addressed by a multidisciplinary approach; (2) scientific rigour and data quality, affecting all steps of research from study design to interpretation and communication of results; (3) standardization and harmonization of practices and methods, accompanied by the development of guidelines, guidance and best practice documents; (4) base research on realistic exposure scenarios including the use of food-grade compounds, doses reflecting consumption estimates and implementation of chronic studies representative of long-term or lifetime exposures; (5) research to evaluate causality and mechanistic explanations; (6) understanding of how gut microbiome information, obtained from surrogate models, translates to human contexts; and (7) development of a framework for the risk assessors to use and evaluate gut microbiome data, especially those obtained with omics technologies.

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CHAPTER 1
INTRODUCTION

FOOD ADDITIVES

A food additive is *any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value. It is intentionally added to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result (directly or indirectly), in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include contaminants, food processing aids or substances added to food for maintaining or improving nutritional qualities* (FAO and WHO, 1995, p. 2).

According to the additive's functional purpose, the Codex Alimentarius Commission (CAC) classifies food additives in one or several of the classes of Table 1 (FAO and WHO, 1989).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA)[1] evaluates the safety of food additives. JECFA serves as an independent scientific expert committee, which performs the risk assessment (aka safety assessment) of food additives (also processing aids, contaminants, natural toxins and residues of veterinary drugs), exposure assessment to chemicals, specifications and analytical methods, as well as guidelines for the safety assessment of chemicals in food. Specifications are documents describing the identity and purity of food additives, ensuring that safety evaluations are conducted on food additives manufactured following such indications. Such specifications are also intended to encourage good manufacturing practices and promote the quality of commercial additives.

JECFA also advises FAO, WHO and the member countries of both organizations, as well as CAC. The advice to CAC on food additives is normally provided to the Codex Committee on Food Additives (CCFA), which performs the risk management role and is responsible for the development and revision of the "Codex General Standard for Food Additives" (GSFA, Codex STAN 192-1995).

[1] JECFA is an international scientific expert committee that is administered jointly by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO). https://www.who.int/groups/joint-fao-who-expert-committee-on-food-additives-(jecfa)/about

TABLE 1 **FOOD ADDITIVE CLASSES AS DEFINED BY THE CODEX ALIMENTARIUS COMMISSION**

CLASS	DEFINITION	FUNCTION
Acidity regulator	A food additive which controls the acidity or alkalinity of a food	acid, acidifier, acidity regulator, alkali, base, buffer, buffering agent, pH adjusting agent
Anticaking agent	Reduces the tendency of particles of food to adhere to one another	anticaking agent, anti-stick agent, drying agent, dusting agent
Antifoaming agent	A food additive which prevents or reduces foaming	antifoaming agent, defoaming agent
Antioxidant	A food additive which prolongs the shelf-life of foods by protecting against deterioration caused by oxidation	antibrowning agent, antioxidant, antioxidant synergist
Bleaching agent	A food additive (non-flour use) used to decolourize food. Bleaching agents do not include pigments.	bleaching agent
Bulking agent	A food additive which contributes to the bulk of a food without contributing significantly to its available energy value	bulking agent, filler
Carbonating agent	A food additive used to provide carbonation in a food	carbonating agent
Carrier	A food additive used to dissolve, dilute, disperse or otherwise physically modify a food additive or nutrient without altering its function (and without exerting any technological effect itself) in order to facilitate its handling, application or use of the food additive or nutrient	carrier, carrier solvent, diluent for other food additives, encapsulating agent, nutrient carrier
Colour	A food additive which adds or restores colour in a food	colour, decorative pigment, surface colorant
Colour retention agent	A food additive which stabilizes, retains or intensifies the colour of a food	colour adjunct, colour fixative, colour retention agent, colour stabilizer
Emulsifier	A food additive which forms or maintains a uniform emulsion of two or more phases in a food.	clouding agent, crystallization inhibitor, density adjustment agent (flavouring oils in beverages), dispersing agent, emulsifier, plasticizer, surface active agent, suspension agent
Emulsifying salt	A food additive which, in the manufacture of processed food, rearranges proteins in order to prevent fat separation	emulsifying salt, emulsifying salt synergist, melding salt
Firming agent	A food additive which makes or keeps tissues of fruit or vegetables firm and crisp, or interacts with gelling agents to produce or strengthen a gel	firming agent
Flavour enhancer	A food additive which enhances the existing taste and/or odour of a food	flavour enhancer, flavour synergist
Flour treatment agent	A food additive which is added to flour or dough to improve its baking quality or colour	dough conditioner, dough strengthening agent, flour bleaching agent, flour improver, flour treatment agent
Foaming agent	A food additive which makes it possible to form or maintain a uniform dispersion of a gaseous phase in a liquid or solid food	aerating agent, foaming agent, whipping agent

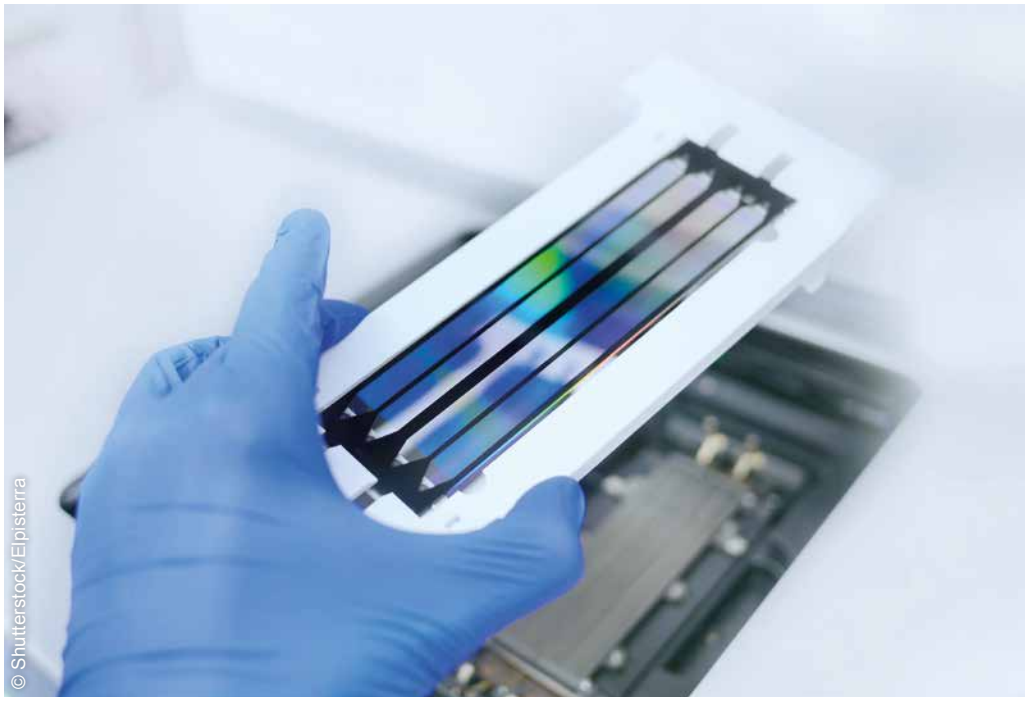
Table 1 (Cont.)

CLASS	DEFINITION	FUNCTION
Gelling agent	A food additive which gives a food texture through formation of a gel	gelling agent
Glazing agent	A food additive, which when applied to the external surface of a food, imparts a shiny appearance or provides a protective coating	coating agent, film forming agent, glazing agent, polishing agent, sealing agent, surface-finishing agent
Humectant	A food additive which prevents food from drying out by counteracting the effect of a dry atmosphere	humectant, moisture/water retention agent, wetting agent
Packaging gas	A food additive gas which is introduced into a container before, during or after filling with food with the intention to protect the food, for example, from oxidation or spoilage	packaging gas
Preservative	A food additive which prolongs the shelf-life of a food by protecting against deterioration caused by microorganisms	antimicrobial preservative, antimicrobial synergist, antimould and antirope agent, antimycotic agent, bacteriophage control agent, fungistatic agent, preservative
Propellant	A food additive gas, which expels a food from a container	propellant
Raising agent	A food additive, or a combination of food additives, which liberate(s) gas and thereby increase(s) the volume of a dough or batter	raising agent
Sequestrant	A food additive which controls the availability of a cation	sequestrant
Stabilizer	A food additive which makes it possible to maintain a uniform dispersion of two or more components	binder, colloidal stabilizer, emulsion stabilizer, foam stabilizer, stabilizer, stabilizer synergist
Sweetener	A food additive (other than a mono- or disaccharide sugar) which imparts a sweet taste to a food	bulk sweetener, intense sweetener, sweetener
Thickener	A food additive which increases the viscosity of a food	binder, bodying agent, texturizing agent, thickener, thickener synergist

Source: **FAO and WHO.** 1989. *Class name and the international numbering system for food additives CAC/GL 36-1989, revised: 2008, amended: 2015.* Codex Alimentarius Commission. Rome. https://www.fao.org/input/download/standards/13341/CXG_036e_2015.pdf

The risk analysis principles applied by CCFA are laid down in the CAC Procedural Manual (FAO and WHO, 2023a). The Standard states the conditions under which permitted food additives may be used in all foods. The Standard includes maximum use levels for food additives in several food groups to ensure that the intake of an additive from all uses does not exceed its Acceptable Daily Intake (ADI).[2]

[2] Acceptable daily intake (ADI). The estimate of the amount of a chemical in food or drinking-water, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk to the consumer. It is derived on the basis of all the known facts at the time of the evaluation. The ADI is expressed in milligrams of the chemical per kilogram of body weight (a standard adult person weighs 60 kg). It is applied to food additives, residues of pesticides and residues of veterinary drugs in food.



Only food additives with assigned ADI or those considered safe by JECFA are included in the Standard.

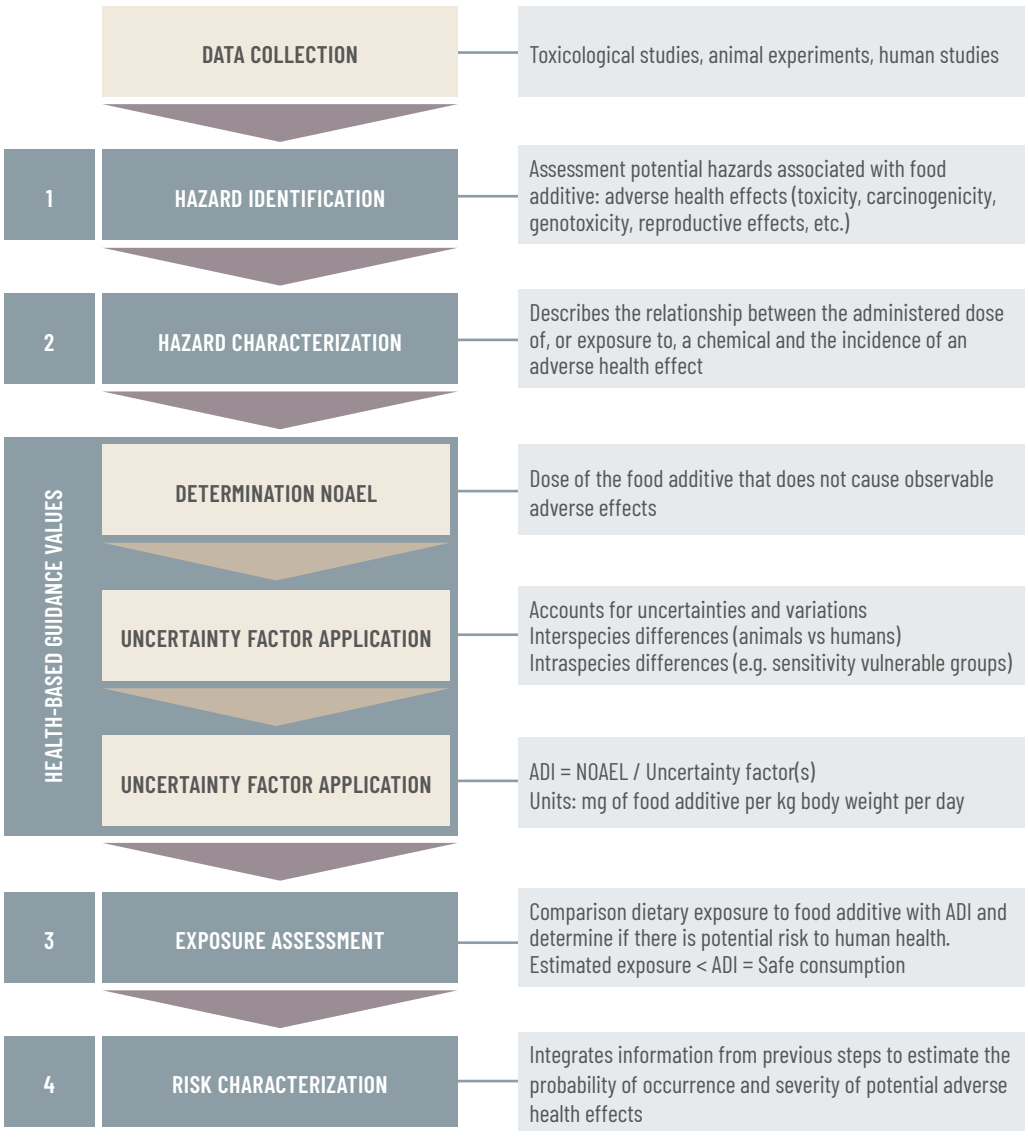
The ADI is *an estimate by JECFA of the amount of a food additive, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk* (WHO, 1987, p111). The process to determine the ADI is shown in Figure 1 and Figure 2. Some food additives may not require a specific ADI as they are deemed to have very low toxicity based on biological and toxicological data, and their dietary intake of the substance at the levels used in food does not pose a hazard.

It's important to note that JECFA periodically reviews and updates the ADI for food additives as new scientific evidence emerges or when there are changes in additive manufacturing. The ADI provides guidance to regulatory authorities worldwide in setting maximum permitted levels of food additives in different food products.

Risk assessment is the first component in a risk analysis process and involves four steps: hazard identification, hazard characterization, exposure assessment, and risk characterization (Figure 1). It is *the process intended to calculate or estimate the risk to a given target organism, system or (sub)population, including the identification of attendant uncertainties, following exposure to a particular agent (e.g. food additives), taking into account the inherent characteristics of the agent of concern as well as the characteristics of the specific target system* (FAO and WHO, 2009c, p. A-31). In this process, risk assessment aims to determine health-based guidance values (e.g. ADI – Figure 2), which are further used for regulatory purposes (e.g. setting maximum permitted levels of additives in foods). Definitions of risk assessment-related terminology are provided in Annex I.

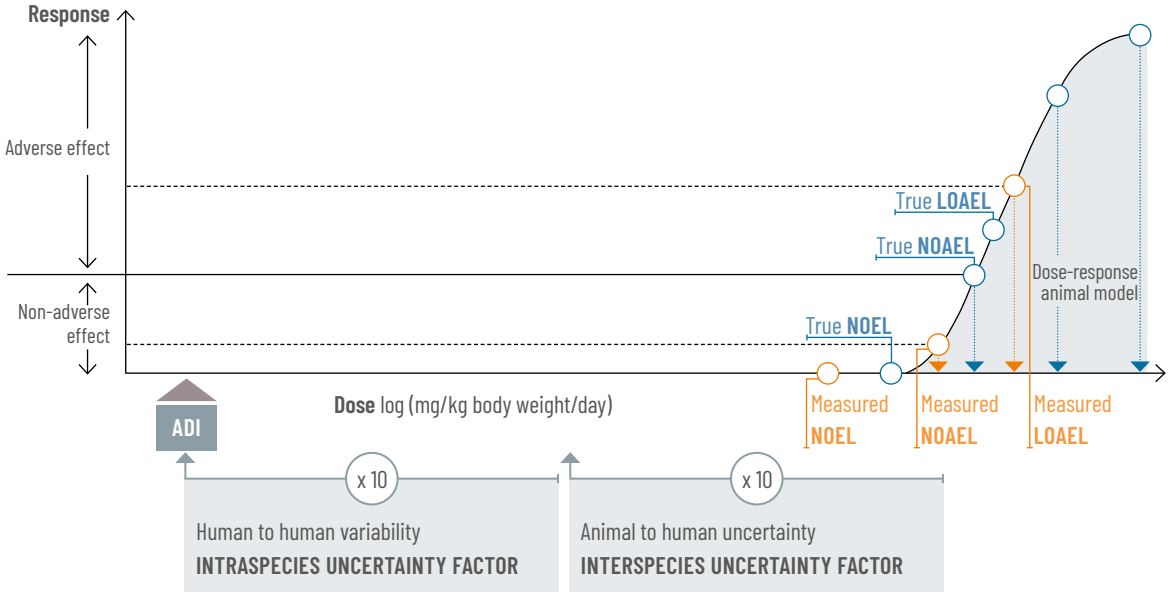
JECFA also has an expert committee responsible for the risk assessment of veterinary drug residues. The evaluation of these compounds, especially antimicrobials, considers two specific gut microbiome-related endpoints for the determination of the microbiological ADI (mADI), i.e. effects related to the functional barrier and the possible selection of antimicrobial-resistant bacteria, which is supported by a stepwise decision-tree (FAO and WHO, 2009c; VICH, 2019). This approach has been recommended by the FAO/WHO Joint Meeting on Pesticide Residues (JMPR) (FAO and WHO, n.d.; FAO and WHO, 2009c, 2017).

FIGURE 1 **SCHEMATIC REPRESENTATION OF THE SAFETY RISK ASSESSMENT CONDUCTED BY THE JOINT FAO/WHO EXPERT COMMITTEE ON FOOD ADDITIVES**



Source: Authors' own elaboration.

FIGURE 2 **ACCEPTABLE DAILY INTAKE DETERMINATION**



Source: Authors' own elaboration.

While food additives are rigorously tested based on existing scientific information and are deemed safe for consumption at the levels typically used in foods, a controversy surrounds these groups of compounds. One controversy is fed by contradictions and uncertainties in scientific studies or emerging research suggesting potential health risks. One of the primary areas of debate is related to the impact of Westernized diets, ultraprocessed foods in particular, which can contain multiple food additives, on human health (Calvo and Uribarri, 2023; Whelan *et al.*, 2024). These foods are linked to about 32 health issues, including obesity, diabetes, heart disease, and certain types of cancer (Lane *et al.*, 2024). Ultra-processed foods are of low nutritional value, often containing high levels of sugar, salt, unhealthy fats, and fewer vitamins, minerals, and fibre than whole or minimally processed foods, contributing to these health risks. Although these studies contribute to the overall body of evidence linking imbalanced diets to disease, interpreting the research results is challenging and needs to be conducted with caution. Studies often show correlations between ultra-processed food intake and health risks but can't definitively prove cause and effect. In addition, most studies in humans are observational, typically relying on self-reporting dietary habits, which can be inaccurate. A limitation of observational studies is that they are often affected by confounding factors[3]

[3] A confounding factor in a study is *a variable which is related to one or more of the variables defined in a study. A confounding factor may mask an actual association or falsely demonstrate an apparent association between the study variables where no real association between them exists. If confounding factors are not measured and considered, bias may result in the conclusion of the study* (EC, n.d.).

(Tulchinsky and Varavikova, 2014), making it difficult to isolate the effects of ultra-processed food consumption or specific ingredients from other factors. Non-communicable diseases, such as obesity, are typically multifactorial and are influenced by overall diet, exercise, and genetics, among other factors.

Due to the evidence showing the close interaction between the gut microbiome and its involvement in physiological processes, including metabolic activity, there are growing concerns about its participation in the etiopathogenesis of chronic disorders (Perler, Friedman and Wu, 2023), following dietary exposure to certain food types (e.g. ultra-processed) or food components (e.g. food additives) (Whelan *et al., 2024). A recent critical review on ultra-processed foods as a risk factor for obesity indicated the lack of data relative to food additives or the gut microbiome to judge the benefits of avoiding ultra-processed foods (Valicente et al., 2023).*

WHAT IS THE MICROBIOME?

The gut microbiome is a dynamic microbial network composed of bacteria, fungi, viruses, protozoa and archaea living in a symbiotic relationship with the host (Durack and Lynch, 2018). More than 99 percent of the genes within the microbiome belong to bacteria (Qin *et al.*, 2010). Microbiota is another term used to refer to these microbial populations. The terms "microbiome" and "microbiota" are commonly used interchangeably due to the lack of consensus definitions. While microbiota refers to the group of individual microbes within the microbial community and its taxonomical structure, the microbiome is a more complex entity. In addition to the notion of microbiota, it also encompasses the function and dynamics within this population. A widely accepted definition describes the microbiome as the collective microbial genomes that, in the case of humans, reside at specific body sites, such as the skin and gastrointestinal tract (Turnbaugh *et al.*, 2007). A more recent proposal defines a microbiome as "a characteristic microbial community occupying a reasonable, well-defined habitat with distinct physio-chemical properties" (Berg *et al.*, 2020, p. 17).

SPATIAL DISTRIBUTION OF THE GUT MICROBIOME

The gut microbiome exhibits a heterogeneous distribution throughout the gastrointestinal (GI) tract, reflecting the influence of diverse host factors such as pH gradients, oxygen levels, antimicrobial peptides, mucus production, and immune responses, creating distinct microenvironments that shape the microbial communities (Donaldson, Lee and Mazmanian, 2016; Kennedy and Chang, 2020). Dietary compounds also influence the composition along and across the GI tract.

The microbial communities of the small intestine are subject to harsh environmental conditions (low pH, enzymes, bile acids, antimicrobial peptides), which determine their lower abundance and diversity than the microbiota of the large intestine (Kastl *et al.*, 2020; Martinez-Guryn, Leone and Chang, 2019; Rowan-Nash *et al.*, 2019).

However, the small intestine microbiota is more dynamic due to the need to adapt to the rapidly changing environment. Digestion and absorption of dietary compounds (e.g. fatty acids, simple carbohydrates) occur primarily in the small intestine, where there is a relevant interaction between microbiota, exogenous chemicals and the host (Kastl *et al.*, 2020). The gradient of oxygen decreases from the proximal to the distal intestine, which leads to a higher presence of facultative anaerobes in the early segments of the intestine. As the oxygen pressure decreases towards the colon, the abundance of strict anaerobes increases (Kennedy and Chang, 2020). The microbiota of the large intestine has a high capacity to ferment complex polysaccharides, which are the primary carbon source reaching the colon as they are not digested by the host (Donaldson, Lee and Mazmanian, 2016). Although most studies target the faecal microbiota (low cost and ease of sampling) and, to a lesser extent, the colonic microbiota, the microbial community of the small intestine should not be underestimated as it is the first to encounter and interact with external dietary chemicals and the host physiology (Martinez-Guryn *et al.*, 2018; Scheithauer *et al.*, 2016).

In addition to distinct longitudinal gastrointestinal ecosystems, there are also cross-sectional differences in the microbiota composition and function (Donaldson, Lee and Mazmanian, 2016; Yang *et al.*, 2020). On one side, the luminal microbiota is relevant for the digestion and absorption of carbohydrates. On the other side, the mucosa-associated microbiota, less abundant and more stable (Donaldson, Lee and Mazmanian, 2016), plays an essential protective role, e.g. maintaining the mucus layer integrity and modulating the immune function of intestinal epithelial and immune cells (Yang *et al.*, 2020).

TEMPORAL FLUCTUATIONS OF THE GUT MICROBIOME

The gut microbiome starts taking shape early in life, with some evidence that in utero influences, such as the placenta, amniotic fluid, and the umbilical cord, contribute to microbial colonization of the infant gut (Ihekweazu and Versalovic, 2018), and it continues to evolve after birth upon exposure to the mother and the environment, forming a complex ecosystem in the gastrointestinal tract (Arrieta *et al.*, 2014; Bäckhed *et al.*, 2015; Wampach *et al.*, 2017). Numerous factors affect the composition and dynamics of the gut microbiome, including host genetics, age, gender, diet, medication, lifestyle, stress, geographical location and environmental factors (Clarke *et al.*, 2019; David *et al.*, 2014; Rothschild *et al.*, 2018). While some reports indicate that the microbiota composition stabilizes in adulthood, population-level analyses reveal that the microbiome remains highly dynamic (Priya and Blekhman, 2019), with high interindividual taxonomical diversity and temporal intra-individual variability (Lloyd-Price, Abu-Ali and Huttenhower, 2016; Shanahan, Ghosh and O'Toole, 2021). Studies comparing the function and composition of the gut microbiome have shown that functional stability is reached early in life and is likely to remain so for a long time afterwards (Kostic *et al.*, 2015). They have also suggested that the overall functional potential of the gut microbiome tends to exhibit more similarity between individuals (Turnbaugh *et al.*, 2009a).

MICROBIOME FUNCTION AND INTERACTIONS WITH THE HOST

The symbiotic relationship between the microbiome and its host is primarily functional. This means that the interaction and benefits derived from the microbiome are not solely based on the presence or abundance of specific microbial phylotypes[4] but on their collective functions. Functional redundancy is a common microbiome feature and an essential aspect of the microbiome-host relationship (Louca *et al.*, 2018; McBurney *et al.*, 2019). Functional redundancy refers to multiple microbial phylotypes that can perform similar functions within the microbiome. The more diverse the microbial population is, the more likely the presence of functional redundancy is. Even if specific taxa are absent or their abundance changes, other microbiome members can maintain essential functions. Although redundancy contributes to the overall stability and resilience of the gut microbiome, making it more robust to perturbations, specialized functions that are carried out by a small number of species are also important. However, these functions are less well-characterized due to challenges in mapping certain metagenomic data to reference databases (Walker and Hoyles, 2023). In addition, keystone taxa, including *Bacteroides fragilis*, have been shown to drive the composition and function of the gut microbiome (Banerjee, Schlaeppi and van der Heijden, 2018).

4 "In microbiology, a phylotype is an environmental DNA sequence or group of sequences sharing more than an arbitrarily chosen level of similarity of a particular gene marker. The most widely used phylogenetic marker is the small subunit ribosomal RNA gene. Two prokaryotic sequences are generally considered as belonging to the same phylotype when they are more than 97–98%. In prokaryotic microbiology, phylotypes, often referred to as Operational Taxonomic Units (OTUs), are a proxy for species" (Moreira and López-García, 2011, p. 1254). Given recent developments, some authors have called for an update to the 97 percent identity threshold (Edgar, 2018).

Depending on the degree of disturbance, changes in microbiota composition may not be relevant if the overall function of the microbiome is not compromised. Therefore, the study of the microbiota composition alone may not be sufficient to fully explain its function and microbiome-host interactions (Lozupone *et al.*, 2012). In addition, the microbiome seems functionally more stable (offering a higher discriminatory power) than its taxonomical composition (Louca *et al.*, 2016; Shanahan, Ghosh and O'Toole, 2021). Overall, it becomes increasingly evident that function holds more significance than mere microbial phylotyping, leading some research groups to raise questions about the suitability of approaches to better study and understand microbiome communities, e.g. characterization phenotypic traits (e.g. molecular or metabolic) versus taxonomical analysis alone (Martiny *et al.*, 2015; Xu *et al.*, 2014).

Microbiome activities play a vital role in host physiology and supporting overall health (Abdelsalam *et al.*, 2020):

1. It assists in digesting and metabolizing food components (e.g. fermentation of complex carbohydrates) and other exogenous compounds (Koppel, Maini Rekdal and Balskus, 2017). The microbiome can metabolize compounds produced by the host, such as intestinal bile acids into secondary bile acids.
2. It produces essential metabolites such as vitamins, amino acids and short-chain fatty acids (SCFAs) (Read and Holmes, 2017). SCFAs, particularly butyrate, result from the fermentation of carbohydrates, and they are particularly interesting as they are used as an energy source by intestinal enterocytes. Moreover, SCFAs can modulate metabolic pathways and neuronal and intestinal functions and participate as modulators of the host immune response (Koh *et al.*, 2016; Neish, 2009; Portincasa *et al.*, 2022). Changes in the levels of SCFAs have been associated with multiple disorders like obesity, metabolic dysregulation, hypertension, intestinal bowel disease, neurological disorders or allergies (de la Cuesta-Zuluaga *et al.*, 2018; Dong and Cui, 2022; Morrison and Preston, 2016; Parada Venegas *et al.*, 2019; Portincasa *et al.*, 2022; Sasaki *et al.*, 2024), although there is still lack of causal demonstration and limited knowledge on the potential mechanisms involved. The potential to modulate the production of SCFA has led to the development of research lines to investigate therapeutical interventions by, for example, conducting faecal microbiota transplants or potential treatments, especially with butyrate, although with mixed results (Hodgkinson *et al.*, 2023).
3. The microbiome offers protection by stimulating the immune system and contributing to its maturation. Also, it participates in maintaining the intestinal barrier. The first line of intestinal defence (colonization resistance or colonization barrier) exerted by the gastrointestinal microbiota is characterized by preventing the colonization of exogenous pathogens and the proliferation of opportunistic commensals (Pilmis, Le Monnier and Zahar, 2020). The host also contributes to maintaining the colonization resistance via the intestinal immune system, for example, by modulating the production of antimicrobial peptides and mucus (Kinnebrew *et al.*, 2010; Mowat and Agace, 2014).

The microbiome can influence physiological processes in other host regions, either directly through microbial products absorbed and distributed systemically or indirectly by promoting local physiological responses in the host (e.g. immune system) with systemic reach. This is how the microbiome can participate in several functional axes connecting the gut with other body regions (e.g. gut-brain, gut-liver, gut-lung) (Haller, 2018).

Therefore, studying and characterizing the functional aspects of the microbiome is crucial for gaining deeper insights into its role in human health and disease. While the functional nature of the microbiome is gaining attention, understanding the specific roles of individual species and their contributions to overall function is still an active area of research. As the microbiome research progresses, it will likely provide more comprehensive insights into the intricate relationship between microbial functions and host health.

HEALTHY MICROBIOME AND DYSBIOSIS

Although there is a substantial amount of scientific information associating the microbiome with human health and disease, there are no consensus definitions for what constitutes a healthy and an unhealthy (dysbiosis) microbiome. A major challenge in defining a healthy microbiome is the high interindividual variability within the healthy population (Lloyd-Price, Abu-Ali and Huttenhower, 2016; Wei *et al.*, 2021). The international cancer microbiome consortium discussed the healthy microbiome not as a stand-alone component but in connection with the health status of the host (Scott *et al.*, 2019), both working in a symbiotic manner to promote beneficial immune responses and metabolic mutualism, also referred to as eubiosis, "balanced host-microbiome interaction" (Berg *et al.*, 2020, p.18), (Belkaid and Hand, 2014; Nicholson Jeremy *et al.*, 2012). The expert consortium also indicated that health-associated microbiomes should be characterized as being diverse and resilient to short-term environmental pressures with sufficient plasticity to adapt to the benefit of the host following long-term stresses (Lozupone *et al.*, 2012). In 2017, a multidisciplinary workshop was organized to explore the question: "Can we begin to define a healthy gut microbiome through quantifiable characteristics"? (McBurney *et al.*, 2019). Due to the difficulties in defining a "healthy microbiome", the group suggested that research should be directed to determine factors (environmental, clinical or nutritional) that diminish symbiotic features and highlight the relevance of the holistic function of the microbiome, its diversity and activity redundancy.

Dysbiosis is another concept lacking a consensus definition (Hooks and O'Malley, 2017), and it is inconsistently interpreted in many research works (Brüssow, 2020). It is often referred to as the imbalance of the microbiota composition and disruption of its complex structure (Petersen and Round, 2014). Gut dysbiosis is also reported as microbial imbalance characterized by decreased diversity, changes in the Firmicutes/Bacteroidetes ratio, reduced relative abundance of beneficial bacteria, and alterations in the normal function of the microbiome (Petersen and Round, 2014; Pilmis, Le Monnier and Zahar, 2020).

However, some of these characteristics and concepts are outdated, such as the Firmicutes/Bacteroidetes ratio (Cani, Moens de Hase and Van Hul, 2021) or result in diverging outcomes in studies evaluating gut microbiome associations with disease (Brüssow, 2020; Walker and Hoyles, 2023). A more modern concept of dysbiosis relates to the notion of pathobiome, which refers to the pathogenic agent integrated within its biotic environment (Vayssier-Taussat *et al.*, 2014). Considering the holobiome[5] (Skillings and Hooks, 2019), dysbiosis is to disease what eubiosis is to health.

Gut dysbiosis has been associated with the disruption of the intestinal barrier function, intestinal disorders, immune-mediated and metabolic diseases (e.g. inflammatory bowel disease, obesity), as well as neurological alterations (Margolis, Cryan and Mayer, 2021; Sanders *et al.*, 2021; Zheng, Liwinski and Elinav, 2020). A recent review has collected and categorized indexes developed to determine gut dysbiosis (Wei *et al.*, 2021), primarily used as markers within the clinical context. Most indexes are based on parameters describing the taxonomic composition and diversity of the microbiota and illustrate the higher weight typically given to the taxonomical structure of the microbial community over the functional aspect.

STUDY OF THE MICROBIOME

Numerous approaches are available to study the microbiome's composition, diversity, function, and their relationship with the host and the environment. However, there is no gold standard, and the selection of the most suitable models and analytical strategies depends primarily on the purpose of the study and the questions that need to be answered.

5 Holobiome: A host plus all of its symbiotic microbiota, or the collective unit made up of all of the host and microbial genomes of the holobiont.

MODELS

Models to study the gut microbiome are essential for understanding the complex interactions between the microbial community, dietary compounds (e.g. food additives) and other environmental factors. They can provide valuable insights about if and how the gut microbiome responds to different exposures and how these responses may impact host health.

In vitro models

One common approach to studying the gut microbiome's exposure to chemical compounds is using *in vitro* models. These models involve culturing specific microbial strains or complex microbial communities in controlled laboratory conditions. *In vitro* systems such as fermentation vessels or bioreactors can mimic the gastrointestinal environment and allow researchers to directly expose the microbiome to specific chemicals at controlled concentrations. They enable the study of changes in microbial composition and function in response to exposure (Nissen, Casciano and Gianotti, 2020) and the ability of the gut microbiome or select microbiome members to digest or biotransform dietary chemicals. These systems differ in complexity. The simplest units (e.g. static batch fermentation models) are chambers run under specific conditions and a defined medium, which is not replaced over time. In continuous culture bioreactors, the medium is replaced periodically, and environmental and nutrient parameters are monitored over time, allowing for extended exposure periods. More modern and complex systems are composed of multiple bioreactors connected in series mirroring the conditions of different sections of the gastrointestinal tract, including peristaltic movements (e.g. simulator of human intestinal microbial ecosystem [SHIME®], TIM-2, SIMGI) or even simulating some intestinal structures by integrating a mucosal compartment (e.g. mucosal SHIME or M-SHIME®) (Guzman-Rodriguez *et al.*, 2018; Nissen, Casciano and Gianotti, 2020; Van de Wiele *et al.*, 2015). However, none of the bioreactors can mimic all key anatomical and physiological gastrointestinal conditions (Roupar *et al.*, 2021).

Cell cultures are also used to evaluate the impact of microbial-derived compounds on epithelial cells of the intestinal mucosa. The monolayer lines Caco-2, HT29, and T84, derived from human colon cancer cells, are commonly used for this purpose (Pearce *et al.*, 2018). They can be used in tandem with bioreactors, where the activity of components present in the media is tested in the cell cultures.

Ex vivo models

More recent advances have permitted the development of *ex vivo* models (e.g. intestinal enteroids and organoids, organs-on-a-chip and microfluidic devices). They consist of functional live tissues with more complex cellular environments than cell cultures, resembling more closely the conditions of *in vivo* systems (May, Evans and Parry, 2017; Pearce *et al.*, 2018). Like *in vitro* models, *ex vivo* systems allow more control of experimental conditions than *in vivo* models.

Although promising, these systems are evolving and their applicability is still limited due to several drawbacks, including short-term culturing capacity, cost and difficulties in obtaining human samples (May, Evans and Parry, 2017; Pearce *et al.*, 2018).

In vivo models

These models allow researchers to explore the effects of chemical exposures on the gut microbiome within a living organism and assess both local gut effects and potential systemic consequences.

When using *in vivo* surrogate animal models to study the human gut microbiome, it is critical that they are physiological- and clinically relevant to the human context. Selecting the most suitable model depends on the research question and the study's objectives. Criteria for choosing an appropriate model for microbiome studies include genetic background, baseline microbiota, or phenotypic expression of diseases (Kamareddine *et al.*, 2020). The gastrointestinal anatomy and physiology of pigs closely resemble that of humans. Both being omnivores, they have similar nutritional requirements and share similar dominant phyla with the human gut microbiome (i.e. Firmicutes and Bacteroidetes) but differ significantly at the genus level (Heinritz, Mosenthin and Weiss, 2013; Hoffmann *et al.*, 2015). Non-human primates (NHP) are genetically and physiologically similar to humans and have been helpful in the clinical context (Shively and Clarkson, 2009), but their use for gut microbiome-diet interactions has been somewhat limited (Amato *et al.*, 2015; Nagpal *et al.*, 2018b). Despite significant differences in bacterial communities (Amato *et al.*, 2015), in a comparative study, the microbial diversity in humans was shown to be more similar to NHP than to rats and mice (Nagpal *et al.*, 2018a). It has been reported that the rat baseline microbiota is more similar to that of humans than that of mice (Flemer *et al.*, 2017; Wos-Oxley *et al.*, 2012), although other studies have concluded that mice microbiota is closer to human microbiota than rat microbiota (Nagpal *et al.*, 2018b). Mice have similar dominant phyla to humans but differ in several health-relevant genera that are absent in mice (Nguyen *et al.*, 2015). Mice and rats have been the predominant models used to study the microbiome. Mice are genetically manipulable, e.g. to mimic human disease conditions, and have more genetic variants than rats, making them more versatile models to study mechanisms, including those involving the microbiome (Turner, 2018). Many mouse and rat strains are available, but a limited number of studies have been conducted to compare microbiota from different strains (Hugenholtz and de Vos, 2018). Moreover, since there are no recommendations of models for dietary interventions, the strain selection is often based on experience or commercial availability (Hugenholtz and de Vos, 2018).

Germ-free mice have been valuable in investigating causal relationships between the microbiome and physiological changes in the host, including, for example, the contribution to metabolic alterations or predisposition to opportunistic infections and disease. In microbiome studies, germ-free animals are inoculated with bacterial cultures or colonized with healthy or altered microbiota from a donor.

Germ-free mice can be humanized when the donor is a human. True germ-free mice are bred and raised free of microorganisms under rigorous environmental conditions. These animals have physiological differences from their conventional counterparts and lack the co-evolution between the host and microbiota, requiring careful consideration when interpreting and extrapolating findings (Anklam *et al.*, 2022). For example, they have a slower epithelial renewal rate, altered immune system, altered gene expression of gastrointestinal cells and a decreased mucus layer (Fritz *et al.*, 2013). In addition, germ-free mice are expensive. A less expensive alternative is the use of antibiotic-treated animals (nearly germ-free), which are given high doses of antibiotic cocktails to deplete the gut microbiota (Kennedy, King and Baldridge, 2018; Reikvam *et al.*, 2011).

Contrary to other regulated substances like pesticide residues, where ethical considerations limit direct testing in humans, the impact of food additives on the gut microbiome can be assessed through interventional human trials. This allows researchers to directly control variables and observe cause-and-effect relationships. Additionally, the gut microbiome can be further evaluated in humans on a larger scale through epidemiological studies. These observational studies investigate existing dietary patterns and gut health data within populations to identify potential correlations between food additive exposure, microbiome composition and function, and non-communicable diseases. Examples of these studies will be presented and discussed later in this review.

In silico models

In addition to *in vitro* and *in vivo* studies in animal models, computational models and predictive algorithms have also been developed to analyse and predict the gut microbiome's response to various chemical exposures and possible interactions with the host (Shokri Garjan *et al.*, 2023). These models use advanced bioinformatics and machine learning techniques to analyse large datasets and predict how different compounds may influence the gut microbial community.

Overall, these models work in tandem, providing a multifaceted approach to studying the gut microbiome and its exposure to chemical compounds. Combining experimental data from *in vitro* and animal models with computational predictions can enhance our understanding of the complex dynamics between the gut microbiome and environmental exposures. These insights have significant implications for fields like toxicology, food safety assessment, and the development of personalized approaches to improve gut health and overall well-being.

THE MICROBIOME ANALYSIS

Sampling, sample handling and sample preparation

Sampling the gut microbiome involves careful consideration of the sampling site, sampling frequency, sample handling procedures, and storage methods. These steps are relevant sources of variability in microbiome studies (Choo, Leong and Rogers, 2015; Gorzelak *et al.*, 2015; Watson *et al.*, 2019). Therefore, rigorous control and adherence to best practices and – ideally – standardized protocols are essential to generate reliable (accurate and reproducible) data (Gorzelak *et al.*, 2015).

Sampling site. Microbiome features and microbiome-host interactions are context-specific, meaning that they depend on the environmental and anatomical characteristics of the intestinal section selected for sampling. Therefore, choosing a sampling site is critical in capturing a representative snapshot of the gut microbiome. Faecal samples are most commonly used due to convenience (non-invasive nature, ease of collection) and are typically used as a proxy for the microbial composition in the distal colon. However, researchers may also target earlier segments of the gastrointestinal tract. Within a specific site, there is the option to sample from the mucosal lining or luminal contents.

Sampling method. There are several methods to sampling the gut, from non-invasive faecal sampling to sitespecific approaches targeting mucosal or luminal contents (e.g. biopsy, luminal brush, laser capture microdissection, catheter aspiration), all having advantages and disadvantages (Tang *et al.*, 2020). Novel sampling methods, such as ingestible capsules, have emerged to enhance the non-invasive collection of gut microbiome samples (Rehan *et al.*, 2024). These capsules are equipped with sensors and technologies that facilitate targeted sampling at specific locations within the gastrointestinal tract. This promising approach offers a unique opportunity to capture site-specific information, providing a more detailed understanding of the composition and dynamics of microbial communities along the gut. These capsules not only reduce the need for invasive procedures but also opens avenues for more extensive and convenient longitudinal studies. However, they still require further developments to be used in routine analysis.

Sampling frequency. The frequency at which samples are collected is determined by the study objectives. Longitudinal studies involve repeated sampling over time, therefore offering valuable insights into temporal variations and responses to internal and external factors like diet, e.g. immune response and diet. In crosssectional studies, samples are collected at one specific time point, e.g. at the end of an intervention in a mouse study.

Sample handling and storage. It is essential to avoid contamination and preserve the microbial composition and microbial analytical targets (DNA, RNA, proteins or metabolites). Proper sample storage is critical to maintaining the stability of microbial DNA and other biomolecules (Smirnov *et al.*, 2016). Additionally, well documented labelling and tracking systems are employed to minimize the risk of sample mix-ups and ensure the reliability of downstream analyses.

Sample preparation includes the extraction of target analytes for further downstream analysis (DNA, RNA, proteins or metabolites). Such protocols should maximize the extraction efficiency of the target analyte while minimizing potential contamination and the presence of other sample components that can interfere with the analysis.

Analytical tools

There are numerous analytical tools to study microbiomes, from classical microbiological and targeted analysis to characterize individual species or strains and their function to more holistic approaches using modern technologies, including omics and untargeted analysis.

The study of the microbiome, microbiome-chemical, and microbiome-host interactions has evolved rapidly over the last decade, parallel to the new advancements in omics technologies, bioinformatics, and machine learning. These technical developments (e.g. sequencing) have allowed for cultivation-independent, DNA- (e.g. metagenomics) and RNA- (e.g. metatranscriptomics) based approaches to investigate the microbial community holistically. The omic techniques (e.g. metagenomics, metatranscriptomics, metabolomics, metaproteomics) provide a unique opportunity to analyse and untangle the complex microbial ecosystem. However, although modern methods have contributed significantly to understanding the microbial community and its environment, more traditional analytical tools are also part of the toolbox to study the microbiome. Selecting the most appropriate method(s) will depend on the scientific question and hypothesis (Allaband *et al.*, 2019).

The most common method to analyse the taxonomical composition and diversity of the microbiota is by sequencing specific genes like the 16S ribosomal RNA (rRNA) gene for bacteria and Archaea, the 18S rRNA gene, and internal transcribed spacer (ITS) regions for eukaryotes like fungi. This involves various steps, including DNA extraction, amplification, sequencing, and bioinformatic analysis (Arrieta *et al.*, 2014). The 16S rRNA gene is highly conserved across bacteria. It contains nine hypervariable regions (V1-V9) that determine the taxa level of analysis, ranging from high-level taxa to genus identification (Yang, Wang and Qian, 2016). However, 16S rRNA gene sequencing faces several challenges. The following are some examples. It has limited resolution and may not always identify at the species level due to some gene regions being identical among species (Jovel *et al.*, 2016; Wang *et al.*, 2007). Also, 16S rRNA gene sequencing provides data as relative abundances of bacteria taxa. This can impact the interpretation of results because the proliferation or loss of part of the population of a specific group will change the relative abundance of other microbiota members, which, in terms of absolute abundance, may not have changed at all. In addition, minority or rare members may not be captured by the analysis.

To address these issues, alternative microbiome profiling approaches allow the integration of absolute quantification of microbial abundances in 16S rRNA gene sequencing data, such as quantitative PCR analysis or quantitative polymerase chain reaction (qPCR). However, they are not free from challenges (Galazzo *et al.*, 2020). Furthermore, there is variation in the number of copies of the 16S rRNA gene among different bacterial genomes (Kembel *et al.*, 2012). This discrepancy can result in overestimating the relative abundance of certain microbial members. To help researchers address this issue, the ribosomal RNA operon copy number database (rrndb) provides annotated information on rRNA operon copy numbers from prokaryotes (Klappenbach *et al.*, 2001; Lee, Bussema and Schmidt, 2009; Stoddard *et al.*, 2015). The 16S rRNA gene has also been used to predict the functional capacity of the gut microbiota by using, for example, tools like the PICRUSt[6] and the KEGG pathway database (KEGG, 2024). However, its predictive value has been questioned by multiple studies (Matchado *et al.*, 2024; Sevigny *et al.*, 2019). The numerous analytical options, including the selection of primers, and computational pipelines, can lead to different microbiome profiles, affecting outcome comparison and reproducibility.

Shotgun metagenomics analysis provides a comprehensive genetic study of the microbiome. Unlike targeted amplicon sequencing (e.g. ITS, 16S, and 18S rRNA genes), shotgun metagenomics sequences the entire genome present in a sample. Compared to 16S rRNA gene sequencing, shotgun metagenomics[7] analyses the genome from the entire microbiome (bacteria, viruses, fungi, archaea, and small eukaryotes) and offers higher resolution down to species and strain levels, depending on the sequencing depth (Allaband *et al.*, 2019; Hu *et al.*, 2022). In addition, shotgun metagenomics is more powerful in identifying less abundant taxa or low biomass microbes – which can be biologically relevant – than 16S rRNA gene sequencing (Durazzi *et al.*, 2021). Shotgun metagenomics allows researchers to investigate the taxonomical profile of the entire microbial population and explore the functional potential of the microbiome (functional profiling) (Joseph and Pe'er, 2021). It enables researchers to identify genetic traits, detect antimicrobial resistance genes, assess genetic biochemical pathways, and analyse other microbiome components. However, there is no consensus on the best sequence assembly approach (Galloway-Pena and Hanson, 2020). While powerful, shotgun metagenomic analysis may introduce errors and biases from experimental and computational factors (Bharti and Grimm, 2021), and it can also face reproducibility challenges similar to 16S rRNA sequencing (Allaband *et al.*, 2019).

In general, each workflow step of both 16S rRNA marker gene and metagenomic sequencing (e.g. DNA extraction, PCR primers) can favour the measurement of some taxa over others, therefore affecting accuracy and reproducibility (Human Microbiome Project Consortium, 2012; McLaren, Willis and Callahan, 2019; Sinha *et al.*, 2017).

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6 Phylogenetic Investigation of Communities by Reconstruction of Unobserved States

7 Shotgun (untargeted analysis), meta (sequencing of "all"), genomic (genome).

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In addition, the analysis of the same sample using different methodologies can lead to different results, making comparisons challenging. Therefore, standardizing protocols is crucial for consistent and reliable results.

Genomics provides information about the presence of genes but does not indicate whether they are being expressed. The transcription of genes is evaluated by analysing the messenger RNA (mRNA). It provides mechanistic insights about which metabolic pathways may be up- or down-regulated. Quantitative real-time PCR (qRT-PCR) or microarray techniques are used to analyse target-specific gene transcription. Similar to metagenomics, metatranscriptomics (mRNA sequencing) targets the entire mRNA content (Shakya, Lo and Chain, 2019). Some of the limitations of metatranscriptomics include short mRNA half-life, difficulties in isolating high-purity RNA, avoidance of contamination with unwanted RNA, and the fact that mRNA does not always equate to the presence of protein or protein activity (Bashiardes, Zilberman-Schapira and Elinav, 2016). Several reviews have published additional information on transcriptomics methodologies, challenges and how microbiome and host transcriptomics relate to health (Bashiardes, Zilberman-Schapira and Elinav, 2016; Nichols and Davenport, 2021; Ojala, Kankuri and Kankainen, 2023; Shakya, Lo and Chain, 2019).

Metaproteomics and metabolomics are analytical methods used to measure microbial function. There are different approaches to metabolomics. Targeted strategies focus on analysing specific groups or families of compounds (e.g. short-chain fatty acids – SCFA), while untargeted analysis aims to detect as many metabolites as possible. Metabolomics can be described using different names depending on the compounds being analysed, for example, lipidomics (lipid profiling) or volatolomics (volatile organic compounds profiling). Technologies for detection include mainly mass spectrometry, although nuclear magnetic resonance spectroscopy is also used (Bauermeister *et al.*, 2022; Smirnov *et al.*, 2016).

Altered metabolite profiles after exposure to dietary compounds may indicate changes in the normal function of the microbiome. As microbial metabolites participate in the physiological and metabolic processes of the host, changes in the microbiome's activity may also potentially induce alterations in the host. Microbial metabolites are typically analysed from colonic content or in faecal samples. However, they are also found in plasma and other tissues after being absorbed by the host. Metabolomics is usually combined with metagenomic or transcriptomic studies.

The information obtained from omic approaches helps us understand microbial structures and processes. However, these technologies present new challenges. They generate a large amount of data that needs to be processed and translated into meaningful information. In addition, there are gaps in our existing knowledge, which means that some information cannot be fully understood. For instance, certain metabolic activities have been identified but cannot be linked to specific genes or enzymes (Koppel, Maini Rekdal and Balskus, 2017). On the other hand, a significant portion of the faecal metagenome (about 86 percent) cannot be associated with known metabolic pathways (Human Microbiome Project Consortium, 2012). An additional challenge lies in the annotation[8] of new molecules or molecules modified by the microbiome that do not match known compounds in reference libraries (Allaband *et al.*, 2019). It is challenging to evaluate the influence of the microbiome on the host, or vice versa, because of shared and intertwined functions (enzymes and metabolites) between the two. However, understanding the interplay of host-microbiome is essential to understand the role of the gut microbiome on health and develop strategies to minimize or control the potential influence of the gut microbiome on adverse health effects (Li and Holmes, 2014; Visconti *et al.*, 2019).

Although omics open new opportunities to understand the complexity of microbial networks and their interactions with their ecosystems, conventional and targeted analytical approaches have specific purposes and will continue to be used. For example, they can complement omics findings to characterize newly discovered microbiota members or metabolic pathways.

STANDARDIZATION AND BEST PRACTICES

The study of the gut microbiome and its interactions with chemicals and the host need validated analytical methods. It is also important to standardize analytical practices to ensure the reliability and reproducibility of results. This standardization applies to all omics technologies, including genomics, metagenomics, proteomics, and metabolomics. Moreover, harmonizing standards is essential to facilitate comparison and integration of data from diverse sources, therefore improving data robustness. Some proposals have been made in this respect, which can be consulted in Annex III. Guidelines and Best Practices.

[8] Here, metabolite annotation means "tentative identification of a metabolite." Also related is ion annotation referring to the "assignation of different metabolic features (adducts, charges, and losses) into a single value" (Godzien *et al.*, 2018, p. 417).

MICROBIOME IN RISK ASSESSMENT

The consideration of the gut microbiota in the evaluation of food additives has been considered in risk assessments since the late 1980s (WHO, 1987). Back then, the two aspects considered included the capacity of the intestinal microbes to transform chemicals and the potential of the food additives to act on the microbiota, specifically looking into antimicrobial activity and increased substrate for the microbial community.

The gut microbiome impact on food additives is relevant from a toxicological standpoint because microbial transformation processes can modify the bioactivity or toxicity of chemicals and alter the chemical's bioavailability and toxicokinetics (Claus, Guillou and Ellero-Simatos, 2016; Koontz *et al.*, 2019; Spanogiannopoulos *et al.*, 2016; Weersma, Zhernakova and Fu, 2020). The development of omic technologies has led to the evaluation of the gut microbiome from a holistic perspective and the emergence of new insights into the role of the gut microbiome in physiological processes and how external factors (e.g. food additives) can influence this relationship (Koontz *et al.*, 2019).

Based on the growing body of evidence indicating that the microbiome can transform chemicals in the gut, potentially altering their toxicity and bioavailability and that it can also impact health (e.g. changes in the gut barrier function, the promotion of antimicrobial resistance or metabolic alterations), it becomes necessary to evaluate whether risk assessments should be updated to incorporate microbiome information, as it is understood today after the omics revolution. However, updating chemical risk assessment procedures to include these new data needs to be approached with caution. The challenges posed by microbiome science and the degree of readiness of different types of microbiome data need to be carefully evaluated. This is crucial because regulatory science requires robust and reliable scientific evidence due to the implications for global health. Some preliminary assessments have already been conducted, indicating that despite the significant progress in microbiome knowledge and emerging omic technologies, microbiome data may not be mature enough to be integrated into risk assessments (Anklam *et al.*, 2022).

This critical review aimed to examine the current state of research – the amount, quality and reliability of scientific information – regarding the impact of food additives on the gut microbiome and, consequently, on host health. Efforts were aimed at identifying limitations, gaps, and research needs, as well as exploring the applicability of microbiome data in food safety risk assessments. Additionally, we investigated whether the gut microbiome could be a valid endpoint in these assessments. It is important to note that we did not evaluate or provide an opinion on whether food additives are beneficial or harmful to the gut microbiome or human health.

CHAPTER 2
SCIENTIFIC LITERATURE RESEARCH METHODOLOGY

The Codex General Standard for Food Additives (GSFA) Online Database (FAO and WHO, 2022), the online version of the Combined Compendium of Food Additive Specifications (FAO, 2022), and the Codex General Standard for Food Additives 192-1995 rev. 2019 (FAO and WHO, 1995) were used as references to identify the food additives to include in the search query of the scientific publication databases.

The scientific literature was screened for sweeteners, emulsifiers, stabilizers, thickeners, gelling agents, foaming agents, sequestrants, humectants, preservatives and colours. The purpose was to identify original peer-reviewed research articles linking the potential effects of these food additive classes on the human gut microbiome and its potential contribution to health effects in the host organism (e.g. animal models and humans). The search was conducted using English terms and targeted articles published between 2010 and the date of database query (September 2021–June 2022). The decision to use 2010 as the starting point was based on the emergence of third generation sequencers (e.g. Illumina system), which made sequencing faster, more affordable and accessible, therefore contributing to a wider adoption. This situation led to a significant increase in sequenced genomes and databases with more abundant and improved data quality.

The scientific publication databases used to perform the defined queries were *PubMed* (www.ncbi.nlm.nih.gov/pubmed/), *Web of Science* (www.webofknowledge.com) and Scopus (www.scopus.com).

A preliminary pilot study was conducted to identify and prioritize target additive classes for further search strategies. Detailed descriptions of the search approaches can be found in *Annex II. Literature search strategy*.

The strategy started by searching using queries using microbiome-related terms (e.g. gut microbiota, gut microbiome) combined with class additives (e.g. sweetener, emulsifier) or using a more restrictive approach using specific food additive names (e.g. sucralose, rebaudioside A) or their synonyms.

The following is an example to illustrate the Boolean syntax used to query the databases:

"Gut microbiome" OR "Human gut microbiome" OR "Microbiome" OR "Gastrointestinal microbiome") AND "Food" AND ("sweetener" OR "keyword related to specific sweetener name" OR "keyword related to specific sweetener synonym".

Inclusion criteria for relevant articles:

> title, abstract or keywords including the term food additive, food additive class, or the name of specific food additive — independent from dose — , and mentions of effects in the gut microbiota of humans or animal models;

> mentions of health effects;

> both *in vivo* and *in vitro* studies were considered. *In vivo* studies focusing on mammal models (ruminants excluded) were especially considered, as they share more physiological and microbiome similarities with humans than other available models (e.g. fish, insects); and

> studies evaluating: (1) intestinal or faecal microbiota, (2) specific microbial groups isolated from intestinal or faecal material or (3) microbiota consortia.

Exclusion criteria for irrelevant articles:

> languages different from English;

> title or abstract did not include both (1) one keyword related to the microbiome and (2) one keyword related to food additives;

> studies evaluating feed additives;

> compounds evaluated as prebiotics or dietary supplements (e.g. curcumin);

> document types different from original research articles (grey literature);

> studies evaluating the oral microbiota (oral, plaque, dental, caries);

> animal studies (exception made for mammal animal model, e.g. pig, rodents);

> studies on the effects of additives on specific bacteria from commercial sources;

- studies on the impact of additives on food and soil microbiota; and
- studies using compounds used in high doses to induce specific conditions in models of disease (e.g. dextran sulfate sodium (DSS) to induce colonic inflammation).

Two studies were added manually because they were often mentioned by scientists and were used in this review to add discussion points. These studies include a paper evaluating sucralose, published before 2010 (Abou-Donia *et al.*, 2008), and a research manuscript investigating a commercial feed additive used in food animal production (Daly *et al.*, 2016). In addition, a recent human interventional trial published after the literature search period, which evaluated different commercial non-nutritional sweeteners, was added due to its relevance (Suez *et al.*, 2022).

The review includes manuscripts investigating silver (E-174). However, silver has not been evaluated by JECFA, does not have an International Numbering System (INS) number, and is not included in Codex General Standard for Food Additives (GFSA). Its use is permitted in some jurisdictions, although with limited food applications. In addition, like the food additive titanium dioxide (also evaluated here), a fraction of the particle distribution falls within nano scale (diameter < 100 nm). The controversy surrounding the potential detrimental health effects derived from nanoparticles intake was the main reason for including this food additive in the review.

Manuscripts were not excluded based on quality, as the evaluation of quality was a crucial aspect of this report. The review aimed to determine the type of information available to scientists, risk assessors, and the general public. Analysing the available information is beneficial for identifying research limitations and pitfalls, which serves as an initial step in developing future research guidance.


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CHAPTER 3
FOOD ADDITIVES EVALUATED

Food additives included in this review are listed in Table 2.

TABLE 2. **LIST OF FOOD ADDITIVES INCLUDED IN THIS REVIEW**

FOOD ADDITIVE	INS OR E-NUMBER	FUNCTIONAL CLASSES	JECFA ADI (MG/KG BW/DAY)	ADDITIONAL INFORMATION
Acesulfame K	950	Flavour enhancer, sweetener	0–15	GFSA JECFA Evaluation
Aspartame	951	Flavour enhancer, sweetener	0–40	GFSA JECFA Evaluation
Saccharin	954	Sweetener	0–5	GFSA JECFA Evaluation
Sucralose	955	Flavour enhancer, sweetener	0–15	GFSA JECFA Evaluation
Steviol glycosides	960 (a, b, c, d)	Sweetener	0–4	GFSA JECFA Evaluation
Neotame	961	Flavour enhancer, sweetener	0–2	GFSA JECFA Evaluation
Erythritol	968	Flavour enhancer, humectant, sweetener	Not specified	GFSA JECFA Evaluation
Sorbitol	420(i)	Bulking agent, humectant, sequestrant, stabilizer, sweetener, thickener	Not specified	GFSA JECFA Evaluation
Xylitol	967	Emulsifier, humectant, stabilizer, sweetener, thickener	Not specified	GFSA JECFA Evaluation
Carboxymethyl cellulose	466	Bulking agent, emulsifier, firming agent, gelling agent, glazing agent, humectant, stabilizer, thickener	Not specified	GFSA JECFA Evaluation
Polysorbate 80	433	Emulsifier, stabilizer	0-25	GFSA JECFA Evaluation

Table 2 (Cont.)

FOOD ADDITIVE	INS OR E-NUMBER	FUNCTIONAL CLASSES	JECFA ADI (MG/KG BW/DAY)	ADDITIONAL INFORMATION
Mono and diglycerides of fatty acids	471	Antifoaming agent, emulsifier, glazing agent, stabilizer	Not limited	GFSA JECFA Evaluation
Lecithin	322(i)	Antioxidant, emulsifier, flour treatment agent	Not limited	GFSA JECFA Evaluation
Carrageenan	407	Bulking agent, carrier, emulsifier, gelling agent, glazing agent, humectant, stabilizer, thickener	Not specified	GFSA JECFA Evaluation
Xanthan gum	415	Emulsifier, foaming agent, stabilizer, thickener	Not specified	GFSA JECFA Evaluation
Curdlan	424	Firming agent, gelling agent, stabilizer, thickener	Not specified	GFSA JECFA Evaluation
Maltodextrin	1400	Carrier, emulsifier, stabilizer, thickener	-	GFSA JECFA Evaluation
Titanium dioxide	171	Colour	Not limited (not specified)	GFSA JECFA Evaluation
Silver*	E-174	Colour	Not evaluated by JECFA	-
Allura red AC (Red 40)	129	Colour	0-7	GFSA JECFA Evaluation
Erythrosine (Red 3)	127	Colour	0-0.1	GFSA JECFA Evaluation
Sunset yellow FCF (Yellow 6)	110	Colour	0–4	GFSA JECFA Evaluation
Brilliant Blue FCF (Blue 1)	133	Colour	0–6	GFSA JECFA Evaluation

* Silver has not been evaluated by JECFA, does not have an INS number, and it is not included in the GFSA. Its inclusion in this review is due to its permitted use in some jurisdictions and the fact that a fraction of the particle size falls within the nano scale (diameter < 100 nm).

Source: Authors' own elaboration.

CHAPTER 4
STUDY SUMMARIES

This section summarizes key findings from the selected studies, with a particular focus on statistically significant results as reported by the research teams. It's also important to acknowledge that these studies may include null results, which play an essential role in interpreting the broader implications of food additive exposure. Some observations are added in this section for select studies. However, a comprehensive critical evaluation of the methodologies, findings interpretation, reporting, and research implications, including the consideration of null results, will be thoroughly discussed later in the discussion section.

Gerasimidis *et al.* (2020) conducted an *in vitro* batch fermentation study to evaluate the impact of eleven food additives on the composition of human faecal microbiota (pooled from 13 healthy individuals), the production of short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA). Food additives were acquired as commercial preparations, food-grade compounds or reagents, including sucralose, ViaSweet™ stevia (95 percent steviol glycosides), Canderel™ (maltodextrin, 1.02 percent aspartame, 0.68 percent acesulflame K), maltodextrin, cinnamaldehyde, κ-carrageenan, carboxymethyl cellulose, polysorbate 80, sodium benzoate, sodium sulfite, and titanium dioxide. The authors selected additives based on published research work reporting on the implication of these compounds on the onset of non-communicable diseases (NCD). The fermentation medium was prepared in-house and supplemented with fibres (e.g. pectin, α-cellulose, high resistant maize starch). Experiments were run for 24h at doses equivalent to 50 percent of the ADI or based on daily intake estimates (Table 3). The microbiota was evaluated by qPCR targeting five bacterial groups positive or negatively associated with NCDs: *Bacteroides/Prevotella, Bifidobacterium, Blautia coccoides, Clostridium leptum* and *Escherichia coli* and by sequencing the V4 region of the 16S rRNA gene. A summary of results is shown in Table 3 and Table 4. Stevia and carboxymethyl cellulose showed no or limited effects on the composition and function of the microbiota. The two methods to evaluate the microbiota (qPCR and 16S rRNA gene sequencing) were not always in agreement, which the authors attributed to the fact that qPCR provides absolute quantification of specific bacteria groups, while 16S rRNA gene sequencing provides a representation of the overall microbial community. The authors indicated that the gut microbiome can be modified differently by the tested food additives and highlight the need to evaluate their impact of the additives not only individually but also in combination in the presence of different macro-, micronutrients and fibre commonly consumed by humans.

TABLE 3. **EFFECTS OF SELECT FOOD ADDITIVES ON SELECT HUMAN FAECAL BACTERIA MEMBERS**

FOOD ADDITIVE	DOSE EQUIVALENCY: % ESTIMATED DAILY INTAKE/ADI (REFERENCE)	BACTERIAL CHANGES								SCFA CHANGES									
		Diversity index	β-diversity	Total bacteria	*E. coli*	*C. leptum*	*Bacteroides/Prevotella*	*Bifidobacterium*	*C. coccoides*	Total SCFA	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Caproic acid	Heptanoic acid	Caprylic acid	Isobutyric acid	Isovaleric acid
Sucralose	50% (FDA)																		
Stevia	50% (FDA)																		
Canderel™*	8% (FDA)																		
Maltodextrin	0.083% (FDA)																		
Cinnamaldehyde	50% (WHO)																		
CMC	27% (JECFA)																		
Polysorbate 80	27% (JECFA)																		
K-carrageenan	8.9% (EFSA)																		
Sodium benzoate	50% (EFSA)																		
Sodium sulfite	50% (EFSA)																		
Titanium dioxide	50% (JECFA)																		

Notes: (absolute concentration by qPCR of 16S rRNA gene copies/ml of major bacteria group) and production of short-chain fatty acids (SCFA) and branched-chain fatty acids (BCFA). Green: concentration higher than control ($p<0.05$), Orange: concentration lower than control ($p<0.05$), Blue: community structure differs from control.

* Canderel™: Maltodextrin, 1.02% aspartame, 0.68% acesulfame K; CMC: Carboxymethyl cellulose.

Source: Adapted from **Gerasimidis *et al.*** 2020. The impact of food additives, artificial sweeteners and domestic hygiene products on the human gut microbiome and its fibre fermentation capacity. *European Journal of Nutrition*, 59(7): 3213–3230. https://doi.org/10.1007/s00394-019-02161-8

TABLE 4. **EFFECTS OF SELECT FOOD ADDITIVES ON HUMAN FAECAL MICROBIOTA COMPOSITION EVALUATED BY 16S (V4) RRNA SEQUENCING**

FOOD ADDITIVE	RELATIVE ABUNDANCE (LOG2 FOLD CHANGE)
Sucralose	↑ *Escherichia/Shigella, Klebsiella, Bilophila*
Canderel™*	↑ *Blautia*, ↓ *Oscillibacter*
Cinnamaldehyde	↑ *Escherichia/Shigella,* ↓ *Subdoligranulum, Faecalibacterium, Collinsella, Dorea*
Sodium sulfite	↑ *Bilophila*, ↓ *Collinsella*
Sodium benzoate	↑ Lachnospiraceae
K-carrageenan	↑ *Escherichia/Shigella*
CMC	↑ Lachnospiraceae
Polysorbate 80	↑ *Bilophila, Bacteroides, Lachnosclostridium, Ruminoclostridium* ↓ *Subdoligranulum, Faecalibacterium*

* Canderel™: Maltodextrin, 1.02% aspartame, 0.68% acesulfame K; CMC: Carboxymethyl cellulose.

Source: Adapted from **Gerasimidis *et al.*** 2020. The impact of food additives, artificial sweeteners and domestic hygiene products on the human gut microbiome and its fibre fermentation capacity. *European Journal of Nutrition*, 59(7): 3213–3230. https://doi.org/10.1007/s00394-019-02161-8

SWEETENERS

Food additives included here are collectively referred to by the research groups as non-nutritional sweeteners (NNS), non-caloric artificial sweeteners (NAS or NCAS), high-intensity sweeteners or low-caloric sweeteners (LCS). The description of these compounds, including the toxicity and toxicokinetics of several of these food additives, has been extensively reviewed by Magnuson *et al.* (2016).

ACESULFAME K

Annex III.1. contains a summary of the studies evaluating acesulfame K.

Uebanso *et al.* (2017b) didn't observe effects in the caecal or faecal microbiota of a mouse model (4-week-old C57Bl/6J) after an 8-week treatment with acesulfame K provided in drinking water at the ADI level (15 mg/kg body weight [bw]/day). The diet consisted of standard chow. The host's lipid profile and bile acids remained unchanged.

Bian *et al.* (2017a) evaluated the effects of 37.5 mg/kg bw/day acesulfame K (gavage dosing) in both genders of CD1 mice for 4 weeks. The dose, higher than the sweetener ADI, led to gender-specific microbiome composition and faecal metabolome changes. In males, the relative abundance of butyrate-producing genera *Bacteroides* and *Anaerostipes*, as well as *Sutterella* were increased. This fact, along with the enrichment of microbial genes related to energy metabolism and the increase in energy-related metabolites, was proposed as a possible reason for the increased body weight in males. The microbiota of females treated with acesulfame K also changed, including the decrease of *Lactobacillus* and *Clostridium*. Unlike males, the abundance of genes related to energy and polysaccharide metabolic pathways decreased. Genes related to microbial pro-inflammatory compounds (e.g. Lipopolysaccharide [LPS] synthesis, flagella in females, and LPS, bacterial toxins, thiolactivated cytolysin in males) were increased in the treatment groups. Although the research group indicated the potential influence of the sweetener-microbiome alterations in the development of obesity and related chronic disorders, they acknowledged the need to characterize the impact of acesulfame K on the host physiology, and to evaluate the effects of the sweetener in human cohort studies. In addition, they also listed the limitations of the study, including small sample size, high acesulfame K dose, short exposure periods, and the lack of food intake and body composition measurements.

Hanawa *et al.* (2021) treated male C57BL/6J mice with a dose of 150 mg/kg bw/day acesulfame K in drinking water for 8 weeks. The diet consisted of standard chow. Food and water consumption were not provided. The evaluation of the caecal microbiota showed a decrease in α-diversity, while the β-diversity was different from the control group. Several phyla were affected, which included a reduction of Proteobacteria and Bacteroidetes, and an increase in Actinobacteria and Verrucomicrobia (mainly *Akkermansia muciniphila*). At the family level, Erysipelotrichaceae increased, while there was a reduction in the families Clostridiaceae, Lachnospiraceae and

Ruminococccaceae. The treated group presented histological alterations of the small intestine (grade 1.6 in a scale with a maximum severity of 5), increased intestinal permeability and expression of proinflammatory cytokine genes. To evaluate whether the disturbed microbiome contributed to intestinal damage, the authors transplanted faecal material from treated mice into microbiome-depleted mice, which didn't elicit any intestinal alterations. Therefore, the scientist excluded the participation of the microbial community in the host alterations. Despite the reported findings, the researchers acknowledged the use of a dose higher than those consumed by humans and, although they proposed some mechanistic explanations, they did not exclude other possibilities involved in the development of intestinal damage.

Wang *et al.* (2018) determined the bacteriostatic effect of 2.5 percent acesulfame K (w/v) *in vitro* on two strains of *E. coli*, resulting in 90 percent and 98 percent growth inhibition of *E. coli* HB101 and *E. coli* K-12, respectively.

In a human cross-sectional study, the short-term consumption of acesulfame-K (also aspartame and a combination of both) was evaluated in 31 healthy participants (Frankenfeld *et al.*, 2015). The diet was not prescribed but monitored and recorded during the 4 days preceding the faecal sample collection for the microbiome study. Acesulfame-K was consumed by 7 individuals at calculated doses ranging between 1.7 and 33.2 mg/day, which is lower than the content of the sweetener in a soft drink (~50 mg). Although the research group reported no differences in the Firmicutes to Bacteroidetes ratio and the median abundance of bacteria (at taxa levels class and order) between consumers and non-consumers, there was a difference in the β-diversity, which the authors attributed to differences in lower abundance species. There were also no differences in the predicted functional composition of the microbial population. The authors acknowledged several limitations of their study, including the small sample size and the lack of information about the participants' consumption of sweeteners.

ASPARTAME

Annex III.2. contains a summary of the studies evaluating aspartame.

Lean and high-fat diet (HFD)-induced obese Sprague-Dawley fed standard chow and HFD, respectively, were given aspartame in drinking water (controls received only water) (Palmnas *et al.*, 2014). Aspartame doses were 5 (HFD) or 7 (normal chow) mg/kg bw/day given for 8 weeks. According to the authors, the doses were equivalent to consuming 2–3 cans of diet soda, which is lower than the sweetener ADI (40 mg/kg bw/day). The microbiota composition, evaluated only at the end of the study by qRT-PCR with primers specific for the S16 rRNA gene, was mostly influenced by the HFD, leading to an increase of total bacteria, Firmicutes, *Clostridium* cluster XI, *Bifidobacterium* spp. and decreased Bacteroidetes, *Bacteroides, Prevotella* spp. However, *Clostridium leptum* and Enterobacteriaceae increased in both aspartame-treatment groups, irrespective of the diet. In addition, *Roseburia* spp. increased as the result of the interaction of the HFD and aspartame.

The sweetener also attenuated alterations caused by the HFD (e.g. increased Firmicutes:Bacteroidetes ratio). The analysis of the serum metabolome showed altered levels of several metabolites, some diet-dependent, others increased in the aspartame-treated groups, and some resulting from the interaction diet x aspartame. The SCFA acetate and butyrate increased in the normal chow group, while propionate increased in aspartame-treated mice fed with both diets. According to the authors, aspartame appeared to mitigate some of the negative outcomes induced by the HFD. However, aspartame-treated groups showed higher fasting blood sugar and impaired insulin tolerance, independent of the diet type. The scientist noted that these effects could have resulted from gluconeogenesis potentially induced by increased microbial SCFA propionate (observed in the two diet groups). Propionate has been reported to be an efficient gluconeogenic substrate (De Vadder *et al.*, 2014, cited by Palmnas *et al.*, 2014). No increase in residual aspartame breakdown products was detected, which was indicative of rapid metabolism.

The same research group evaluated the generational effects of aspartame (also rebaudioside A in a separate treatment group; see Steviol glycosides section) in diet-induced obese Sprague-Dawley rats dams during gestation and lactation (Nettleton *et al.*, 2020). Mothers were fed a high-fat/high-sucrose diet for 10 weeks prior to breeding to induce obesity. After weaning (week 3), the offspring continued on a control diet until the end of the study (week 18). The control group were obese rats given water. The research also included a lean reference group but was not used for statistical comparisons. Body weight and body fat appear to be higher than the control group in female offspring at weaning (no differences after weaning). Similar results were seen for males, although there were no changes in body weight. Insulin tolerance seemed affected (reduced) in the aspartame dam group during gestation only. In male offspring, insulin was reduced during the insulin tolerance test (ITT) and blood glucose increased during the oral glucose tolerance tests (OGTT) at W8 but not at W17. The expression of several genes of the mesolimbic reward was altered in the offspring, which may have influenced food consumption and palatability of food. Regarding caecal SCFA, propionate, butyrate, isobutyrate, isovalerate and valerate increased in the aspartame-treated dam group (no differences in offspring). Quantitative polymerase chain reaction (qPCR) was used on faecal samples to monitor the evolution of select bacteria linked to obesity over time. Caecal microbiota was evaluated by 16S rRNA gene sequencing. Aspartame-treated dams showed a reduced abundance of Enterococcaceae, *Enterococcus* and *Parasutterella*, while *Clostridium* cluster IV increased. Male and female offspring had a higher abundance of Porphyromonodaceae compared to controls. Pooled caecal content collected from offspring at weaning was transferred by gavage to an unknown strain of germ-free (GF) mice. After 14 days, the mice treated with caecal samples from the two sweetener groups showed higher body weight and body fat than the control, as well as signs of glucose intolerance. The microbiota of the aspartame group had an increase in Porphyromonodaceae family, similar to the sweetener groups (dams and offspring).

> NOTE: Although the content of research works will be discussed later in the discussion section, it is useful to show, at this point, some of the issues identified, for example, in this study. Despite the authors' claims, this study does not support that disturbances of the gut microbiota as the cause of the observed alterations are due to the sweetener treatment. Although this study – feeding dams a high-fat/high sucrose diet – includes a lean control, it is not used in statistical comparisons and is excluded in multiple experiments (e.g. insulin tolerance, microbiota, glucose tolerance in transplanted animals). Therefore, it is not possible to discard the diet as a confounding factor and only contributor to the metabolic changes reported by the authors. Moreover, there are several parameters that, according to the methodology, were evaluated but not reported at all. These included glucose metabolism-related markers (e.g. serum leptin, ghrelin, GLP-1, and pro-inflammatory markers, e.g. serum IL-6 and TNF-α), which were not mentioned in the results and discussion. It can be assumed that they were part of the null results. All in all, the findings from Nettleton *et al.* (2020) do not provide sufficient evidence to support the increased risk of metabolic disease in the offspring of obese mothers fed HFSD exposed to low levels of aspartame (below ADI).

The short-term consumption (4 days) of aspartame was evaluated in human subjects by Frankenfeld *et al.* (2015). As for acesulfame-K, described above, there were no differences in the composition and function of the microbiome between consumers and non-consumers, except for the β-diversity. In this study, 7 individuals consumed aspartame in the range of 5.3–112 mg/day (mean 62.7mg/day), which is less than the content of the sweetener in one soft drink (192 mg).

The human interventional study conducted by Suez *et al.* (2022) to evaluate commercial aspartame and three other non-nutritional sweeteners has been reported under the saccharin section.

SACCHARIN

Annex III.3. contains a summary of the studies evaluating acesulfame K.

Suez *et al.* (2014) aimed to demonstrate that saccharin-induced dysbiosis has a causal role in glucose intolerance, a risk factor for type-2 diabetes (T2D). The manuscript reported the results of different study conditions and designs, summarized in Table 5. Animal experiments were carried out in male C57BL/6 mice. The following were experimental conditions: In the first experiment, mice were given a 10 percent commercial saccharin (5 percent saccharin, 95 percent glucose) solution in drinking water (controls were water, 10 percent glucose and 10 percent sucrose) and fed normal chow or a HFD for 11 weeks. The authors claimed that the dose was "well below" the toxic saccharin dose (6.3 g/kg bw/day) (Taylor, Richards and Wiegand, 1968). However, after considering the liquid intake (~20 ml per day) of 20 g mice provided in the supplemental information, the daily intake was estimated as 5 g/kg bw, 1 000 times higher than the saccharin ADI (5 mg/kg bw/day). The liquid intake of the glucose control was also higher than the water control (~18 ml and ~2–3 ml, respectively). Other commercial sweeteners were also included in this evaluation in the normal chow group only (no microbiota evaluated for these sweeteners), with estimated intakes of 2.5 g/kg bw/day for both aspartame and sucralose.

Another group of mice was treated for 5 weeks with pure saccharin in the drinking water at a dose equivalent to the saccharin ADI, with *ad libitum* access to HFD. There was no difference in the reported water and food intake between these two groups. An additional study was conducted *in vitro* by culturing pooled faecal material from naïve C57BL/6 mice in Chopped Meat Carbohydrate Broth containing 5 mg/ml saccharin or phosphate-buffered saline (PBS) for 9 days. Faecal microbiota transplant (FMT) was conducted using faecal material from mice treated with commercial saccharin (normal chow) and pure saccharin (HFD), and *in vitro* cultured microbiota groups. A different mouse strain, germ-free Swiss Webster fed normal chow, was used for these experiments. There was no information about the number and selection of microbiota donors and if the faecal material used for the transplant was pooled or not. Moreover, the manuscript didn't indicate whether the microbiota was evaluated before and after the transplant, which is critical to assessing the microbial engraftment. All treatment and control groups were subject to the glucose tolerance test at the end of the treatment. The authors reported glucose intolerance in all saccharin-treated groups (including mice recipients of faecal materials from these mice). However, there were no differences in glucose levels at 0 and 2 h after the test initiation. The only exceptions were treatment groups fed with HFD. Here, even for the controls, the glucose levels within the first hour were much higher than values observed for mice fed normal chow. Fasting plasma insulin and insulin tolerance tests were normal in all tested groups. The microbiota of the commercial saccharin group (high dose) fed normal chow had over 40 operational taxonomic units (OTUs) different from controls, with an increased abundance of the genus *Bacteroides*. Although some Clostridiales increased in abundance, the majority decreased, such as *Lactobacillus reuteri*, also observed in transplanted mice. The shotgun metagenomic analysis of faecal DNA showed changes in 115 KEGG pathways among treatment groups. The authors focused the discussion on the increased glycan degradation pathways – attributed to the increased *Bacteroides* – and elevated levels of faecal SCFA propionate and acetate (but not butyrate). The authors also reported dysbiosis in the pure saccharin/HFD group.

In addition to the higher liquid intake in the commercial saccharin and control groups mentioned above, food consumption was clearly lower than that of the water control group. These intake values should not be ignored, considering that the diet has a major impact on the microbiota composition and function, especially if they are maintained for the length of the treatment (11 weeks). Moreover, it would be expected that this consumption pattern had a significant impact on the mice' metabolism, regardless of the potential contribution of the microbiome.

A fraction of mice in the commercial sweeteners/normal chow and pure saccharin/HFD groups were kept under the same treatment conditions for another 4 weeks. During this period, animals received antibiotics also in the drinking water. Considering the liquid intake, mice received much higher antibiotic doses than planned. As a result of this experiment, all sweetener groups had glycaemic responses that were no different from those of the controls. This led the authors to link the disturbed microbiota with elevated glucose responses. Also, in connection with the diet, the authors did not comment on the potential effects of the HFD in the study outcomes or how the differences in diets between donors (HFD) and faecal recipients (standard chow) could have influenced the composition of the transplanted microbiota.

Suez *et al.* (2014) also reported the results of an interventional study on only seven healthy individuals who took part in a previous cross-sectional cohort study (n=381, non-diabetic). The research team did not specify if these individuals were selected randomly or the type of diet they followed during the 6 days they consumed commercial saccharin (5 mg/kg bw/day). Following blood glucose response tests, four subjects ("responders") had a response statistically different from the other three ("non-responders") only on the last 3 days of treatment. The authors reported pronounced microbial compositional changes after saccharin consumption in the "responders" group. It should be noted that the microbiota composition of responders and nonresponders was different before the treatment [assessed from the provided figures]. To evaluate whether dysbiosis could cause glucose intolerance, faecal material from only two of the four responders and two of the three nonresponders were transplanted into germ-free mice. Glucose intolerance was reproduced in the mice. The microbiota composition of one donor had similarities to those observed in the mice studies described above, including increased *Bacteroides fragilis* and *Weissella cibaria*, and decreased *Candidatus arthromitus*.

Suez *et al.* (2014) suggested that, based on their findings, non-caloric sweeteners could contribute to the global rise in the prevalence of diabetes and obesity.

This study and other research investigating non-nutritional sweeteners (NNS) has been addressed in several critical reviews, although displaying different positions on the potential safety risk of these additives (Hughes *et al.*, 2021; Lobach, Roberts and Rowland, 2019; Schiffman and Nagle, 2019).

TABLE 5. **COMPONENTS AND CONDITIONS IN THE STUDY REPORT BY SUEZ *ET AL.***

TEST MATERIALS / CONTROLS[A]	EXPERIMENTAL CONDITIONS[B]	TESTS[C]
Commercial aspartame Commercial sucralose Dose: 2 500 mg/kg bw/day (estimated based on liquid intake, 20 g mice) Controls: water, glucose, sucrose	Mice (n=20) Normal chow 11 weeks	Glucose and Insulin tolerance test Fasting plasma insulin
Commercial saccharin Dose: 5 000 mg/kg bw/day (estimated based on liquid intake, 20 g mice) Controls water, 10% glucose, 10% sucrose	Mice (n=20) Normal chow 11 weeks	Glucose and insulin tolerance test Fasting plasma insulin MB: composition, predicted functional genes FMT[D]: Glucose tolerance test, MB (composition)
Commercial saccharin Dose: not estimated (no liquid intake provided: 10% solution [5% saccharin, 95% glucose]) Control: 10% glucose	Mice (n=10) HFD 11 weeks	Glucose tolerance test
Pure saccharin 5 mg/kg bw/day Control: water	Mice (n=20) HFD 5 weeks	Glucose tolerance test and fasting plasma insulin MB: composition, predicted function FMT: Glucose tolerance test, MB (composition)
Saccharin 5 mg/ml (assumed pure) Control: PBS	9 days in bioreactor (faecal material from naïve mice)	MB: composition, predicted function) FMT: Glucose tolerance test, MB (composition, predicted function)
Commercial saccharin 5 mg/kg bw/day	Interventional study in humans (7 individuals) 6 day-treatment	Glucose and insulin tolerance test MB composition

[A] Sweeteners in drinking water available *ad libitum* (mice studies).

[B] Mice model: adult male C57BL/6 mice.

[C] Different tests have different sample sizes.

[D] FMT: Faecal microbiota transplant in adult male Swiss Webster fed with normal chow and no sweetener in water (Tests performed 6 days after transplant).

Source: Adapted from **Suez *et al.*** 2014. Artificial sweeteners induce glucose intolerance by altering the gut microbiota. *Nature*, 514(7521): 181–186. https://doi.org/10.1038/nature13793

Recently, Suez *et al.* (2022) conducted another human interventional study (randomized non-blinded) to evaluate the effects of four different commercial non-nutritive sweeteners (NNS) containing either saccharin, sucralose, aspartame or stevia, on blood glucose levels as a primary endpoint, and the oral and faecal microbiotas as secondary outcomes. The authors did not disclose the commercial brand names of the sweeteners; they only disclosed that they contained glucose as a bulking agent. One experimental group was exposed to glucose only. The control group did not receive any of the sweeteners. Each group consisted of 20 non-obese healthy individuals, both males and females, who needed to report daily activities and the amount of standardized and real-life meals consumed throughout the study. The study consisted of three phases: (1) a 7-day baseline period, (2) a 14-day treatment with six sachets/person/day given in the drinking water (daily intake of 180 mg stevia, 102 mg sucralose, 180 mg saccharin or 240 mg aspartame, which equates to 75 percent, 34 percent, 20 percent and 8 percent of the corresponding ADI [in a 60 kg person]

established by the United States Food and Drug Administration (FDA) [i.e. 4, 5, 15, 50 mg/kg bw/day, respectively]), and (3) 7-day clearance period. Oral glucose tolerance tests (OGTT) were conducted by the participants at home, who needed to fast between 7–14 hours. Results showed person-to-person variability in the baseline. Each treatment group was compared to its baseline. In the absence of changes in the levels of insulin (except for the stevia group) or glucagon-like peptide-1 (GLP-1), the glycaemic response (iAUC) was significantly elevated only in the saccharin and sucralose groups but returned to baseline during the clearance period. Saccharin and sucralose exerted changes in the faecal microbiota composition, while some features related to the microbial function and plasma metabolome were affected by all four non-nutritive sweeteners in a compound-specific fashion. The authors found some correlations between the changes in microbial composition (genera and species), gene abundance (and corresponding pathways) and the OGTT-iAUC response. Regardless of the statistical significance, the research group further expanded the evaluation selectively on the individuals with the highest or lowest OGTT-iAUC (i.e. top and bottom responders[9] after treatment) from each treatment group to identify metabolites that could mediate in the glycaemic response and the microbial signatures positively or negatively associated with it. This study found correlations between non-nutritive sweeteners and the glycaemic response.

To evaluate the potential causative involvement of the gut microbiome on the glycaemic response, Suez *et al.* (2022) conducted microbiota transplants using faecal material collected on day 1 of baseline period and day 21 of treatment from seven select donors per group (the four top responders and three lower responders[10]) in germ-free (GF) Swiss-Webster mice (one donor–one recipient). These GF mice underwent OGTT, and the iAUC was evaluated by comparing – for each treatment group – the response of animals transplanted with faecal materials from day 21 against animals transplanted with faecal material from baseline. Top responders from all treatment groups showed significant alteration of the glycaemic response, as well as the low responders of the saccharin group. The microbiota of top and bottom sucralose responders in the GF mice was different (e.g. higher α-diversity) not only at day 21 but also in the baseline. Based on these findings, the authors suggested individual-specific responses to the sweetener, which can be influenced by the initial microbiota configuration. According to the research group, further correlations between the microbiome (composition and function) and glycaemic response in sucralose GF mice suggested that the altered OGTT could be partially mediated by the capacity of bacteria to metabolize dietary and/or host-derived carbohydrates and their utilization for energy production. However, this preliminary information would require further causative validation.

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9 The authors of this study called all selected individuals "responders" (highest or lowest OGTT-iAUC responses), also those from groups that did not show statistically significant differences in OGTT, which are in fact "non-responders".

10 Same comment as 9.

Suez *et al.* (2022) reported several study limitations, which were generalized to all NNS. Individuals with metabolic disorders may respond to NNS differently than the healthy participants of this study. Pure test compounds or commercial NNS containing fillers different from glucose (as used in this study) may lead to different microbiome and glycaemic responses. They also proposed conducting future research on extended exposure periods at lower doses and controlled diets, e.g. carbohydrate-rich or carbohydrate-restricted.

Becker *et al.* (2020) used saccharin as a positive control group (based on the findings from Suez *et al.*, 2014) in a mouse study aimed to evaluate the potential use of stevia to improve high-fat diet-induced glucose intolerance and alter the microbiota (see details later under the stevia section). Diet-induced obese male and female C57BL/6J mice fed HFD were given 5 mg/kg bw/day saccharin or stevia in the drinking water for 10 weeks. Two negative controls in this study were fed a high- or low-fat diet. Increases in body weight, changes in glucose response and lower microbiota richness were driven by the diet and not by the sweetener. The mouse gender influenced differences in β-diversity found between low-fat and the HFD groups. Specifically, an increase in *Akkermansia muciniphila* in the female-saccharin group and the increased Lactococcus in the stevia-female group accounted for the differences in β-diversity. The Firmicutes/Bacteroidetes ratio was significantly higher in saccharin and stevia compared to the HFD samples, and this one was higher than the low-fat diet group. Based on the findings, the authors concluded that HFD had more influence in the alterations found at the phylum level than the sweeteners. The authors also acknowledged the low statistical power of their study due to the small sample size (n=10, five male and five female, per treatment group).

Labrecque *et al.* (2015) evaluated the impact of ethanol, saccharin and pregnancy status on the microbiome of female C57BL/6J mice. For two weeks, animals were given access to drinking water containing 0.066 percent saccharin, 10 percent ethanol or a combination of 10 percent ethanol and 0.066 percent saccharin only for 4 hours a day. Faecal samples were collected from the pregnant females between 11–15 days after mating. The faecal microbiota was evaluated at the end of the study by qPCR using primers designed by the team to target the 16S rRNA gene. The presence of saccharin and the pregnancy status of the mice influenced how ethanol affected the abundance of certain bacterial groups. *Clostridium* decreased in pregnant mice exposed to ethanol+saccharin and in non-pregnant mice consuming ethanol, but no change was observed in non-pregnant mice consuming ethanol+saccharin. *Eubacterium* increased in pregnant mice consuming ethanol+saccharin and decreased in non-pregnant animals drinking ethanol+saccharin. *Helicobacter* was elevated in nonpregnant mice exposed to ethanol with or without saccharin. *Bacillus, Bacteroides* and *Lactobacillus* were not affected. No differences were observed in fluid intake or weight. No other host parameters were evaluated. Based on the reported daily fluid intake (~1 ml/4 h) and a 31 g mouse, the saccharin dose was estimated as 20 mg/kg bw/day, which is higher than the theoretical experimental dose and slightly higher than the JECFA ADI.

Daly *et al.* (2016) evaluated the effects of 2-week supplementation of SUCRAM (containing the sweeteners saccharin and neohesperidin dihydrochalcone [NHDC, E-959]) in the feed (0.015 percent, w/w) on the caecal microbiota of 28-day-old, weaned piglets (Landrace X Large White). Of note, SUCRAM is a feed additive used in animal production, specifically a flavour enhancer. It has been reported to promote health, reduce postweaning enteric disorder and reduce mortality in pigs (Daly *et al.*, 2016; Sterk *et al.*, 2008). Feed additives are evaluated and regulated independently from food additives. This study was included primarily because it was often cited by the researchers investigating food additives and to emphasize the need of scientists to consider the context of the work they reference. The treatment induced changes in the microbial community structure as indicated by the analysis of β-diversity. It increased the abundance of Lactobacillaceae, mainly *Lactobacillus* 4228, while reducing Ruminococcaceae and Veillonellaceae. This finding was observed in parallel to the increase in lactic acid, which reduces the luminal pH, and helps control the proliferation of pathogenic bacteria. Similar findings – increases in the abundance of caecal *Lactobacillus* and intraluminal lactic acid – were also observed in a previous study by the same research group (Daly *et al.*, 2014). The abundance of SCFA was not affected by the treatment. An additional *in vitro* study conducted with isolated *Lactobacillus* 4228 in the presence of different sugars, identified NHDC as the key factor in shortening the lag phase of *Lactobacillus* growth. This finding suggested the rapid adaptation of the bacteria to changes in carbohydrate sources. Moreover, the *Lactobacillus* strain did not degrade NHDC, requiring the presence of other sugars to promote its growth.

Bian *et al.* (2017c) evaluated if 0.3 mg/ml saccharin in drinking water (estimated ~18–26 mg/kg bw/day) given to male C57BL/6J mice for 6 months could alter the microbiota and influence the inflammatory response of the host. The sequencing of the 16S rRNA gene was used to monitor the faecal microbiota composition at day 0, and after 3 and 6 months of exposure. The abundance of several genera changed with respect to the control group (water without saccharin) at month 3 or month 6. The abundance of *Ruminococcus* was consistently reduced throughout the study, *Anaerostipes* only in month 3, and *Dorea* and *Adlercreutzia* only in month 6. *Akkermansia* and *Oscillospira*, increased only in month 3, while *Corynebacterium, Roseburia* and *Turicibacter* increased only in month 6. Although the authors did not report it, the figures in the manuscript show that the bacteria's abundance in the control group fluctuated over time in a pattern different from that of the treatment mice. This difference complicates comparing the groups and interpreting the results. Only data from month 6 were reported for the other parameters evaluated in this study. The functional analysis of the 16S rRNA sequence showed enrichment of genes related to lipopolysaccharide (LPS) synthesis, flagella assembly, fimbriae, microbial toxins and antimicrobial resistance. The transcription genes of inflammatory markers from liver samples showed increased inducible nitric-oxide synthase (iNOS) and tumour necrosis factor-alpha (TNF-α), but not IL-1β and IL-6. The authors reported changes in the faecal metabolome of saccharin-treated mice, affecting 1 743 metabolites. They limited the discussion to alterations of functional microbial metabolites related to the inflammatory response.

The primary focus was on daidzein (an isoflavone) metabolism, specifically, the decrease of equol (with antioxidant and anti-inflammatory activity) and increase of daidzein itself and its metabolites O-desmethylangolensin (O-DMA) and dihydrodaidzein. Several other anti-inflammatory compounds also decreased, while the pro-inflammatory metabolite quinolinic acid increased. Taken altogether, the authors suggested that consumption of saccharin could alter the gut microbiota, increasing the risk of inflammation in the host.

Sunderhauf *et al.* (2020) conducted several studies to evaluate the effects of 5 mg/kg bw/day saccharin (in drinking water) on healthy C57BL/6J mice and a model of dextran sulfate sodium (DSS)-induced colitis. In a preliminary *in vitro* test, saccharin influenced the growth of select bacteria in a dose-dependent manner, with effects observed at 5 mM, but not at the lower concentrations tested (2.5 and 0.5 mM). These were a bacteriostatic effect on the Firmicutes *Staphylococcus aureus* and the Proteobacteria *Klebsiella pneumonia* and *Pseudomonas aeruginosa*, and almost no growth of *Bacillus cereus* (Firmicutes). The first *in vivo* experiment, which did not evaluate the microbiota, showed that a seven-day supplementation of saccharin in healthy mice did not alter the intestinal barrier (no sign of intestinal inflammation and lowered the expression of inflammatory markers). After inducing acute experimental colitis with DSS treatment, a short-term supplementation of saccharin (2–7 days) led to protective "therapeutical" effects (improved colonic histology score, molecular markers of inflammation). The microbiota was not evaluated either. In the next study, the authors evaluated the potential "prophylactic" effect of saccharin (administered 5 weeks before colitis induction) in the subsequent 30 days of chronic colitis.

While α-diversity did not differ from the control group after 5 weeks of saccharin consumption, there were changes in β-diversity, increased Bacteroidetes and Proteobacteria and decreased Firmicutes, as well as a reduction in the faecal bacterial load. The improved intestinal disease activity index (DAI)[11] in the saccharin-consuming group observed at the early stages of colitis declined towards the end of the 30 days. The authors suggested that saccharin-induced changes in the microbiota, which promoted an anti-inflammatory response, tended to disappear in the absence of saccharin. However, after 30 days of colitis, the IgA levels (related to inflammatory activity) were significantly lower in the saccharin group. The microbiota was not evaluated after inducing colitis nor at the end of the study.

Serrano *et al.* (2021) reported null effects of saccharin on glucose homeostasis, gut microbiota and faecal metabolome in humans and mice. The human trial consisted of a randomized, double-blind, placebo-controlled, interventional study with 46 healthy participants. The authors aimed to target the high-end JECFA ADI, giving the participants a daily dose of 400 mg pure saccharin (in capsules administered twice a day) for 2 weeks. Measurements were carried out before and at the end of the 2-week treatment, and after a 2-week clearance period to monitor for potential delayed effects. This study included two additional groups given lactisole[12] or lactisole plus saccharin. The animal study used two different genotype mice, wild-type (WT) C57BL/6J and T1R2-deficient[13] mice. They were treated with higher doses of saccharin (250 mg/kg bw/day in the drinking water) for longer (10 weeks) than in the human study. According to the authors, the dose was equivalent to four times the human ADI adjusted for mouse body surface area following the indications described in Nair and Jacob (2016). In addition to the between-treatment comparisons, the inclusion of a pre-treatment baseline allowed the study of intra-individual variations over time and the identification of age-dependent increases in SCFA levels and body weight in the WT group. Also, in the wild type, the results from the β-diversity analysis suggested that saccharin induced fewer overall changes in microbial profiles over time. The authors acknowledged some limitations of their study. These include: (1) the inability to extrapolate findings to other sweeteners; (2) the short exposure period (two weeks) used in the human study, which makes it difficult to predict the effect of chronic saccharin consumption; (3) the potential for having missed other physiological parameters more suitable in the identification of saccharin-driven adverse effects; and, (4) relatively small sample size.

Wang *et al.* (2018) determined the bacteriostatic effect of 2.5 percent saccharin (w/v) *in vitro* on two strains of *E. coli*, resulting in 98 percent and 99.5 percent growth inhibition of *E. coli* HB101 and *E. coli* K-12, respectively.

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11 The disease activity index (DAI) measures changes in body weight, rectal bleeding and stool consistency.

12 Lactisole is a human-specific inhibitor of human sweet taste receptors (STRs) (Serrano *et al.*, 2021, p. 2).

13 T1R2 is a sweet taste receptor. Intestinal STRs are involved in the regulation of metabolic responses to sugars. (Serrano *et al.*, 2021, p. 2).

SUCRALOSE

Annex III.4. contains a summary of the studies evaluating sucralose.

Abou-Donia *et al.* (2008) evaluated the effects of Splenda (1.1 percent sucralose, 1.1 percent glucose, 93.6 percent maltodextrin) administered to male Sprague Dawley rats in solution via gavage. The doses, 100, 300, 500 or 1 000 mg/kg bw/day (1.1, 3.3, 5.5, 11 mg sucralose/kg bw/day) were given to male Sprague Dawley rats for 12 weeks. Half of the rats were subjected to an additional clearance period of 12 weeks. The control group received water without Splenda. Select cultured bacterial groups (total aerobes, total anaerobes, Lactobacilli, Enterobacteria, Clostridia, Bifidobacteria and *Bacteroides*) isolated from stools were included in the microbial evaluation. Enterobacteria were not affected by Splenda in this study. There was a general count reduction in the other bacterial groups compared to the control group after the 12-week treatment period, except for the lowest dose, which only affected total anaerobes, Bifidobacteria, Lactobacilli and *Bacteroides*. After the 12-week clearance period, only total anaerobes were reduced in all treatment groups, and Bifidobacteria at the two mid doses. Treatment groups showed increased faecal pH and presented an apparent dose-dependent colonic histological change. After the clearance period, all animals, including the controls, showed some degree of histological alteration, which authors attributed to ageing. With the exception of the lowest Splenda dose, intestinal P-glycoprotein (P-gp) and the cytochrome P-450 (CYP)[14] evaluated by Western immunoblot were elevated after the treatment and only in the highest doses after the clearance period. Although this study focused on Splenda, there is an underlying reference to sucralose throughout the discussion of findings. The science and interpretation of the findings of Abou-Donia's work were later criticized by Brusick *et al.* (2009).

Uebanso *et al.* (2017b) evaluated the effects of two doses of sucralose, 1.5 and 15 (ADI level) mg/kg bw/day, administered in drinking water, in four-week-old C57BL/6J mice for a period of 8 weeks. The researchers observed dose-dependent effects in several of the parameters studied, including the reduction of the butyrate-producing *Clostridium* XIVa cluster in faeces and luminal butyrate levels, as well as an increased ratio of secondary to primary bile acids. The authors suggested that sucralose could disturb the microbiome, cholesterol and bile acid metabolism.

Wang *et al.* (2018) studied the impact of pure sucralose on five-week-old C57BL/6 mice, which were fed either a high-fat diet (HFD) or standard chow. The daily sucralose intake was calculated as 3.3 mg/kg bw for the standard chow group and 1.5 mg/kg bw for the HFD group. No changes in α-diversity were observed in either group. Firmicutes increased in both sucralose groups, although transient in animals fed standard chow. *Bifidobacterium* increased only in the sucralose+standard chow. It was noted that in the control groups not receiving sucralose, the HFD alone increased the abundance of Firmicutes and decreased Bacteroidetes.

[14] P-glycoprotein (P-gp) and the cytochrome P-450 (CYP) are enzymes involved in the detoxification of exogenous compounds.

The only host parameter evaluated was the body weight, which, as expected, increased in the HFD control group compared to the standard chow. In the sucralose treatment groups, body weight decreased in the standard chow-fed animals, while no change was observed in the HFD group. Lastly, the study found that sucralose had a dose-dependent bacteriostatic effect on the *E. coli* strain HB101, at concentrations of 1.25 and 2.5 percent (w/v).

Sanchez-Tapia *et al.* (2020) treated a total of 16 groups of lean male Wistar rats with different sweeteners in the drinking water (1.5 percent sucralose, 2.5 percent steviol glycosides, or 10 percent of each of the following: steviol glycosides+sucrose, sucrose, glucose, fructose, honey, brown sugar) for four months. Animals were fed either standard chow or HFD. There was one control per diet with no sweetener added to the drinking water. As fluid intake was not reported, it was challenging to accurately estimate the daily exposure to the sweeteners. For sucralose, the estimated daily intake, based on an average 500-g rat (taken from the publication chart) and daily fluid intake of 20–40 ml, would be about 600–1 200 mg/kg bw, which is between 40–80 times higher than the JECFA ADI. In general, the HFD significantly impacted the parameters evaluated, inducing signs of a pro-inflammatory response. Some of the effects observed were also dependent on the sweetener type. Non-caloric sweeteners, such as sucralose, were associated with a reduction in the α-diversity of the gut microbiota based on analysis of faecal samples. When considering β-diversity, these sweeteners accounted for only 4.6 percent of the changes. In contrast, caloric sweeteners accounted for 18.4 percent of the variation, and a high-fat diet (HFD) contributed to 30 percent. In the presence of HFD, sucralose significantly decreased the Bacteroidetes/Firmicutes ratio, with an increased abundance of *Bacteroides* (*B. fragilis*). *Lactococcus, Mucispirillum,* and *Bifidobacterium* were the most affected genera (reduction) by sucralose supplementation. Most of the observations were related to HFD consumption. In animals on an HFD, there was a decrease in the abundance of *Akkermansia* and an increase in *Desulfovibrio*. Shotgun metagenomics showed that gene richness was lowest in the two non-caloric sweetener groups (HFD only). Sucralose, in particular, increased the abundance of genes related to the synthesis of SCFA and LPS (HFD only). Such findings were consistent with the increased abundance of colonic toll-like receptors (TLR) TLR2 and TLR4[15] and plasma lipopolysaccharide (LPS), which would indicate metabolic endotoxemia. Sucralose groups, especially combined with HFD, led to high levels of faecal SCFAs, particularly acetate, which is correlated with the increased abundance of the receptor G-protein coupled receptor 43[16] (GPR43). This group developed a fatty liver. In addition, there was a shortening of epithelial length and decreased colon occludin, which is indicative of alterations in intestinal permeability. Sucralose groups, especially mice fed HFD, showed insulin resistance (high serum insulin levels) and glucose tolerance (high fasting glucose levels and altered GTT). HFD in combination with glycoside steviosides or sucralose significantly reduced body weight.

15 TLR4 – Lipopolysaccharide receptor.

16 GPR43 – SCFA receptor.

Although the authors indicated that they had demonstrated the implication of the gut microbiota in the development of metabolic endotoxemia, and lipid and carbohydrate metabolism after exposure to the sweeteners, the fact is that the study was not designed to evaluate either causality or the underlying mechanisms.

A mouse study, which primarily aimed to investigate the hepatoprotective effects of rebaudioside A in beverage-induced non-alcoholic steatohepatitis (NASH), also included an evaluation of sucralose (Xi *et al.*, 2020). The steviol glycosides section includes additional details about the effects of rebaudioside A from this study. The study was conducted in an obese mice model (strain and gender not reported) receiving 97 mg sucralose/L (~5 mg/kg bw/day) in the drinking water and fed an HFD for 15 weeks. The dose was a third of the JECFA ADI for sucralose. Findings were compared to other HFD groups, including the control and a treatment group exposed to sucrose+fructose in drinking water. All the HFD groups were also compared against a control diet group. The only aspect of the microbiota composition discussed by the authors for the sucralose group was the high inter-individual variability. The histopathology analysis of the liver revealed hepatic fibrosis, with a degree similar to the high carbohydrate/HFD group. However, like rebaudioside A, the treated animals presented signs of improved glucose homeostasis and insulin sensitization.

Adult male C57BL/6J mice were given 0.1 mg/ml sucralose in drinking water for 6 months (Bian *et al.*, 2017b). The authors reported that this dose was equivalent to the ADI set by the United States FDA for sucralose (5 mg/kg bw/day). Although fluid intake and body weight were monitored, no data were provided or discussed. Based on a 20 g mouse and 2 ml of daily fluid intake, we estimated the ADI to be 10 mg/kg bw/day, which is twice the United States FDA ADI, but lower than the JECFA ADI for sucralose (15 mg/kg bw/day). The faecal microbiota was assessed by sequencing region V4 of the 16S rRNA gene before the treatment and after 3 and 6 months of the experimental phase. Microbial diversity was not evaluated.

A total of 14 genera had significant differences in relative abundances at 3 or 6 months. After 6 months, Christensenellaceae, Clostridiaceae, *Akkermansia, Roseburia* and *Turicibacter* had increased relative abundance, while Erysipelotrichaceae, *Dehalobacterium, Streptococcus* and *Ruminococcus* were reduced. The authors reported increased enrichment of genes related to LPS, flagella, fimbriae, bacterial toxins and antimicrobial resistance. The analysis of the faecal metabolome resulted in the tentative identification of 66 altered compounds, some related to the quorum sensing system, amino acids and derivatives, lipids, fatty acids, and bile acids. Liver samples were used to evaluate the expression of seven pro-inflammatory markers, of which only iNOS and MMP-2 were significantly increased. No anti-inflammatory markers were targeted in this study. Based on these findings, the authors indicated that sucralose alters the microbiota composition and function and increases the risk of liver inflammation. However, they acknowledged a few limitations and how to approach newer sucralose evaluations: (1) the need for a better characterization of host effects, e.g. determination of circulating LPS and histological evaluation; (2) the need for a dose–response evaluation at sucralose levels relevant to human exposure; (3) the use of a more holistic approach to further assess functional alterations of the microbiome, including shotgun metagenomics and metatranscriptomics; and (4) the need for more accurate quantitative analyte detection and validation of relevant metabolites.

Three concentrations (1, 3.5 and 35 mg/ml) of the commercial sweetener Splenda (99 percent maltodextrin and 1 percent sucralose) were given to SAMP1/YitFc and AKR/J mice in the drinking water for 6 weeks (Rodriguez-Palacios *et al.*, 2018b). SAMP mouse is a model of Crohn's Disease-like ileitis and AKR mouse is its parental ileitis-free control strain. Both mouse strains had their controls without Splenda. The authors selected the middle sweetener concentration based on the maximum recommended dose by the United States FDA. The gut metagenome of SAMP mice was characterized and compared to the AKR before the Splenda treatment. It was found that SAMP ileitis has a Bacteroidetes-rich microbiome phenotype that favours the probability of enterobacterial growth. In both SAMP and AKR mice, Splenda supplementation promoted the growth of Proteobacteria classes (α- β- γ- δ- ε-proteobacteria). Bacteroidetes and Firmicutes were not altered. Moreover, *Escherichia coli* and maltodextrin-utilizing enterobacteria were enriched in SAMP mice. The ileum of Splenda-treated SAMP mice exhibited large clusters of bacteria infiltrated in the ileal lamina propria, including *E. coli* and gene enrichment related to maltose/maltodextrin metabolism. In addition, there was an increase in pro-inflammatory myeloperoxidase (MPO) activity in the ileal tissue (not in the colon) starting at the middle dose. Although mice showed intestinal inflammation at the high dose, the middle dose did not induce histological alterations, indicating that Splenda did not alter the ileitis phenotype. No systemic effects were observed (TNF-α, glucose tolerance). The authors concluded that although Splenda could promote gut dysbiosis in healthy and CD-prone mice, it exacerbated MPO activity only in individuals with pro-inflammatory predisposition. Moreover, they proposed measuring faecal Proteobacteria and MPO activity as biomarkers to monitor human inflammatory bowel disease (IBD) susceptibility and gut health status.

This could help the development of dietary guides for susceptible individuals. Although it was not possible to identify the individual contribution of Splenda components to the observed effects, there is evidence that maltodextrin (the most abundant compound of the commercial sweetener) favoured the proliferation of maltodextrin-utilizing bacteria and the presence of genes related to the maltodextrin metabolism in the villi infiltrate. The use of maltodextrin as component of placebo samples in gut microbiome studies has been questioned by others, due to its potential influence in the microbial community (Almutairi *et al.*, 2022). However, the specific influence of sucralose in the observed outcomes was unclear.

Dai *et al.* (2020) evaluated the effect of maternal sucralose exposure on the intestinal barrier, hepatic parameters, inflammatory markers and gut microbiota of offspring. C57BL/6 mouse mothers were fed standard chow and exposed during gestation and lactation to 0.1 mg/ml sucralose in the drinking water (a dose equivalent to 5–15 mg/kg bw/day, according to the authors). After weaning (week 3 post-partum), mice were fed standard chow for 5 weeks, and then HFD for 4 additional weeks. Parameters were evaluated at weaning and at the end of the study (week 12). At weaning, the faecal microbiota showed a decreased α-diversity and the β-diversity differed from the control. The abundance of the phyla Verrucomicrobia and Proteobacteria increased, while Bacteroidetes decreased. The genera with increased abundance were *Alistipes, Blautia, Akkermansia, Escherichia/Shigella* and *Anaerostipes,* while *Parabacteroides, Streptococcus, Ruminococcus* and the butyrate-producing groups Prevotellaceae and *Clostridium* XIVa decreased. Butyrate was the only SCFA (reduction) altered at this age. At the end of the study (12-week-old mice), α-diversity was no different from the control, while β-diversity was different and, contrary to the observation in younger mice, the abundance of Proteobacteria decreased. Effects at the intestinal level were only evaluated at weaning. The intestinal barrier was altered in the sucralose group, with several parameters affected, including shorter villi, decreased goblet cells, reduced expression of MUC2,[17] GPR43, several junction proteins, and IgA. Although inflammation was not apparent in the small intestine or the colon, pro-inflammatory cytokines were more elevated in the treatment group, indicating a low-grade inflammation. Although there was no significant macroscopic hepatic inflammation, several pro-inflammatory cytokines were increased. After a 4-week HFD, both groups of 12-week-olds showed a fatty liver, although the steatosis was more pronounced in the sucralose group. This group also showed increased pro-inflammatory cytokines, disturbed hepatic lipid metabolism, and increased serum total cholesterol, triglycerides and hepatic markers (ALT, AST). The authors concluded that maternal sucralose exposure has long-lasting effects on the offspring, contributing to the exacerbation of HFD-induced hepatic steatosis in adulthood through mechanisms involving gut dysbiosis and GPR43 down-regulation. Mothers were not evaluated, and food and fluid intakes were not reported.

[17] Mucin 2, oligomeric mucus/gel forming protein.

Li *et al.* (2020a) evaluated the effects of sucralose in a common model for colorectal cancer (CRC). In this model, based on C57BL/6J mice, CRC is induced with azoxymethane (AOM) and dextran sulfate sodium (DSS). Sucralose was given in the drinking water at a concentration of 1.5 mg/ml, which we estimate to be about 150 mg/kg bw/day (based on a 20 g mouse with 2 ml daily fluid intake). This concentration is ten times higher than the JECFA ADI, and 30 times higher than the FDA ADI. Sucralose was given for 6 weeks before and 36 days after inducing the disorder. Sucralose was also given to a group of mice not treated with AOM and DSS for the entire duration of the study. The analysis of the faecal microbiota was conducted at the end of the study by qPCR targeting specific bacteria groups and species. All sucralose groups showed decreased abundance of total faecal bacteria. In the disease model, sucralose reduced the abundance of Proteobacteria, while it increased the abundance of Firmicutes (also in the sucralose alone group) and Actinobacteria. Both sucralose groups increased the abundance of *Clostridium symbiosum, Peptostreptococcus anaerobius*, and *Peptostreptococcus stomatis* substantially (this one not in the sucralose/healthy mice), while decreasing the abundance of *Bifidobacterium*. Sucralose aggravated the disease parameters (decreased body weight, survival rate, size, and number of colonic tumours). These parameters did not differ from the control in the sucralose/healthy animals. Sucralose also induced colonic tissue damage, and altered tight junctions, inflammatory response, protease activity and tumour-associated signalling pathway molecules. Some of these effects were also affected – to some degree – in the healthy animals given sucralose. Based on these findings, the authors proposed that the exacerbated effects caused by the artificial sweetener could be potentially driven by the impaired inactivation of digestive proteases, which is consistent with the reduced bacterial β-glucuronidase (enzyme inhibitor) resulting from the altered microbiota.

The same research group conducted a similar study but focused on DSS-induced colitis without the cancer component (Guo *et al.*, 2021). The model (C57BL/6 mice) and sucralose exposure (1.5 mg/kg bw/day in the drinking water for 6 weeks before inducing ulcerative colitis with DSS for 7 days) were the same. No food or fluid intake was reported. There were two sucralose groups, of which one underwent colitis induction. One control had DSS-induced colitis (no sucralose exposure) and the other one was a normal control (no sucralose, no colitis). Sucralose alone led to changes in the microbial community – evaluated by qPCR with primers specific for select taxa – with respect to the normal control. These changes affected Bacteroidetes, *Bifidobacterium, B. breve, B. bifidum, Parabacteroides distasonis, Faecalibacterium prausnitzii,* and *Lactobacillus*, which decreased, while *Akkermansia muciniphila, Pseudomonas aeruginosa, Prevotella copri, Fusobacterium nucleatum,* and *Bacteroides fragilis* resulted in increased abundance. Animals exposed to sucralose showed an exacerbated form of colitis, compared to the colitis control, with additional body weight loss, and a more pronounced expression of the pro-inflammatory markers and intestinal barrier parameters. Sucralose+colitis and sucralose-only groups shared several altered taxa. Sucralose alone did not change the immune response, although the intestinal barrier was slightly disturbed. The authors concluded that sucralose likely increased the susceptibility of individuals to DSS-induced colitis by inducing gut dysbiosis and altering the intestinal barrier.

Shi *et al.* (2021) evaluated the effects of sucralose supplementation on bile acids and the gut microbiota in the context of non-alcoholic fatty liver (NAFL). C57BL/6 female mice fed standard rodent chow were given 0.1 mg/ml sucralose in drinking water for 11 weeks. With this concentration, the authors wanted to achieve the United States FDA ADI (5 mg/kg bw/day). Although food and fluid intake were monitored, no data was provided. Parallel groups were given sucralose with metformin (MET) or prebiotic fructo-oligosaccharides (FOS). Although caecal microbial α-diversity remained unaltered, there were differences in β-diversity among the groups. Sucralose-fed animals showed several altered phyla, including Bacteroidetes, Firmicutes, Verrucomicrobia, Actinobacteria, and Proteobacteria.

At the genus level, sucralose groups had an increased abundance of *Bacteroides* and *Clostridium*. These genera were positively correlated with elevated levels of deoxycholic acid (DCA) found in several samples (faeces, liver, serum). The authors suggested that the DCA accumulation in the liver inhibited the expression of hepatic enzymes leading to non-alcoholic fatty liver disease (NAFL). Also, in the sucralose group there were a number of altered parameters related to hepatic lipid metabolism, including increased total cholesterol, triglycerides and fatty acids. The authors also reported an altered glucose tolerance test (of note: blood glucose among groups was only significant between 15–30 min after the test initiation). In the groups combining sucralose with MET or FOS, most parameters related to caecal microbiota and bile acids were comparable to the control. Although the authors indicated that their findings "highlighted the effects of gut microbiota and its metabolite DCA on sucralose-induced NAFLD of mice", there was no proof of causality.

This study did not describe some methodologies, including the hepatic histological evaluation and glucose tolerance tests. Another reporting limitation was that figure captions did not correspond to the figure content. In addition, the study contained numerous null results for many of the tested parameters that were not discussed.

Thomson *et al.* (2019) conducted a 7-day interventional study (randomized, double-blind) in human healthy male individuals given sucralose in capsules equivalent to 75 percent of the high end of the JECFA ADI (260 mg, 3 times/day). The baseline was determined before the intervention for all parameters evaluated (body weight, glucose response, insulin resistance, microbiota composition and β-diversity). No differences in body weight or glycaemic and insulinaemic responses were observed between the treatment and placebo groups. Also, the treatment did not modify the microbial communities. Interestingly, individuals in the placebo control had a different microbiota baseline with increased Firmicutes and decreased Bacteroidetes (compared to the sucralose group), which correlated with subjects having higher BMI and cholesterol levels. Regardless of whether individuals consumed sucralose or a placebo, those who exhibited a higher insulin response after the intervention showed lower abundances of Bacteroidetes and higher abundances of Firmicutes. Based on this observation, the authors noted that "initial metabolic differences could have been more important than the intervention itself in terms of altering the gut microbiome", an aspect that should be considered in future studies.

The human interventional study conducted by Suez *et al.* (2022) to evaluate commercial sucralose and three other non-nutritional sweeteners has been reported under the saccharin section.

STEVIOL GLYCOSIDES

Annex III.5. contains a summary of the studies evaluating steviol glycosides.

Li *et al.* (2014) evaluated the impact of rebaudioside A in BALB/c mice for 4 weeks. Doses were 5 or 50 mg/kg bw/day, which are 1.25 and 12.5 times higher than the steviol glycoside ADI, respectively. The authors highlighted the limited effect of the sweetener on bacterial growth. Only the high dose increased α-diversity and the abundance of *Lactobacillus* species. Li *et al.* (2014) also conducted an *in vitro* study for 24 hours, in which select bacteria (*Escherichia coli* O157:H7, *Salmonella typhimurium, Staphylococcus aureus, Listeria monocytogenes, Lactobacillus plantarum, Bifidobacterium longum*) were treated with 0.01, 0.1, 0.5, and 1 percent rebaudioside A (w/v) in the media. These tests supported the limited effects of bacterial growth observed *in vivo*. Here, concentrations ≥ 0.5 percent inhibited the growth of *S. aureus*, while they promoted the growth of the probiotic *L. plantarum*.

Nettleton *et al.* (2019) evaluated the effects of 2–3 mg/kg bw/day rebaudioside A provided in drinking water to young male Sprague-Dawley rats for 9 weeks. Two additional groups were treated with 10 percent oligofructose-enriched inulin in the chow, alone or in combination with rebaudioside A. The chow composition used in the control and rebaudioside A was the same but slightly different from the two groups treated with the prebiotic. Moreover, chow composition was modified when rats were 8 weeks old.

Compared to the control, rebaudioside A did not affect body mass, body fat, glucose tolerance and insulin sensitivity. The prebiotic group reduced food intake and intestinal permeability, and improved body composition and insulin sensitivity. While there were no changes in α- and β-diversity, the caecal microbiota of sweetener-treated rats showed changes in the abundance of some bacterial groups compared to the control. These include reduced Clostridiales family XIII and Ruminococcacceae and the increased so-called "beneficial bacteria" *Akkermansia muciniphila, Bacteroides goldsteinii* and *Bacteroides thetaiotaomicron*. These findings were accompanied by increased microbial caecal SCFAs acetate and valerate. The prebiotic groups had a higher impact on the microbiota than the sweetener alone. Interestingly, results showed a reduction in α-diversity, accompanied by an increase in the relative abundance of *Bifidobacterium* and *Lactobacillus*. Some of the microbial alterations found in the prebiotic groups were similar to those observed in the sweetener group but were more pronounced. These included the increased abundance of *Akkermansia muciniphila*, and almost absent Clostridiales family XIII and *Ruminococcacceae*. Despite the increased *Bifidobacterium* abundance, a taxon with a relevant role in the production of SCFA, there was a decrease in the SCFA acetate, valerate, isovalerate, butyrate, and isobutyrate.

The same research group evaluated generational effects of 2–3 mg/kg bw/day rebaudioside A administered in drinking water (also aspartame in a separate group, see aspartame section) in diet-induced obese female Sprague-Dawley rats during gestation and lactation (Nettleton *et al.*, 2020). Mothers were fed a high-fat/high-sucrose diet for 10 weeks to induce obesity. After weaning (week 3), offspring continue on a control diet and water until the end of the study (week 18). Body weight and body fat appeared higher than the control group in females at weaning only (no differences after weaning). Similar results were seen for males, although there were no changes in body weight. According to the authors' interpretation of findings, insulin tolerance was impaired in dams during gestation only. Unlike aspartame, glucose and insulin tolerances did not differ from the controls in the rebaudioside A offspring group. The expression of several genes of the mesolimbic reward pathways was altered in the dams and offspring, which may have influenced the consumption and palatability of food. However, they didn't seem to modify food intake. Regarding caecal SCFA, propionate, butyrate and isobutyrate increased in the obese-rebaudioside A-treated dams compared to the obese-water control group. Quantitative polymerase chain reaction (qPCR) was used on faecal samples to monitor the evolution of select bacteria linked to obesity over time. Caecal microbiota was evaluated by 16S rRNA gene sequencing. Alpha diversity increased only in the dam stevia-treatment group. Dams and offspring (male and female) had a higher abundance Porphyromonadaceae compared to controls. The abundance of *Sporobacter* increased in females and was altered in male offspring. Pooled caecal content from offspring was transferred to an unknown strain of GF mice. After 14 days, the two sweetener groups showed higher body weight and body fat than the control, as well as signs of glucose intolerance. The microbiota of the aspartame group had an increase in *Porphyromonodaceae* family, similar to the sweetener groups (dams and offspring) (Note: Some comments about the limitations of this study are mentioned in the aspartame section).

The study by Becker *et al.* (2020) evaluating stevia and using saccharin as a positive control group is summarized under the saccharin section.

As part of the study mentioned above in the sucralose section, Sanchez-Tapia *et al.* (2020) also treated lean male Wistar rats with 2.5 percent steviol glycosides or 10 percent of the combination steviol glycosides and sucrose in the drinking water (other groups were exposed to sucralose, sucrose, glucose, fructose, honey, brown sugar) and fed either a control diet or a high-fat diet for 4 months. They had one control per diet with no sweetener added to the drinking water. As fluid intake was not reported, the daily steviol glycoside intake was calculated to be approximately 1 000–2 000 mg/kg bw/day, based on an average 500-g rat (taken from the publication chart) and a standard daily fluid intake of 20–40 ml. These doses are between 250–500 times higher than the JECFA ADI. As mentioned above, the HFD significantly impacted the parameters evaluated. Some of the effects observed were also dependent on the sweetener type. In general, the high doses of steviol glycosides seemed to affect the microbiota and physiological parameters to a lesser degree than the high doses of sucralose. Non-caloric sweeteners, including steviol glycosides, led to a decrease in α-diversity based on analysis of faecal samples, and only 4.6 percent explained changes to microbiota variation (β-diversity), compared to 18.4 percent by caloric sweeteners and 30 percent by the HFD. Like sucralose, *Bifidobacterium* was significantly reduced in the steviol glycoside groups, except for the steviol glycosides+control diet group. *Faecalibacterium prausnitzii* increased in both diet groups. Among all treatment groups in the control diet, the relative abundance of *Akkermansia* was highest in the steviol glycosides group, but it was reduced in the presence of HFD. Deep shotgun metagenomics showed that gene richness was lowest with the two non-caloric sweeteners (HFD only). Steviol glycosides led to an immune response favouring an anti-inflammatory scenario (with reduced LPS and pro-inflammatory markers). However, when combined with sucrose, the response was pro-inflammatory. Of the groups fed the control diet, the steviol glycosides group led to the lowest production of SCFAs. When given along with HFD, both steviol glycoside and sucralose significantly reduced body weight. Slight alterations of the glucose and insulin test as well as fasting glucose and insulin were observed only in the combination of sucrose+steviol glycosides, independently from the diet.

Xi *et al.* (2020) evaluated the impact of rebaudioside A on sugar-sweetened beverage-induced non-alcoholic steatohepatitis in an obese mice model (strain and gender not reported). The mice received 194 mg/L rebaudioside A (estimated to be ~10 mg/kg bw/day) in the drinking water while fed an HFD for 15 weeks. The dose was about 2–3 times higher than the JECFA ADI for steviol glycosides. Sucralose and a combination of sucrose and fructose were also evaluated. In addition to the HFD control, there was a group fed control chow. Several host parameters were monitored in addition to the faecal microbiota. In general, rebaudioside A treatment (+HFD) led to a lesser impact on glucose alterations, insulin sensitization, liver dysfunction, and hepatic steatosis compared to the high carbohydrate/HFD or HFD control groups, although not to the level of the control chow.

Also, the impact of rebaudioside A on hepatic fibrosis was lower than that observed in the high-carbohydrate/HFD group. The authors reported a microbiota composition different from the high carbohydrate/HFD group. They observed high inter-individual variability in the number of individual bacterial species and noted that no specific species could explain the complex mechanisms of non-alcoholic steatohepatitis (NASH). However, they suggested the potential beneficial role of the *Akkermansia:Bacteroides* ratio, which was higher in the Rebaudioside A group compared with the high carbohydrate group (sucrosefructose in drinking water). The authors indicated that their preliminary results on the potential role of microbiota diversity on NASH require further investigation and elucidation of underlying molecular mechanisms.

Mehmood *et al.* (2020) evaluated the potential renoprotective effects of stevia extracts on a Kunming mouse model of adenine-induced chronic kidney disease (CKD). During the 3 weeks of study, the sweetener was provided daily via oral gavage at two doses, 200 or 400 mg/kg bw, while the mice were fed on chow containing adenine. The stevia doses were 50 and 100 times higher than the JECFA ADI. Stevia ameliorated the renal condition in a dose-dependent manner, as shown by the improvement of several renal, serum and urinary biomarkers and modulation of the inflammatory response, and it prevented renal fibrosis by suppressing the expression of marker genes. The microbiota of the stevia groups showed no differences in α-diversity only when compared to the normal control (not to the disease control). Stevia seemed to improve the alterations of adenine-induced CKD and changes in the phyla (decreased relative abundance of Bacteroidetes and increased Tenericutes, Proteobacteria and Cyanobacteria) observed in the disease control group. At the family level, the relative abundance of Streptococcaceae and Enterobacteria decreased in the stevia group.

Gatea, Sârbu and Vamanu (2021) studied the effects of stevioside (~4 mg/kg bw/day) on the composition and metabolic activity of faecal microbiota from three healthy children. The research was conducted *in vitro* for 12 months in a bioreactor simulating the colonic tract (unicompartmental gastrointestinal simulator – GIS1). Of note, this study had significant design and methodological limitations. For example, these included lack of methodological details (e.g. unclear doses), absence of a negative control group without stevioside (although comparisons seemed to be conducted against the baseline at time 0, before treatment), and statistical analyses not performed for all variables studied (e.g. SCFA). The microbiota was evaluated by qPCR using primers targeting select microbial groups. While Firmicutes and *Bacteroides* remained constant throughout the study, Actinobacteria, Enterobacteriaceae and *Bifidobacterium* increased, while the abundance of *Lactobacillus* fluctuated over time. SCFA also fluctuated, and there was a drop in the production of lactic acid only in the middle of the study. The authors attributed such changes to a dysbiotic microbiota and altered microbial metabolic function (fermentative capacity). The authors also indicated that changes in the microbial composition and function could explain the altered antioxidant status (i.e. reduced total antioxidant activity and antiradical activity, increase of the inhibition of the lipid peroxidation), observed only in the middle of the study.

Wang *et al.* (2018) observed selective effects of 2.5 percent rebaudioside A (w/v) *in vitro* on the growth of *E. coli* strains. While the sweetener reduced the growth of *E. coli* HB101 by 83 percent, it did not affect *E. coli* K12.

The human interventional study conducted by Suez *et al.* (2022) to evaluate commercial stevia and three other non-nutritional sweeteners has been reported under the saccharin section.

NEOTAME

Annex III.6. contains a summary of the studies evaluating neotame.

Chi *et al.* (2018) evaluated the impact of neotame on the faecal microbiota and faecal metabolome of CD-1 mice. Mice were gavaged daily for four months with a neotame dose of 0.75 mg/kg bw, which is 2.5 times higher than the United States FDA ADI (0.3 mg/kg bw/day) but lower than the JECFA ADI (2 mg/kg bw/day). The sequencing of the region V4 of the 16S rRNA gene of bacteria isolated from faecal samples demonstrated reduced α-diversity and changed β-diversity in the treatment group compared to the control. The abundance of the phylum Firmicutes was reduced, affecting Ruminnococcaceae (*Ruminococcus*) and Lachnosphiraceae (*Blautia, Dorea, Oscillospira*). On the contrary, the abundance of Bacteroidetes increased, which had a major impact on *Bacteroides*. The analysis of the 16S rRNA gene indicated an enrichment of pathways related to amino acid metabolism, as well as LPS, antibiotic and folate biosynthesis pathways. However, the enrichment was reduced in the case of genes related to carbohydrate, fatty acid, and lipid metabolism, as well as butyrate fermentation pathways. The authors speculated that the altered microbiota could be the cause of the altered metabolite profiles, e.g. reduced lipids and fatty acids metabolites and increased cholesterol, campesterol and stigmastanol. Some limitations noted by the authors included the small animal sample size (n=5) and the short exposure period (4 weeks), which may not reflect the current neotame use. The authors also indicated that alternative approaches could have provided more information, e.g. shotgun metagenomics and targeted SCFA analysis. This study did not evaluate effects on the host, other than body weight, which was not affected by the sweetener.

SWEETENER COMBINATIONS

Annex III.8. contains a summary of the studies evaluating sweetener combinations.

Male and female C57Bl/6 mouse pups were exposed to acesulfame-K and sucralose during gestation and nursing until postnatal day 20 (PND 20) (Olivier-Van Stichelen, Rother and Hanover, 2019). Mothers received a combination of the two sweeteners in the food at doses equivalent to 1x and 2x their ADIs (Acesulfame-K: 15 mg/kg bw/day; Sucralose 5 mg/kg bw/day in the United States) from post-coital day until PND 40. The actual food consumption was not reported. Acesulfame-K was detected in the urine of newborns, which, according to the authors, confirmed prenatal exposure to the sweetener. Moreover, both sweeteners were detected in the mother's milk in lower concentrations than in faeces and blood.

Metabolomic analysis revealed dosedependent altered metabolites involved in amino acid, carbohydrate and lipid metabolism. Intermediary metabolites of detoxification pathways were also changed, which was consistent with the down-regulated transcript of enzymes involved in liver conjugation. There were no differences in α- and β-diversity in the mothers' microbiota, with no compositional changes between treatment and control groups. All changes were seen in the pups only, with increased α-diversity, increased Firmicutes (e.g. Clostridiales, Lachnospiraceae and Rumminococcaceae), Firmicutes:Bacteroides ratio and depleted Verrucomicrobia (exclusively *Akkermansia muciniphila*). Therefore, there was no possible transmission of altered microbiota from mothers to children. A decreased correlation between mothers' and pups' microbiomes could suggest the sweeteners as a potential reason for the difference. *A. muciniphila*, which could grow *in vitro* in the presence of sweeteners and was present in dams and pups at birth, did not colonize the gut as expected in healthy individuals. The metabolome also showed the altered abundance of several bacterial metabolites. According to the authors, the findings suggested that the disturbed microbiome in the pups could explain the metabolic imbalance.

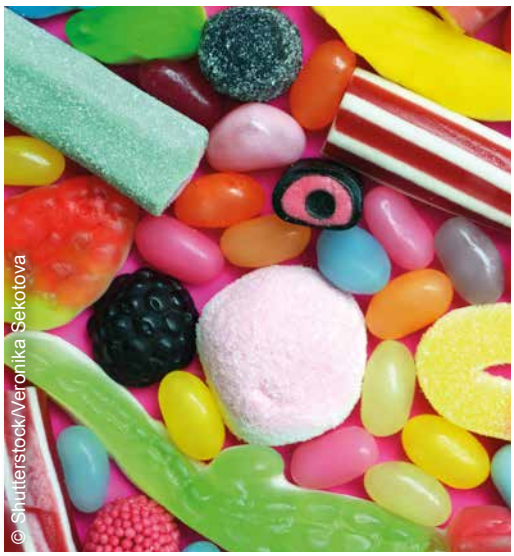
The short-term consumption (4 days) of aspartame and acesulfame-K was evaluated in human subjects by Frankenfeld *et al.* (2015). The results are similar to the findings observed for the individual consumption of acesulfame-K or aspartame previously described, i.e. there were no differences in the composition and function of the microbiome between consumers and non-consumers, except for the β-diversity. In this study, only three individuals consumed the two sweeteners.

An interventional study evaluated the impact of aspartame and sucralose in glucose metabolism and the faecal microbiota of healthy human individuals (Ahmad, Friel and Mackay, 2020a, 2020b). The two sweeteners were provided sequentially for 2 weeks each with a washout period of 2 weeks in between (two groups: aspartame > sucralose and sucralose > aspartame). Doses corresponded to 14 percent of the aspartame ADI (Canada: 40 mg/kg bw/day) and 20 percent of the sucralose ADI (Canada: 9 mg/kg bw/day). These figures correspond to the daily consumption in Canada of approximately three 355 ml cans of beverages (Garriguet, 2008). The authors reported no changes in the faecal microbiota, levels of SCFA, glucose metabolism and insulin sensitivity. They also indicated that the trial period may not have been long enough to observe changes in the microbiota.

Martínez-Carrillo *et al.* (2019) exposed weaned CD-1 mice to sucrose or two commercial sweeteners, Splenda® (unknown sucralose content, dextrin, maltodextrin) and Svetia® (2.5 percent steviol glycoside, 0.6 percent sucralose, sucrose and isomalt) in the drinking water (5 hours/day + 19 h without the sweetener). Based on the information provided, the estimated daily intake of steviol glycosides and sucralose in the Svetia® product was about 7–9 mg/kg bw and 2 mg/kg bw, respectively. The content of sucralose in Splenda® was not specified in this study. After 6 or 12 weeks of treatment, the researchers evaluated parameters related to the immunity of the small intestine and the microbiota of this location (*in vitro* culture: Heart Brain Infusion Agar and Blood Agar). Body mass index (BMI) did not change, and the microbial profiles of identified species differed among groups, including the controls.

Bacillus, followed by *Pseudomonas*, were the genera with the most identified species. Pro-inflammatory cytokines, IL-6 and IL-17A, increased after the consumption of both commercial sweeteners. There were also changes in the abundance of mucosal lymphocyte subsets CD3+, CD4+ and CD8+, depending on the sampling location and sweetener. The researchers did not elaborate on the potential physiological consequences of such changes. Of note: animals on sweeteners, especially Splenda and Svetia®, consumed more fluid than the controls, which should have been considered to estimate the actual sweetener intake.

Mahalak *et al.* (2020) evaluated the impact of a commercial stevia product (Splenda Naturals plus Stevia [SN Stevia] containing erythritol + ~ 1 percent rebaudioside D) or erythritol. The experiments were conducted *in vitro* by culturing select bacteria or human microbiota inoculated in a bioreactor and *in vivo* using a monkey model. Select bacteria strains, including *Escherichia coli, Enterococcus caccae, Lactobacillus rhamnosus, Ruminococcus gauvreauii, Bacteroides galacturonicus,* and *Bacteroides thetaiotaomicron* were exposed for 24 h to SN Stevia (25 µg/ml), erythritol (50 µg/ml), steviol (12.5, 25 and 50 µg/ml), stevioside, reabaudioside A (each at 12.5, 25 and 50 µg/ml) and glucose (concentration not reported). Only *B. thetaiotaomicron* showed increased growth in the presence of steviol, while the other bacterial strains didn't have any significant alterations, including *E. coli*. The second *in vitro* study consisted of faecal microbiota from one human individual (health status not reported) inoculated in bioreactors and exposed either to SN Stevia or erythritol at a dose of 6.2 mg/kg, based on adult average weight (68 Kg). This experiment did not change microbial diversity markers or composition. Regarding microbial function, the treatments increased butyric and pentanoic acids, but bile acid transformation was unaffected. This research was complemented with the *in vivo* evaluation of SN Stevia in one monkey (*Cebus apella*) at a 62 mg/kg dose in drinking water for 2 weeks. There were no control animals in this study. The longitudinal evaluation of the faecal microbiota increased α-diversity and β-diversity, although no changes were observed at low taxa levels (family, genus). This study did not evaluate the effects on the host.



The researchers noted that erythritol was the major component of the commercial sweetener and, therefore, likely responsible for the observed changes in the monkey faecal microbiota. In addition, they suggested the higher dose used in the monkey study as a possible explanation for the differences in microbial diversity observed between the *in vivo* and the *in vitro* (bioreactor) models. However, it should be noted that such disagreements could have also been due to differences between human and monkey faecal microbiotas evaluated in the two models.

Falcon *et al.* (2020) used adult male Wistar rats to compare the effects of low-fat yoghurt sweetened with either a commercial non-caloric sweetener Zero-Cal (0.17 percent sodium saccharin and sodium cyclamate) or 11.4 percent sucrose on the gut microbiota after 17 weeks of treatment. According to the researchers, the amount of sweetener was *equivalent to the daily recommended dose of commercial NNS in adult humans* (no additional information provided). Part of the data of this research (BW and energy expenditure) was published earlier (Pinto *et al.*, 2017). Of note, this study did not have a control group that was not exposed to the evaluated sweeteners. The animals received a standard chow and water *ad libitum*. The findings showed no differences in α- and β-diversities between the two experimental groups. Although caloric intake was similar in both groups, the NNS-consuming animals gained more body weight, probably due to lower energy expenditure at rest (Pinto *et al.*, 2017). The authors rejected the hypothesis that, under experimental conditions, a prolonged intake of commercial NNS is associated with gut microbiota alterations.

Vamanu *et al.* (2019) evaluated several commercial sweeteners individually, including preparations containing sucralose, saccharin, sodium cyclamate and steviol glycosides, in the GIS1 *in vitro* model. This model simulates the human colonic transit. The 500 ml vessels were inoculated with faecal samples from 5 healthy donors and sweetener doses equivalent to two tablespoons of sugar (9 g). Genome copy numbers of select groups of bacteria (Enterobacteriaceae family, *Bacteroides–Prevotella–Porphyromonas* group, *Lactobacillus–Lactococcus–Pediococcus* group, Firmicutes phylum, *Bifidobacterium* genus) were determined by qPCR. In general, except for *Bacteroides*, which decreased only in the presence of one of the three preparations containing steviol, all sweeteners led to significant changes in the different bacterial groups compared to the control. Similarly, all sweeteners led to changes in all tested organic acids (formic, lactic, benzoic phenyllactic and HO-phenylactic acids). Steviol power mainly changed SCFA levels (acetic and butyric acids). NOTE about this study: The study design and methodologies used were unclear and not detailed. For example, the study duration was not reported.

ERYTHRITOL

Annex III.7. contains a summary of the study evaluating erythritol.

In the context of obesity and T2D, two studies by the same research group used erythritol in one of the treatment groups (Han, Kwon and Choi, 2020; Han *et al.*, 2020). However, the main research focus was to evaluate the anti-obesogenic and T2D-protective effects of D-allulose in animals consuming a high-fat diet.

C57BL/6J mice were fed HFD containing 5 percent erythritol or 5 percent D-allulose for 16 weeks in both studies. The two control groups were fed HFD or standard rodent chow. In general, animals given erythritol and HFD were not different from the HFD control. However, the microbiota composition was more similar to that of the mice fed the standard diet than the HFD control. Although some positive effects were induced by erythritol (improved body weight and glucose tolerance), it did not elicit other anti-obesogenic effects (e.g. improved plasma and hepatic lipid profile), and the overall anti-diabetic outcome (improved glucose-, insulin resistance and hepatic glucose-regulating enzymes) seen with the D-allulose treatment.

SUGAR ALCOHOLS

Annex III.7. contains a summary of the studies evaluating xylitol and other sugar alcohols.

Xylitol and other sugar alcohols (polyols) can cause osmotic diarrhea at high concentrations by pulling body water into the intestinal lumen (Mäkinen, 2016). In this case, osmotic diarrhea is not a symptom of disease, but rather a physicochemical response of the intestinal tract to the presence of polyols in the gut lumen, which stops after the elimination of the compound from the diet (Mäkinen, 2016).

Tamura, Hoshi and Hori (2013) evaluated the effects of xylitol supplementation in the intestinal microbiota and isoflavonoid metabolism of mice. CD-1 mice were fed rodent chow containing 5 percent xylitol and 0.05 percent daidzein (an isoflavone) for 28 days. Controls were given 0.05 percent daidzein only. The caecal microbiota was evaluated using a terminal restriction fragment length polymorphism, and it was shown that xylitol decreased the occupation ratio[18] of *Clostridium* cluster XIVa and *Bacteroides*. Daidzein, equol and its precursor dihydrodaidzein were found in the urine of treatment mice at higher concentrations than in the control group. This finding led the authors to suggest that xylitol-induced changes in the microbial population could be responsible for the increased equol[19] metabolism. The researchers also suggested that xylitol and isoflavonoids could synergistically improve bone health, potentially preventing osteoporosis.

Uebanso *et al.* (2017a) evaluated the effects of xylitol on the faecal microbiota and lipid metabolism of young male C57BL/6J mice in two different studies. The first study was conducted for 16 weeks on animals fed a control diet and given xylitol in solution (drinking water) at two dose levels: 40 and 194 mg/kg bw/day. The second study lasted 18 weeks, in which mice were fed an HFD and exposed to the higher xylitol dose only. Specific microbiota members were analysed from faecal samples collected at the study's midpoint (week 7) by qPCR and denaturing gradient gel electrophoresis (DGGE). Changes to the microbiota composition were observed primarily at the higher dose and independent from the diet, with a decrease in the

[18] Occupation ratio: number of specific bacteria/total number of bacteria detected (Rocco *et al.*, 2021, p.2).

[19] Equol is *a metabolite of daidzein produced by intestinal microbiota* (Bowey, Adlercreutz and Rowland, 2003, cited by Tamura, Hoshi and Hori, 2013, p. 23994).

relative abundance of Bacteroidetes, one *Clostridium* species and *Barnesiella*, while two *Clostridium* species and *Faecalibaculum* increased. Increases in Firmicutes and *Prevotella* were observed only in HFD-xylitol-fed animals. The HFD diet was the primary cause of lipid metabolism alterations (e.g. hepatic hypertriglyceridemia and hypercholesterolemia), as seen in both the control and treatment groups consuming an HFD. No changes were observed in glucose tolerance or the expression of inflammation makers, which were evaluated in the second experiment. In addition, there were no or limited changes in the profiles of caecal metabolites. The research group conducted a faecal microbiota transplant (FMT) with donors of the HFD groups only (with or without xylitol). Differences in microbiota composition between the HFD-xylitol and HFD control groups after the transplant were transient and disappeared at the end of the 18-day experimental period. The caecal metabolome did not change between the two groups. However, serum cholesterol was slightly higher in the transplanted HFD-xylitol group compared to the control. The authors attributed this finding to alterations in the microbiota composition induced by xylitol.

In the context of osmotic diarrhoea, Zuo *et al.* (2021) observed dose-dependent effects of xylitol given to male Sprague-Dawley rats for 15 days, which improved by the end of a 7-day clearance period. Xylitol was given by gastric gavage at three different doses: 1, 3, and 10 percent (0.9, 3.15, and 9.9 g/kg bw/day, respectively). The high dose induced diarrhoea with disturbances of colonic microvilli (with inflammatory cell infiltration) and gut microbiota. At this concentration, there was a reduction of the microbial α-diversity. Alterations of the microbial composition – already significant at day 1 of treatment – included an increase in the relative abundance of *Bacteroides*, and decreased Lachnospiraceae, *Alloprevotella*, Ruminococcaceae and Prevotellaceae at the end of the 15-day treatment. The reduction of SCFA-producing bacteria was consistent with the decreased production of acetate, propionate and butyrate. The effects of the medium dose were less severe, including light diarrhoea with the absence of histological and inflammatory alterations and limited changes in the microbial population. No effects were observed at the lowest dose of xylitol tested.

Hattori *et al.* (2021) observed that the gut microbiota exerted a protective effect against sorbitol-induced diarrhoea after giving 5 percent sorbitol in drinking water for 4 days to specific pathogen-free (SPF) and germ-free ICR mice. Only germ-free mice developed diarrhoea. Antibiotics with different antimicrobial spectra were given to C57BL/6J mice to alter the gut microbiota. Only mice with microbiotas disturbed by vancomycin (increased abundance of Enterobacteriaceae – *Escherichia, Klebsiella, Enterobacter, Proteus*) and erythromycin (increased Clostridiaceae – *Lachnosclostridium*) did not present diarrhoea after a 4-day exposure to 5 percent or 10 percent sorbitol in the drinking water. The abundance of these microbial groups was dose-dependent. Additional *in vitro* studies suggested that the Enterobacteria *Escherichia coli*, *Citrobacter farmeri, Klebsiella penumoniae* and *Enterobacter* spp. were involved in the sorbitol degradation. The authors hypothesized that the abundance of sugar-alcohol-degrading intestinal bacteria could be a factor explaining differences in susceptibility to osmotic diarrhoea among individuals.

Other studies have looked into the prebiotic effect of xylitol and the mechanisms involved in the utilization of the sweetener by the gut microbiota. Xiang *et al.* (2021) conducted a study with two components: an *in vivo* model (male C57BL/6 mice) and *in vitro* colon simulation (in the Changdao Moni simulation system – CDMN). For three months, animals were given rodent feed supplemented with 2 and 5 percent xylitol (w/w), corresponding to 2.2 and 5.4 g/kg bw/day, respectively. Based on FDA Guidance to estimate the maximum safe starting dose in clinical trials, these doses were equivalent to 0.18 and 0.44 g/kg bw/day in humans or daily consumption of xylitol of 11 and 26 g (60 kg person). In general, the changes observed were dosedependent. Xylitol did not alter the α-diversity significantly. But some community shifts were observed at the higher concentration (5 percent xylitol), i.e. increased relative abundance of Actinobacteria, Bacteroidetes, *Bifidobacterium, Lactobacillus*, and Erysipelotrichaceae, while Firmicutes, Proteobacteria, *Blautia* and *Staphylococcus* decreased. Lachnospiraceae was not affected. Also, the higher xylitol dose had more pronounced effects on the production of SCFA (increase in propionate) and amino acid metabolism. Xylitol did not alter the host-related parameters evaluated (body and organ weight, colon length). The *in vitro* component of Xiang's research was carried out using human faecal microbiota inoculated in a 3-vessel colonic simulator, which included mucin-coated beads (mucosal beads). For seven days, the microbiota was exposed to 3 percent xylitol (human daily intake: ~0.27 g/kg bw). In general, the sweetener increased the relative abundance of Firmicutes and Lachnospiraceae while decreasing the abundance of Proteobacteria and *Escherichia-Shigella* in the three colonic regions. The abundance of other microbial groups showed more dependency on the intestinal (vessel) section. The fungal community was also evaluated. Xylitol decreased the relative abundance of *Saccharomyces* and increased *Trichosporon*. The study showed that xylitol led to differences in SCFA production between the lumen and mucosa of the *in vitro* system (increased propionate in the lumen and butyrate in the mucosa), which were correlated to specific bacterial groups (e.g. butyrate production correlated with *Bifidobacterium*). The researchers indicated that increases in propionate could lower intestinal pH, which could help restrict the growth of bacteria such as *E. coli*. The metabolome and transcriptome analysis revealed the microbial capacity to utilize xylitol mediated by three different enzymes from some bacteria (*Bacteroides*, Lachnospiraceae). Interestingly, the results from co-culturing specific bacteria (*Lactobacillus reuteri, Bacteroides fragilis* and *Escherichia coli*) suggested that xylitol is utilized by the gut microbiota by cross-feeding[20] mechanisms.

Sato *et al.* (2017) conducted a 24-hour *in vitro* study in which human faecal material (5 healthy male individuals) was cultured with different dietary low-digestible carbohydrates, including xylitol and D-sorbitol. Xylitol promoted SCFA production – of which butyrate accounted for about 50 percent– which was attributed to the increased abundance of *Anaerostipes* spp. (Lachnospiraceae family).

[20] Cross-feeding is the *relationship in which one organism consumes metabolites excreted by the other* (Xiang *et al.*, 2021, p. 1).

The findings resulting from the investigation led the authors to suggest the potential of a synbiotic[21] combination of xylitol (or L-sorbose) and *Anaerostipes* spp. to contribute to improving colonic dysbiosis and potentially ameliorating colonic diseases. Of note: The study and discussion focused only on the production of SCFA by bacteria from a few faecal samples that lacked host data.

Beards, Tuohy and Gibson (2010) conducted an interventional study to evaluate the effects of maltitol alone or in combination with other food additives (polydextrose or resistant starch) on select gut bacteria, SCFA and alterations of intestinal physiology (i.e. stool frequency and consistency, pain, bloating and gas). Healthy individuals consumed chocolate containing the sweetener corresponding to a daily dose of 22.8 g for the first two weeks, 34.2 g for the next two weeks, and 45.6 g for the last two weeks. Stool samples were collected for analysis one day after the end of each separate treatment period. Slight effects on the gut bacteria were observed only after six weeks of treatment (higher dose), which increased the number of Lactobacilli, *Bacteroides*, and Bifidobacteria in all treatment groups. Acetate and propionate were also increased by the treatments. There were no significant intestinal changes. To help reduce the energetic value of confectionery products, the authors suggested the combination of maltitol and polydextrose at a dose of 34.2 g as promoters of key gut bacteria while minimizing abdominal discomfort.

CROSS-SECTIONAL STUDIES INVESTIGATING THE GENERAL IMPACT OF SWEETENERS

Annex III.9. contains a summary of the cross-sectional studies evaluating the general impact of sweeteners.

Ramne *et al.* (2021) conducted a cross-sectional epidemiological study involving 1 371 Swedish participants to evaluate associations between the consumption of sugar-added or artificially sweetened beverages and the gut microbiota composition (evaluated by 16S rRNA [V1–V3] gene sequencing). After applying corrections, only two associations remained valid for the sugar-sweetened drinks (not for artificial sweeteners): (1) negative association with *Lachnobacterium* and (2) positive with the Firmicutes:Bacteroidetes ratio. The authors identified several limitations to this study: (1) the study included beverages only, not other sources (e.g. confectionary foods); (2) measurements of the gut microbiota and diet were performed at a single time point; (3) due to the cross-sectional nature of the study, it was not possible to evaluate causality; (4) limited understanding of confounding factors and the impact of residual confounding; and (5) highly skewed distribution of microbial abundances, which challenges the interpretation of observed associations. The authors concluded that their findings very modestly support the influence of the gut microbiota on the increase of cardiometabolic risk associated with consuming added sugars and sweetened beverages.

[21] Synbiotic agent is defined as a *mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host* (Swanson *et al.*, 2020, p. 687).

Laforest-Lapointe *et al.* (2021) investigated the association between the intake of artificially sweetened beverages (ASB) during pregnancy and the faecal microbiota of offspring (n=50 exposed, n=50 unexposed controls) at ages 3 and 12 months. The mothers' microbiota was not evaluated. The microbial community analysis clustered the samples into four groups with different α- and β-diversities and taxonomical compositions. In one-year-old kids, findings showed the association between maternal ASB consumption and increased BMI, which in turn was associated with microbial community alterations, with several *Bacteroides* spp., which were either enriched or depleted. Although the results suggested the influence of gestational exposure to ASB on the maturation of the gut microbiome during the first year of life, the estimate of such impact was markedly smaller than other drivers, including the infant age, breastfeeding, ethnicity, intrapartum antibiotics and mode of birth. The results also suggested that the increase of BMI in one-year-old infants might be mediated by succinate. The researchers found some limitations to their study: (1) risk of errors in self-reported dietary exposures, inability to distinguish the different artificial sweeteners and consideration of artificial sweetener to beverages only, not in food; (2) maternal diet considered only during gestation (prenatal exposure) but not during the lactation period (post-natal exposure); (3) limited resolution of 16S amplicon sequencing; and (4) physiological parameters not evaluated for a more complete assessment of ASB exposure (e.g. lipid profiles, insulin resistance, weight gain, and so son). Following up on this last point, the authors highlighted the need to investigate further the causal contribution of the infant gut microbiome to the physiological effects (e.g. energy metabolism dysregulation) of artificial sweeteners.

EMULSIFIERS, STABILIZERS, THICKENERS

CARBOXYMETHYL CELLULOSE AND POLYSORBATE 80

Annex III.10. contains a summary of the study evaluating carboxymethyl cellulose (CMC) and polysorbate 80 (P80). CMC and P80 were often evaluated in parallel and influenced by the experimental conditions and findings of Chassaing *et al.* (2015).

In a large study, Chassaing *et al.* (2015) evaluated the impact of CMC and P80 on the gut microbiota, the microbiota pro-inflammatory potential (faecal and serum LPS, flagellin) and metabolic responses in different types of mice. The experiments included different types of mice: wild-type (WT) C57BL/6 and the deficient variants Tlr5- and Il-10-, which are susceptible to developing colitis (Kuhn *et al.*, 1993; Vijay-Kumar *et al.*, 2007). Other mice used in this study were wild-type and germ-free Swiss Webster. Starting at 4 weeks of age, mice received 1 percent CMC or P80 in the drinking water for 12 weeks. Food consumption was monitored for 3 weeks, but not the fluid intake. Both emulsifiers altered the gut microbiota of treated animals – with gender-specific clusters – showing reduced microbial diversity and Bacteroidales. Such alterations were more evident in *Il-10*-deficient mice, showing increased *Clostridium perfringens* and *Akkermansia muciniphila* (mainly with CMC). CMC and P80 led to low-grade inflammation, increased food intake, adiposity and altered glucose homeostasis in WT C57BL/6. The emulsifiers induced more severe alterations in predisposed *Tlr5*- and *Il-10*-deficient mice, including colitis. The altered barrier function in these mice was accompanied by increased proinflammatory microbial markers (flagellin and LPS) and reduced distance between bacteria and the epithelium, which was referred to by the researchers as encroachment. This shortened distance was due to the reduced thickness of the mucus layer, which, in the absence of altered *Muc2* expression, could be explained by the proliferation of mucolytic species (e.g. *Ruminococcus gnavus*). Metabolic alterations were only reported for the *Tlr5*- strain but not in *Il-10*-deficient mice. Similar results were observed when the emulsifiers were given in the rodent chow instead of provided in the drinking water. Dose–response effects were observed after treating mice with 0.1, 0.5 and 1 percent CMC or P80 in the drinking water for 12 weeks, with low-grade inflammation starting at 0.1 percent P80 or 0.5 percent CMC. Mild metabolic alterations were also observed at such low levels, including mild dysglycaemia and increased adiposity. Animal age was also considered, and the same treatments were applied to 4-month-old mice for 8 months, resulting in decreased α-diversity and metabolic alterations, even 6 weeks after treatment cessation. One percent CMC or P80 treatments for 12 weeks also led to altered microbiota and metabolic syndrome in Swiss Webster mice, which, unlike C57BL/6, are considered obesity-resistant (Wong *et al.*, 2007). This treatment was also given to GF Swiss Webster, which didn't develop any of the effects observed in the wild type. However, some effects appeared after these animals received faecal transplants from emulsifier-treated mice, suggesting that the gut microbiota may mediate the effects posed by CMC and P80. Of note, the emulsifiers increased food intake in both wild-type mice, C57BL/6 and Swiss Webster, but not in GF. Bile acids and

SCFAs were evaluated only in WT and GF Swiss Webster, with most alterations observed in WT only. Even though this study evaluated only two out of the many emulsifiers, the authors closed the conclusions with three generalizations by saying:

> (1) *"emulsifiers can disturb the host-microbiota relationship resulting in a microbiota with enhanced mucolytic and pro-inflammatory activity that promotes intestinal inflammation, which can manifest as colitis or metabolic syndrome"; (2) "dietary emulsifiers may have contributed to the increased incidence of inflammatory bowel disease, metabolic syndrome, and perhaps other chronic inflammatory diseases" and (3) "hyperphagia may be driven, in part, by food additives and other factors that might alter gut microbiota and promote low-grade inflammation"* (Chassaing *et al.*, 2015, p. 96).

In a follow-up study, Chassaing *et al.* (2017) also evaluated 1 percent CMC or P80 for 13 days *in vitro* using the mucosal simulator of the human intestinal microbial ecosystem (M-SHIME®) inoculated with faecal material from only one human donor (no further information provided about this individual). The results were similar to those reported *in vivo* (Chassaing *et al.*, 2015). Before treatments started, the microbiota α-diversity decreased to 50 percent during the stabilization period. P80, but not CMC, changed the microbiota composition and both emulsifiers increased the pro-inflammatory potential of the microbial community, characterized by increased transcription of flagella genes, and active flagellin (faster production by CMC than P80). In addition, the proinflammatory cytokine IL-6 increased in $RAG^{-/-}$-deficient mice[22] administered M-SHIME® luminal suspension intraperitoneally. When evaluating a range of doses 0.1, 0.25, 0.5 and 1 percent in the M-SHIME® system, flagellin was detected at all doses (except the lowest CMC) but not following a dose-dependent relationship. P80 also increased LPS levels, primarily at higher doses. SCFA and BCFA levels were not affected *in vitro*. In an additional experiment, 3–4-week- or 5–10-week-old germ-free C57BL/6 mice were transplanted with M-SHIME® luminal microbiota suspension, and effects were evaluated 12–13 weeks post-transplant. After 12 weeks, the α-diversity changed in both P80 and CMC groups, and the microbiota showed an enrichment of Proteobacteria and Enterobacteriaceae, which are associated with inflammatory processes, and decreased Bacteroidaceae, compared to the control group (water). The rest of the findings were consistent with those observed in M-SHIME and previous *in vivo* studies (Chassaing *et al.*, 2015), i.e. increased pro-inflammatory microbiota (flagellin, LPS), microbiota encroachment, low-grade inflammation and what the authors considered an indication of metabolic syndrome (higher fasting glucose levels). The research team attributed these changes to the emulsifier-altered microbiota. When fed an HFD, mice transplanted with microbiota from emulsifier-treated M-SHIME® developed higher body weight, fasting blood glucose, and inflammation than the water control group. Food and fluid intake was not monitored in this study. In a separate study, the same treatment (1 percent CMC or P80 in the drinking water for

[22] $Rag^{-/-}$ mice lack mature B and T cells are necessary for adaptive responses (e.g. to bacteria and bacterial components) (Chassaing *et al.*, 2017), therefore leaving these animals with a compromised immune system.

11 weeks) provided to Altered Schaedler Flora (ASF)[23] C57BL/6 mice (mice with a synthetic microbiota community composed of eight different bacteria species) did not elicit microbial or host alterations. According to the authors, these results suggested the need for a complex microbiota to mediate the emulsifier effects observed *in vivo* and *in vitro* (M-SHIME®). They also suggested the microbiota as a direct target of CMC and P80 and highlighted the usefulness of *in vitro* systems like the M-SHIME® model to evaluate the mechanisms involved in the effects of compounds like CMC and P80 on the microbiota.

Of note: In these two studies (Chassaing *et al.*, 2015; Chassaing *et al.*, 2017), both male and female mice were used to evaluate the gender factor. However, there was no discussion about it, possibly due to the lack of significant differences. Regarding the doses, in the absence of data on fluid intake and unclear body weight increases (as they are reported as relative, not absolute, values), the daily intake estimate corresponding to 1 percent CMC or P80 would be > 1 000 mg/kg bw, which is higher than reported consumption estimates for these compounds (EFSA, 2018b; Shah *et al.*, 2017; Vin *et al.*, 2013).

Based on these results in mice and *in vitro* (Chassaing *et al.*, 2015; Chassaing *et al.*, 2017), Chassaing *et al.* (2021) conducted a short interventional trial (11 days) using a daily dose of 15 g CMC on 16 human health individuals (nine controls, seven treatment). The authors acknowledged that this dose, which is lower than those evaluated in mice and *in vitro*, is likely higher than a typical daily intake in most individuals. All subjects received a CMC-free diet for 3 days preceding the treatment. The comparison between treatment and control led to non-statistically significant (null) results for many of the host parameters evaluated (e.g. body weight, serum inflammatory cytokines, gut permeability and glycaemic response). The researchers reported that interindividual variation had a higher impact on the microbiota composition than the effect of short-term diet variation. Normalized data showed greater changes in the microbial composition of CMC-treated individuals, with no changes at phylum and order levels. At a lower taxonomical level, there was a decrease in the relative abundance of *Faecalibacterium prausnitzii* and *Ruminococcus* spp. and an increase of *Roseburia* spp. and Lachnospiraceae, with unknown functional consequences, according to the authors. In addition, shotgun metagenomic showed some altered microbial metabolic pathways. The faecal metabolome showed some alterations in the CMC-treatment group, such as decreased SCFAs or essential amino acids, which returned to baseline after the treatment. Unlike the results from the studies *in vitro* (Chassaing *et al.*, 2017) and *in vivo* (Chassaing *et al.*, 2015), the treatment in humans did not influence the levels of LPS, flagellin, or the inflammatory marker lipocalin-2. High amounts of CMC were detected intact in faeces, while it was undetectable in the urine of treated individuals.

[23] Synthetic microbiota community composed of eight different bacteria species belonging to three different phyla: (1) Firmicutes: *Clostridium* sp. (ASF356), *Clostridium* sp. (ASF502), *Lactobacillus intestinalis* (ASF360), *Lactobacillus murinus* (ASF361), *Eubacterium plexicaudatum* (ASF492), Firmicutes bacterium (ASF500); (2) Bacteroidetes: *Parabacteroides* sp. (ASF519); and (3) Deferribacteres: *Mucispirillum shaedleri* (ASF457).

Encroachment of gut microbiota in the inner part of the mucus layer closer to the epithelium (associated with chronic inflammatory diseases) was only observed in two individuals from the CMC treatment group. These same subjects (men, oldest in the CMC group) showed greater disturbances of the microbial composition and levels of faecal LPS, while other clinical markers studied did not differ from the controls. Based on these results, the authors suggested that some individuals may be more sensitive than others to CMC, although long-term effects need to be evaluated. The authors also suggested the need for larger studies with longer follow-ups and in determining underlying mechanisms.

The same research group expanded the *in vivo* study led by Chassaing *et al.* (2015) to evaluate the role of CMC and P80 in intestinal inflammation and colon carcinogenesis (Viennois *et al.*, 2017). In the first stage of the study, 1 percent CMC or 1 percent P80 were given to C57BL/6 mice in drinking water for 13 weeks. Of note, most parameters were evaluated in samples obtained in week 9, except histological evaluations conducted from samples collected in week 13. Afterwards, colitis-associated cancer was induced by treating the animals with the carcinogen azoxymethane (AOM) followed by dextran sodium sulfate (DSS). The gut microbiota was evaluated only before the induction of cancer. Microbiota composition analysis revealed a strong clustering following treatment. α-Diversity decreased after 9-week P80 and CMC treatment, and changes in bacterial community composition included an increase in the relative abundance of Bacteroidales (Bacteroidetes) and a decrease in Firmicutes members (e.g. Clostridiales, *Lactobacillus*). No alterations were observed for γ-Proteobacteria, Enterobacteriaceae, *Escherichia coli*, or colibactin-related genes (a pro-carcinogenic toxin). The number of tumours per mouse was higher in the P80 and CMC groups treated with AOM-DSS compared to the control AOM-DSS group control. P80 or CMC had limited effect on the expression of pro-inflammatory makers, affecting only the expression of 1 or 2 chemokines in the AOM-DSS or control treatment groups. Inflammatory markers (MPO and Lcn2), flagellin and LPS were elevated before cancer induction with limited differences after cancer-colitis induction (Lcn2 increased with P80, and flagellin with CMC). Although cell proliferation and apoptosis increased in all groups after AOM-DSS treatment, it was also observed in the P80 and CMC-groups before cancer induction. The authors investigated further the impact of P80 and CMC in apoptosis, cell proliferation and the potential role of the microbiota. However, this experiment used a different mouse strain, Swiss Webster (wild-type and germ-free), which followed the same experimental conditions of the previous experiment. Like C57BL/6 mice, only some genes related to cell proliferation (but not apoptosis and angiogenesis) were dysregulated in the CMC and P80-treated WT Swiss Webster, but not in GF. These results were partially observed after FMT, mainly in the P80 group, leading the authors to suggest that the emulsifier-altered microbiota (not directly evaluated) is a pre-requisite and sufficient to drive perturbations in proliferation and apoptosis processes (of note: apoptosis-related gene expression showed no significant results, and the histological evaluation to show signs of apoptosis was not conducted in this group of mice).

Since dietary induction of cancer is a sensitive and controversial topic, some comments are highlighted at this point in this review, describing some study challenges and limitations:

> This study contains numerous null results, which are understated and not integrated into the broader evidence supporting the findings and conclusions.

> The biological relevance of statistically significant results remains unclear.

> P80 and CMC are given for 13 weeks prior colon cancer-colitis induction with AOM-DSS. Except for histological evaluations, all other parameters are analysed/reported with samples from week 9 (not week 13). These are used as a baseline to compare results from before and after colon cancer-colitis induction.

> The large variability shown in the figures would have been more informational in showing the distribution of individual data points.

> Authors often generalize results in the text, not distinguishing differences in significance between P80 and CMC for a given parameter.

> No justification for using two different mouse strains.

> Microbiota was not evaluated after AOM-DSS-induced carcinogenesis in C57BL/6 mice.

> Also, researchers did not evaluate the microbiota of Swiss Webster mice. Therefore, they could only assume that the microbiota was altered when they stated that the altered microbiota was sufficient and that it was a prerequisite to drive apoptosis and proliferation processes after conducting FMT experiments.

> Methodology indicates that animals were kept in *Helicobacter*-positive rooms (except for GF mice). This could open the possibility for an environmental infection, which could interfere with the reliability of research outcomes (Chichlowski and Hale, 2009; Kim *et al.*, 2022). However, no further analysis or comment was made by the authors targeting this opportunistic pathogen. The infection of mouse colonies with *Helicobacter* spp. is raising concerns among the scientific community due to its potential to confound research results, as they have shown to be involved in inflammation and associated with different cancer types (Chichlowski and Hale, 2009; Kim *et al.*, 2022).

> The authors did not state any study limitations.

The same research team expanded their research to evaluate the impact of dietary intake of CMC and P80 in the development and progression of spontaneous intestinal adenoma (Viennois and Chassaing, 2021). The parameters evaluated and methodologies used were similar to the ones reported in their previous studies. A concentration of 1 percent CMC or 1 percent P80 in the drinking water was provided to male and female C57BL/6 WT and APC^{min} mice for 15 weeks. APC^{min} mice are susceptible for the development of spontaneous intestinal adenomas and are used to simulate human familial adenomatous polyposis and colorectal tumours (Mouse Models of Human Cancer, 2022).

All genetically predisposed mice (APC^{min}) developed intestinal adenomas (with limited inflammatory changes), especially in the small intestine, where the tumour number and size were larger in groups consuming CMC or P80. Cell proliferation was statistically increased in all treatment groups in both the small intestine and colon, except in the small intestine of the WT-CMC group. Microbiota disturbances were greater in AP^{Cmin} than WT mice after CMC and P80 consumption. In WT mice, both genders showed a decrease in Actinobacteria, while other changes were gender-specific. APC^{min} had a decreased relative abundance of Clostridia in both genders, with increases in Proteobacteria in males. Treatment male APC^{min} groups were the only ones showing differences in β-diversity compared to the control group. Contrary to what was shown in previous research reports by the team of Viennois and Chassaing, pro-inflammatory microbiota markers were not altered, i.e. faecal LPS and flagellin levels. Only female groups exposed to P80 showed elevated LPS values. The researchers concluded that CMC and P80 could be risk factors for colorectal cancer, pointing at alterations of gut microbiota-host interactions as an influential factor in gastrointestinal carcinogenesis in individuals with genetic predisposition to these types of disorders. Although not mentioned by the authors, one major limitation of the study was the variable sample size among different groups in each test and the low sample size in many of them, even as low as two or three samples per group in several instances.

In the same line of research and using similar methodologies, Viennois *et al.* (2020) conducted a series of experiments using GF C57BL/6 mice or GF C57BL/6 colonized with Altered Schaedler Flora (ASF). This study aimed to evaluate if chronic exposure to CMC or P80 targeted microbiota pathobionts (here adherent-invasive *E. coli* LF82 or adherent invasive *E. coli* [AIEC]) and could potentially promote an inflammatory response. AIEC has been associated from individuals with Crohn's disease (Barnich and Darfeuille-Michaud, 2007). Mice described above were colonized with AIEC in the drinking water for 1 week, followed by a 12week administration of 1 percent CMC or 1 percent P80 in the drinking water. The consumption of fluid and food was not monitored. The control group was also exposed to the pathogen but not to the emulsifiers (there was no control without the pathogen). In many analyses, the two emulsifiers led to multiple differing results. For the AIEC-ASP mice, the authors reported an increased susceptibility to emulsifier-induced low-grade inflammation based on the statistical significance of some features (i.e. colon length and weight, expression of two – IL-1β, IL-10 – of the cytokines evaluated), histological alterations, and glucose tolerance test. Many indicators were evaluated on days 28 and 56 of exposure, with no mention of whether they were also evaluated at the end of treatment. Compared to the control, only the CMC group had significantly elevated levels of lipocalin-2 and flagellin in some of these mid-points, and increased expression of the Lypd8[24] gene at the end of treatment.

[24] Lypd8 is a protein produced by intestinal epithelial cells that binds to flagellated microbiota, limiting the bacteria's motility and their access to the inner mucus layer, therefore preventing the invasion of colonic epithelium (Okumura *et al.*, 2016).

The abundance of AIEC remained unaltered but encroached in the inner mucus layer, while the ASF composition changed, with a complete loss of *Clostridium* members and clear clustering of the P80 group. Similar results were observed in GF and GF-IL-10-deficient C57BL/6 mice subjected to the same treatment with AIEC, P80 and CMC. The researchers introduced an additional control group (not colonized with AIEC) in the study using IL-10-deficient mice. This experiment contained numerous null results and no changes in lipocalin-2. The evaluation of the transcriptome of AIEC isolates (*in vitro*) showed a dose–response increase in the expression of virulence factors (e.g. flagella and fimbriae-related genes) after exposure to CMC. The collection of findings led the authors to conclude that the pathobiont AIEC was sufficient to increase the susceptibility of mice to emulsifier-induced intestinal inflammation. Doses used were 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016 percent. These *in vitro* studies also showed increased adhesion capacity (not invasion) when exposed to both CMC and P80, at concentrations of 0.25 percent and above. By colonizing GF mice with or AIEC- Δ*fliC* mutant, the impact on inflammation was limited, which authors used to suggest the involvement of flagella in promoting inflammation after the treatment with emulsifiers. The same scientific publication included the results of one last experiment using a model of colitis-associated cancer. Similarly, GF C57BL/6 mice were first colonized with AIEC for one week via drinking water, followed by a 4-week treatment with 1 percent CMC or 1 percent P80, before inducing cancer with AOM (intraperitoneal 10 mg/kg bw). After 5 days, animals received two 7-day DSS cycles, separated by two weeks. The experiment finished 3 weeks after the second cycle. In this case, there was one control only (i.e. mice receiving the pathogen not exposed to the emulsifiers). The CMC group developed a higher number and larger tumours than water or P80. However, epithelial cell proliferation was higher in the P80 group, while cell apoptosis did not differ between groups (but observed in animals consuming emulsifiers, no AOM/DSS). No study limitations were mentioned for this study.

In a later study, the same research team conducted a study *in vitro* using a MiniBioReactor Array (MBRA) model inoculated with faecal material from only one individual to evaluate the impact of 20 food additives on the microbiota, microbial pro-inflammatory compounds (LPS and flagellin) and gene expression by metatranscriptomic analysis (Naimi *et al.*, 2021). The additives were selected based on their properties to function as emulsifiers, although some of them are primarily used by the industry for other purposes (e.g. thickeners and stabilizers). These included: Sodium carboxymethylcellulose (CMC, E466), polysorbate 80 (P80, E433), soy lecithin (E322), sunflower lecithin (E322), propylene glycol alginate (E405), agar agar (E406), iota carrageenan (E407), kappa carrageenan (E407), lambda carrageenan (E407), locus bean gum (E410), guar gum (E412), gum arabic (E414), xantham gum (E415), diacetyl tartaric acid ester of mono- and diglycerides (DATEM, E472e), hydroxypropyl methyl cellulose (HPMC, E464), sorbitan monostearate (E491), mono- and diglycerides (E471), glyceryl stearate (E471), glyceryl oleate (E471) and maltodextrin (E1400). Three days after inoculating the gut (faecal) microbiota in the mini reactor, 0.1 percent of each food additive was added to its corresponding vessel, and the exposure was carried out for 6 days, followed by a 3-day clearance period. The authors reported that most compounds led to "detrimental" effects, and they differed on the extent of the outcome. Soy Lecithin and mono- and diglycerides were the only emulsifiers that did not impact any of the parameters tested. At the other end, the authors reported significant detrimental effects led by some carrageenans (especially kappa carrageenan), gums (especially guar gum), and glycerol stearate, affecting the microbial load, microbiota composition and the expression of pro-inflammatory compounds, flagellin in particular, and other genes. Limited effects were seen for CMC and P80.

Although the authors reported "detrimental" effects of some of the emulsifiers on the microbiota, it is challenging to support their argument based on the limited evidence they provided. For example, the discussion ignores the possibility of utilization of the additives (e.g. carrageenans and gums) by the gut microbiota, which could favour the proliferation of some microbial groups and the expression of genes involved in these processes. This argument is supported by a clear upregulation of certain pathways in gut microbiota members, i.e. *Ruminococcus* and *Bacteroides*, previously reported to be involved in the uptake and breakdown of glycans, including gums (e.g. xantham gum) and carrageenans (La Rosa *et al.*, 2022; Zheng, Chen and Cheong, 2020). Therefore, changes in the abundance of certain taxa and changes in the transcriptome may not necessarily imply a negative or relevant outcome.

When looking at the data provided by the authors (more comprehensive in the supplementary files), concerns arise based on how the data has been processed and presented. Non-normalized data obtained for each emulsifier at the different time points show no or limited statistically significant results. Significant differences appear only after data (bacterial load, diversity, evenness, flagellin, LPS) undergo a two-step normalization: the measurement for each emulsifier relative to (1) the control and (2) the measurement of the emulsifier at the 24-hour time point.

A major issue related to selecting the 24-hour time point as a reference is that the microbiota had not yet reached stability after inoculating the mini bioreactors with the faecal material. Microbiota stability is reached later at 48–72 h. Therefore, it should have been more appropriate to use the 72h time point, or a later point when the microbiota stabilizes, as a reference for data normalization. Another limitation of the study is the use of faecal microbiota from a single healthy but uncharacterized individual.

A further expansion of the initial study by Chassaing *et al.* (2015) was conducted by Holder *et al.* (2019). In this case – and based on the hypothesis that gut microbiota and intestinal health could influence behaviours – the research focused on evaluating behavioural patterns in male and female C57BL/6 mice given 1 percent CMC or 1 percent P80 in drinking water for 12 weeks. No water or fluid intake was monitored. The authors reported that the development of chronic intestinal inflammation (based on changes in the colon length and size), increased adiposity and microbiota disturbances were dependent on gender and the emulsifier tested. Similar dependencies were observed for changes in behavioural patterns. Of note, not all parameters evaluated resulted in significant alterations. Although microbial compounds have been reported to influence the gutbrain axis (Morais, Schreiber and Mazmanian, 2020), functional microbial activity was not evaluated in this study.

Rousta *et al.* (2021) also evaluated CMC and P80 in a germ-free 129SvEv IL-10$^{-/-}$ mouse model of colitis colonized with pooled faecal material from three individuals diagnosed with different inflammatory bowel diseases. The treatment was similar to the one described by Chassaing *et al.* (2015), i.e. 1 percent CMC or 1 percent P80 in drinking water, but for a shorter period (4 weeks). Food and fluid intake were not monitored. Although CMC led to some alterations, P80 did not differ from the control group in all tests performed, except for the histological evaluation of colitis, showing even a lower score than the control group. In this study, the effects of P80 or CMC on the microbiota – evaluated by shotgun metagenomics – were limited, with decreases in some members of the viral population (phylum Uroviricota, primarily the bacteriophages Caudoviricetes) after CMC exposure. Carboxymethyl cellulose (CMC) also led to increased histologic inflammation scores in the large intestine (only cecum and rectum) and alterations of some of the tested inflammatory biomarkers (increased Lcn2 and cytokine – IFN-γ and IL-12p40 – gene expression, but not detected in serum). Based on these findings, the authors concluded that CMC exposure, but not P80, could exacerbate inflammation in susceptible individuals.

The next three studies evaluated the impact of P80 only. In addition to the gut microbiota, Singh, Wheildon and Ishikawa (2016) evaluated the development of intestinal inflammation and liver dysfunction in C57BL/6 mice (gender and age not specified). P80 was given to the mice via gavage at an unclear dose ("1 percent per kg") for 4 weeks. It is also worth noting that it was challenging to evaluate the methodology because it was incomplete. Many parameters tested resulted in statistically significant alterations. These include increases in adiposity, altered glucose homeostasis (fasting hyperglycemia and insulin intolerance), intestinal

dysfunction (increased permeability), signs of low-grade inflammation (increased markers Lcn2 and myeloperoxidase), hepatic dysfunction (steatosis, elevated hepatic enzymes). The faecal microbiota, evaluated by sequencing the regions V1–V4 of the 16S rRNA gene, showed an increase in gram-positive bacteria strains, as reported by the authors, who suggested their role in promoting non-alcoholic fatty liver disease (NAFLD). Microbiota changes included increased Porphyromonadaceae, *Campylobacter jejuni* and *Helicobacter* and decreased *Bacteroides*. Of microbial compounds tested, SCFA (acetate, propionate, butyrate) decreased, while serum deoxycholic acid and LPS and flagellin in faeces and serum (methods not described) were significantly higher than levels found for the controls.

In another study, C57BL/6 male mice were given 1 percent P80 in drinking water for 8 weeks (Furuhashi *et al.*, 2020). Compared to the control group, microbial α-diversity was significantly lower in the small intestine but not in the colon, while β-diversity was different between the two groups and in both locations. Based on these findings, the authors conducted a second experiment to evaluate whether P80 (same treatment condition as before) makes the small intestine more vulnerable to the nonsteroidal anti-inflammatory drug (NSAID) indomethacin (5 mg/kg bw) given intraperitoneally in the last two days of P80 treatment. While P80 alone did not result in histological alterations or changes in the expression of the cytokine IL-1β, it exacerbated the ileitis and cytokine expression caused by indomethacin. Regarding the microbiota, γ-Proteobacteria was the only phylum impacted (increased) in mice treated with P80, with or without indomethacin. *Bacteroides* decreased in the P80 group. An additional investigation was conducted *in vitro* to further characterize γ-Proteobacteria alterations. In the homogenized ileum, *Proteus mirabilis* was identified in the P80 and P80-indomethacin groups. *P. mirabilis*, cultured in a medium containing 0.02 percent P80 for 6 h, did not proliferate, but colonies grew in diameter, which authors explained as an increase in motility. Ileitis caused by P80+indomethacin was reversed by antibiotics, suggesting the involvement of the altered microbiota in the intestinal inflammation.

Li *et al.* (2020b) evaluated if a short (7-day) treatment with 1 percent P80 (gavage, ~100 mg/kg bw based on a 20 g mouse) could exacerbate radiation-induced enteritis in male C57BL/6 mice. Animals underwent radiation after P80 treatment and were monitored for 30 days. The statistical power of this study was estimated as 80 percent with 12 animals per group. However, only a fraction of animals was included in some tests. For example, the microbiome evaluation was based on n=5. The 7-day treatment with P80 (before the radiation treatment) had a limited impact compared to the longer studies by the other research groups mentioned above. Although the intestinal epithelium did not show histological damage, the expression of some markers of epithelial integrity and some pro-inflammatory markers increased. Compared to the baseline (before treatment), the microbiota displayed some changes, including a reduced number of species, increased abundance of *Lactobacillus* and reduced *Allobaculum*. Of note is that the microbiota was not compared to that of a control group in this experiment. P80 worsened the effects of the seven-day radiation treatment, including reduced survival rate, colon length, histology of the small

intestine, levels of intestinal proinflammatory cytokines and expression of genes related to intestinal integrity. The radiation alone did not affect diversity – which was altered in the radiation+P80 group – but reduced the relative abundance of one Lachnospiraceae group while increasing *Bacteroides*. Radiation+P80 increased the relative abundance of Bacteroidetes, including *Rikenella* and Firmicutes members such us *Lactobacillus, Roseburia*, and *Anaerotruncus*. However, there was a decrease in the Proteobacteria *Parasutterella* and the Verrucomicrobia *Akkermansia*. Additional studies were conducted to further evaluate the potential role of the microbiota in the aggravation of radiotherapy by P80. First, C57BL/6 mice were transplanted with stools from P80-treated mice and then radiated. Of note, the researchers did not specify if transplanted mice were germ-free; also, the control group of this experiment was not transplanted with the stools from the control group (non-P80-treated mice), but just received saline instead, making comparisons difficult. In addition, the microbiota was not evaluated. Only two out of the several assessed parameters were altered. These include a shortening of colon length and reduced expression of an intestinal integrity maker. After the radiation treatment, P80-treated animals received an antibiotic cocktail to eliminate the microbiota. The researchers expected an improvement of the alterations. However, except for a slight improvement of inflammatory markers, the antibiotics aggravated some parameters, including BW, colon length, and expression of intestinal integrity markers. The authors suggested that using antibiotics might not be suitable for mitigating the effects resulting from the combination of radiation and P80. Finally, the decreased butyrate levels in the radiated+P80 mice led the authors to investigate the therapeutic effect of butyrate supplementation to minimize the impact of radiation+P80. After radiation, 10 consecutive days of butyrate treatment (7.5 mg/mL, gavage) was shown to ameliorate inflammatory markers and epithelial damage and recovered the bacteria composition.

Another study evaluated if maternal exposure to 1 percent P80 influenced the microbiota, intestinal homeostasis and susceptibility of F1 generation to colitis (Jin *et al.*, 2021). Female C57BL/6 mice were given 1 percent P80 in the drinking water 3 weeks before mating until weaning. Offspring were evaluated at weeks 3 (weaning, seven animals/group were euthanized at this point) and week 8 (five animals euthanized). After weaning, the animals received water with no P80. At the end of treatment, colitis was induced by DSS in the remaining mice. At week 3, the treatment group showed: (1) perturbed intestinal development (reduced length and depth of intestinal villi and colonic crypts, respectively, increased proliferation of ileal cells and reduction of goblet cells in the colon); (2) disrupted intestinal barrier (reduced transcription of MUC2 and expression of ZO1, CLND3) and intestinal low-grade inflammation (reduced sIgA, increased transcription of pro-inflammatory cytokines) in the absence of microscopic inflammation and changes in body weight. Similar results were observed after conducting FMT into C57BL/6 mice previously treated with an antibiotic cocktail. These endpoints were not evaluated at week 8. DSS-induced colitis was more severe in mice from the P80 group, leading the authors to suggest that P80 might increase the susceptibility of animals to colitis in

adulthood. Regarding the microbiota, the authors reported the development of gut dysbiosis in the P80 group. Despite the differences in β-diversity, α-diversity did not differ between groups at a given time point (3 or 8 weeks) but differed within each group between the two check time points. Some differences were observed in the microbiota composition. At week 3, results from faecal samples from the treatment group showed increased *Bacteroides*, whereas *Alloprevotella*, *Clostridium* XIVa, and *Alistipes* decreased. Taxa associated with IBD and inflammation – Proteobacteria, Desulfovibrionales and Helicobacteraceae – remained elevated at 3 and 8 weeks. The authors speculated with the possibility that the alterations observed in the offspring resulted from vertical transmission of dysbiotic microbiota from mothers exposed to P80, given the unlikelihood of intact P80 present in the milk. However, the microbiota and other endpoints were not evaluated in mothers. Although there were male and female mice in F1, gender-dependent effects were not reported in this study.

Five different emulsifiers – CMC, P80, soy lecithin, sophorolipids and rhamnolipids – were evaluated *in vitro* in batch fermenters in the presence of individual faecal microbiota from ten human donors (eight omnivores, one vegan and one vegetarian) (Miclotte *et al.*, 2020). Sophorolipids and rhamnolipids are natural emulsifiers of microbial origin that have not been approved yet as food additives. The doses, 0.005, 0.05 and 0.5 percent (mass/volume), were selected based on the maximum legal concentration in food products by the European Food Safety Authority (EFSA) and the United States FDA. The microbiota composition, predicted metagenome (microbial function), SCFA production and flagellin were monitored for 0, 24 and 48 h. Microbial shifts and changes in SCFA profiles were donor-, dose-, and emulsifier-dependent. Treatments increased the abundance of *Escherichia/Shigella* and *Bacteroides*, while they decreased *Faecalibacterium* and *Prevotella*.

Stronger effects (e.g. lower total and viable cells, SCFA profiles) were caused by sophorolipids and rhamnolipids (followed by soy lecithin), which were attributed to their higher emulsifying capacity. Limited effects were observed in the CMC and P80 groups. Although the predicted metagenome indicated an increase in the levels of motility genes; this result was in discordance with flagellin levels. The researchers highlighted the need to validate these results using *in vivo* models. Such experiments could lead to different outcomes due to the interaction of the emulsifiers with food components and the influences of physiological conditions and activities of the gastrointestinal tract.

MONO AND DIGLYCERIDES OF FATTY ACIDS

Annex III.11. contains a summary of the study evaluating mono and diglycerides of fatty acids.

Jiang *et al.* (2018) fed male C57BL/6 mice with basal (low-fat) rodent chow containing 150 mg/kg glycerol monolaurate (GML) for 8 weeks. Our estimation of the daily intake is approximately 22–26 mg/kg bw. The authors reported microbial dysbiosis in the GML group, with alterations in β-diversity but not in α-diversity. The more relevant finding was the decreased abundance of Verrucomicrobia phylum in the GML group. At the genus level, the relative abundance of *Akkermansia muciniphila* and *Lupinus luteus*[25] decreased, while *Roseburia, Turicibacter, Escherichia coli* and *Bradyrhyzobium* increased.[26] The authors claimed that GML induced metabolic syndrome in the host, based on increased body weight, body and epididymal fat, triglycerides, LDL and decreased HDL. However, fasting insulin and the homeostasis model assessment-insulin resistance index (HOMA-IR) did not differ from the control group. Of note, food intake was significantly higher in the GML group. In addition, the authors also reported a significant increase in serum LPS levels in the GML group as well as low-grade inflammation based only on increased levels of circulatory pro-inflammatory cytokines (IL-1β, IL-t and TNF-α).

Later, the same research group also evaluated the impact of glycerol monolaurate (GML) on the metabolism and gut microbiota of male C57BL/6 mice fed a high-fat diet (HFD) (Zhao *et al.*, 2019). Mice received the HFD for 10 weeks before they were given the same feed supplemented with 150, 300 and 450 mg GML/kg for 10 additional weeks. Our estimation of the daily intake was approximately 22, 44, and 66 mg/kg bw, respectively. This study included two control groups, one given standard rodent chow, and one on the HFD. In general, supplementation with GML ameliorated some of the effects of HFD in a dose–response fashion, with the high dose leading to more noticeable results. Although body weight gain, caloric consumption and glucose intolerance did not differ between the HFD groups, GML improved several alterations induced by the diet, including body and liver fat, elevated serum LPS and TNF-α levels, hyperlipidemia and HOMA-IR.

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25 *Lupinus luteus* is a plant species, not a bacteria.

26 *Bradyrhyzobium* is a gram-negative soil bacteria.

Regarding the gut microbiota, α-diversity did not differ between the HFD groups, but controls and treatment groups clustered separately in the β-diversity analysis. GML, especially the high dose, reverted some of the changes in the microbial community composition caused by HFD. These included an increase in the abundance of Verrucomicrobia, *Akkermansia, Bifidobacterium, Lactobacillus,* and *Bacteroides uniformis* and a decrease in *Lactococcus, Flexispira* and *Escherichia coli*. In addition, changes in the gut microbiota were correlated with the metabolic outcomes observed in the HFD-fed mice. Although the authors highlighted the beneficial effects of GML (450 mg/kg), they indicated the need for additional research to optimize the dose.

The same research group conducted another study to evaluate the dose–response effects of 4-month GML treatment (400, 800 or 1 200 mg/kg supplemented in standard rodent chow) on the gut microbiota, intestinal barrier function, glucose and lipid metabolism and inflammatory response in C57BL/6 mice (Mo *et al.*, 2019). These doses corresponded to approximate daily intakes of 60, 120, and 180 mg/kg bw. Regarding host outcomes, there were no differences for most of the parameters evaluated. The few exceptions were: increased feed intake by animals fed 400 and 800 mg GML/kg, higher triglycerides in the 400 mg GML/kg group, increased circulatory TGF-β-1 and IL-22 levels in the 1 600 mg GML/kg group, and decreased faecal acetic acid in all treatment groups. β-Diversity differed between treatment groups and control, but α-diversity decreased only in the highest two doses. Some microbiota changes were common to all doses, these included a decrease in Tenericutes (mainly Anaerosplasmataceae), Desulfovibrionaceae, *Anaeroplasma*. However, other changes were dose-dependent: the 1 600 mg/kg dose increased the abundance of Proteobacteria (mainly Sutterellaceae), *Clostridium* XIVa and *Oscillibacter*, while Baceroidaceae and Erysipelotrichaceae decreased in the 800 and 1 600 groups, and Porphyromonadaceae and *Barnesiella* increased only in the 400 and 800 groups. The authors interpreted these findings as a development of *"favorable microbial taxa after exposure to GML, without inducing systemic inflammation, dysfunction of glucose and lipid metabolism"* (Mo *et al.*, 2019, p. 1).

Zhao's research team continued investigating the metabolic effects of GML in the context of HFD and obesity (Zhao *et al.*, 2020). C57BL/6 male mice were given an HFD supplemented with 1 600 mg GML/kg for 16 weeks (theoretical daily intake estimation: 240 mg/kg bw). The control groups were fed HFD or LFD without GML. Animals were not obese and fed normal chow before beginning the treatment. While HFD led to increases in body weight, fat deposition, hyperlipidemia, inflammation, altered hepatic lipid metabolism and glucose homeostasis, these effects were not seen in animals fed HFD supplemented with GML. In this treatment group, most parameters monitored did not differ – or were very close – to the LFD control. Of note: The authors concluded that GML *ameliorated* the effects of HFD (obesity). However, as animals were not obese or fed HFD prior to treatment, a more valid conclusion should have been that GML *prevented* the development of features of obesity when fed HFD. The microbiota of the GML group differed from the controls but was more similar to the group fed normal rodent chow.

β-Diversity differed between the three groups, which clustered separately. Compared to the HFD control, the microbiota in the GML group improved α-diversity. The composition also differed between the groups. Compared to the HFD control, GML increased the phylum Verrucomicrobia, and at the genus level, it decreased *Dorea, Bacteroides, Eggerthella* and *Parabacteroides* and increased *Bifidobacterium, Allobaculum* and *Streptococcus*. The non-targeted serum metabolomics and hepatic transcriptomics analysis showed clustering of the three experimental groups, with several differences attributed to metabolites involved in lipid metabolism. Correlations between the omic analyses revealed associations between up-/down-regulated pathways and metabolites with several bacterial groups, including *Bifidobacterium pseudolongum*. In a separate experiment, when giving a 16-week antibiotic cocktail in drinking water to mice fed HFD or HFD supplemented with GML, there were no significant differences between the two groups in all parameters evaluated (this study lacked controls without antibiotics or fed a normal diet). These included body weight, fat pads, glucose homeostasis, circulatory pro-inflammatory cytokines, LPS and the gut microbiota. Based on these results, the authors concluded that the effects of GML on glucose homeostasis, lipid metabolism and systemic inflammation partially depended on the gut microbiota.

Similar results were obtained by the research team after they replicated the study conditions for GML (Zhao *et al.*, 2020), but added two other groups treated with 1 169 mg lauric acid (LA)/kg or 1 243 mg lauric triglyceride (GTL)/kg (results for these compounds are not discussed as they are not relevant as food additives) (Zhao *et al.*, 2022). Again, HFD supplemented with 1 600 mg GML/kg did not induce many of the effects observed in the HFD control (hyperlipidemia, alterations of glucose homeostasis and systemic inflammation), performing better than LA and GTL groups. Based on metabolomics and lipidomics findings, the researchers indicated that GML had a regulatory effect on phospholipid metabolism and bacterial-derived metabolites, promoting the endogenous synthesis of unsaturated fatty acids. Regarding the microbiota, α-diversity in the GML group was lower than the HFD control, which authors attributed to the antibacterial properties of this compound. β-diversity analysis showed clustering of mice fed HFD and LFD. A decrease in the abundance of Bacteroidetes and increase in Firmicutes was observed in all HFD-fed groups. However, the abundance of Proteobacteria was lower in the GML group than in the HFD control. Compared to the HFD control, the relative abundance of *Desulfovibrio* was lower in the GML group (also LA and GTL), similar to the lean LFD control group. The abundance of *Allobaculum, Bifidobacterium, Bacteroides, Streptococcus, Ruminococcus, Lactococcus* and *Sutterella* increased in the treatment groups, compared to the HFD control.

The same research group expanded their investigations to evaluate the effects of glycerol monocaprylate (GMC) also on glucose and lipid metabolism, inflammation and the gut microbiota (Zhang, Feng and Zhao, 2021). C57BL/6 male mice were fed a standard rodent diet supplemented with two doses GMC (150 and 1600 mg/kg) for 22 weeks. Compared to the control group, the effects of both doses on the host's metabolism and inflammatory markers were very limited (e.g. no changes in

body weight, adipose tissues, glucose homeostasis and most circulatory cytokines). There were only slight dose-dependent effects in the lipid biochemistry and the transcription of a few genes related to the hepatic lipid and glucose metabolism. Regarding the microbiota, GMC increased α-diversity. Also, β-diversity from treatment groups differed from the control. Changes in microbiota composition varied with the dose. While the abundance of Firmicutes, Lactobacillaceae and Bacilli increased at the low dose, Clostridiales, Lachnospiraceae, *Ruminococcus* did so at the high dose. The production of several SCFAs also increased in both groups, including propionic acid.

Elmén *et al.* (2020) evaluated the effect of five emulsifiers (glycerol monoacetate, glycerol monostearate, glycerol monooleate, propylene glycol monostearate, and sodium stearoyl lactylate [SSL]) on pooled human microbiota in culture media (brain–heart infusion broth or chemically-defined medium). The concentration tested was about tenfold lower (0.025 percent) than the levels permitted by the United States FDA for SSL (0.2–0.5 percent weight of finished product). The authors reported that only SSL induced changes in the microbiota, and these were independent of the culture media used. For this reason, they focused on the evaluation of this emulsifier. The reduced butyrate production was consistent with decreases in the abundance of butyrate-producing Clostridia belonging to the families Clostridiaceae, Ruminococcaceae and Lachnospiraceae, e.g. *Dorea, Anaerostipes, Faecalibacterium, Coprococcus, Flavonifractor* and *Pseudoflavonifractor*. Other effects on the microbiota included increased abundance of Bacteroidaceae (*Bacteroides*), Enterobacteriaceae (e.g. *Escherichia*) and *Desulfovibrio*, while *Bifidobacterium* abundance decreased. The authors also reported the potential pro-inflammatory effect of SSL based on the observed increases in LPS and flagellin. SSL also reduced or suppressed the growth of several representative Clostridia species obtained from commercial and non-commercial sources, with species-specific sensitivities to the range of concentrations tested (0.00078–0.025 percent). Glycerol monostearate and propylene glycol monostearate also affected the bacteria tested but to a lesser degree than SSL. The authors concluded that their findings (proliferation of potentially pathogenic microbiota members, reduced microbial groups previously reported as beneficial, butyrate reduction, and production of pro-inflammatory microbial compounds) might contribute to the detrimental effects of Western diets on the gut microbiota and human health, supporting the findings of *in vivo* studies.

LECITHIN

Annex III.13. contains a summary of the study evaluating lecithin.

Robert *et al.* (2021) conducted a short-term study to evaluate the impact of lecithins from two different origins, soy (SL) and rapeseed (RL), on Swiss mice's gut microbiota and lipid metabolism. Both compounds were given in the feed for 5 days. The dose levels were 10 percent SL (~97 percent mg/kg bw/day) and 1, 3 or 10 percent RL (~10, 29 or 97 mg/kg bw/day, respectively). This exposure was followed by 1-time oral gavage administration of the same compounds and concentration levels equivalent to 3, 10 and 33 mg/kg bw/day. The authors referred

to EFSA estimates of daily exposure levels of lecithin as food additives: 32–1777 mg/kg bw/day in adolescents and 70–118 mg/kg bw/day in adults (EFSA, 2017). The evaluation of the faecal microbiota was conducted by real-time PCR. It targeted Bacteroidetes, Firmicutes, Bifidobacteria, *Escherichia coli, Akkermasia muciniphila, Clostridium coccoides, Clostridium leptum* group, Lactic acid bacteria and *Faecalibacterium prausnitzii*. All treatment groups increased the abundance of *C. leptum*, while microbial parameters remained unchanged. In the host, only the high dose RL led to increases in postprandial abundance α-linolenic acid in plasma and beneficial changes in the bile acid profile. In the context of obesity, the authors reported that the doses of the emulsifiers tested did not increase lipemia, therefore reassuring their use as emulsifiers. However, they also recommended to further confirm these findings in studies with human subjects.

CARRAGEENAN

Annex III.12. contains a summary of the study evaluating carrageenan.

Carrageenan is a high molecular weight (HMW) polysaccharide (polygalactan) naturally found in several species of red seaweeds. Variations in the conformation of the galactose backbone, number and location of sulfate groups lead to the different forms, lambda- (λ), kappa- (κ) and iota- (ι) carrageenans (McKim *et al.*, 2019). These carrageenan types occur naturally as copolymers, but one of them is enriched in commercial preparations with size distribution ranging from 200–800 kDa in > 95 percent of the product and about < 5 percent 10–50 kDa (McKim *et al.*, 2019). The capacity of anionic sulfate groups to bind charged groups present in food proteins is responsible for the textural functionalities of carrageenan as additives in food products, including gelling and thickening, as well as stabilizing properties (Hotchkiss *et al.*, 2016; McKim *et al.*, 2019). The different forms of carrageenan are used in various food types, including meat products (e.g. sausages, reformed meats), dairy (e.g. ice cream) and dairy alternative beverages (e.g. almond, soy) (Liu *et al.*, 2021).

Humans cannot degrade and absorb carrageenans, which pass through the digestive system and reach the colon intact. Although some marine bacteria have been shown to utilize these seaweed compounds (Chauhan and Saxena, 2016), little is known about the potential of gut microbes to degrade carrageenan.

Yin *et al.* (2021) evaluated *in vitro* (batch fermentation) the capacity of human gut microbiota from eight healthy individuals to utilize commercial κ-carrageenan polysaccharide (KCP, 450 kDa), mild-acid-degraded κ-carrageenan (SKCO, 100 kDa) and κ-carrageenan oligosaccharide (KCO, 4.5 kDa). The last two were prepared in the laboratory using the commercial carrageenan as starting material. Only KCO was degraded by the microbiota in seven faecal samples, with increased concentrations of propionic and butyric SCFAs. However, no desulfation was observed in these samples. The authors identified *Bacteroides xylanisolvens* as the main degrading-KCO bacteria, and *Escherichia coli* as utilizer of resulting products, which in turn increased the degradation efficienty of *B. xylanisolvens*,

a typical synergistic cross-feeding activity. Genome sequencing analysis revealed the presence of κ-carrageenase precursor genes. In a later *in vivo* experiment to assess the inflammatory response to KCO degradation, 5 percent KCO was given to GF Kunming mice in drinking water for 8 weeks alone or after intragastric administration of the two bacteria (5 x 10^8 CFU/0.5ml). In addition to the control (water only), a fourth group was given the bacteria in the absence of KCO. The histological evaluation did not show surface erosions and crypt damage in any of the intestinal segments evaluated (duodenum, jejunum, ileum, colon and rectum). However, only the colon and rectal samples showed inflammation at submucosal level in the three treatment groups, although higher in the rectal samples of the animals treated with both KCO and KCO-degrading bacteria. The transcriptome from rectal samples showed the most pronounced up- or downregulated genes in the combination treatment. Genome analysis identified differently expressed genes associated with carbohydrate and polysaccharide binding proteins and pathways associated with the immune and inflammatory responses. These results were in agreement with the results of RNA sequencing of inflammatory markers.

Shang *et al.* (2017) evaluated the impact of κ-, ι- and λ-carrageenans on colon health and the gut microbiota of adult C57BL/6J mice. Each treatment group was given one type of carrageenan at a dose of 20 mg/L in drinking water for 6 weeks. According to the researchers, this dose was equivalent to the human daily consumption of 250 mg/day reported elsewhere (Bhattacharyya *et al.*, 2012). Water and food intake were not reported. The authors did not clarify whether the three carrageenan types used in the study were food-grade. At the end of the treatment, the research team reported the induction of colitis by κ-, ι- and λ-carrageenans (infiltration of inflammatory cells in the proximal and distal colon and increased TNF-α, while other cytokines – IL-1β, IL-6, IL-10 – remained unaltered). The colonic microbiota differed from the control in the three treatment groups, but changes were dependent on the compound. Regarding diversity makers, richness and α-diversity estimators increased in λ- and ι- carrageenan groups (richness was not altered in λ-carrageenan) but decreased in the κ-type. The principal component analysis (PCA) also showed the clustering of all groups. Carrageenans altered several taxa, and, in many cases, the κ-type had an opposite effect than λ- and ι- carrageenans. The treatments decreased the relative abundance of Bacteroidetes and Verrucomicrobia and increased Firmicutes. Proteobacteria increased in the groups given ι- and λ-carrageenans, while decreased in the κ-group. There were more differences at lower taxa levels. The authors highlighted the reduction of *Akkermansia muciniphila* – negatively associated with chronic inflammatory diseases (Cani *et al.*, 2022) – by all treatments. *Desulfovibrio*, a gut commensal known to reduce sulfate groups, remained unaltered, which led the authors to conclude that only fermentable sulphated polysaccharides – not carrageenan – can promote the growth of *Desulfovibrio*. Based on the findings, the authors suggested the possible involvement of carrageenan-altered microbiota – with a focus on decreased bacteria with anti-inflammatory properties like *A. muciniphila* – in the development of colitis. However, they also indicated the need for additional research to investigate this possibility further.

Mi *et al.* (2020) evaluated the effects of κ-carrageenan delivery mode (drinking water or feed) – in the context of a high or low-fat diet – on colitis development and the gut microbiota of C57BL/6J mice. κ-Carrageenan was extracted in the lab from fresh red algae (*Kappaphycus alvarezii*). There were two sets of experiments. In the first set, mice were fed a high-fat diet (HFD) starting 10 weeks before treatment until the end of the experiment. In the second set, mice were on a low-fat diet (LFD). Each set consisted of three groups: (1) 0.5 percent κ-carrageenan in the drinking water, (2) 0.5 percent κ-carrageenan in the feed and normal water, and (3) control (no carrageenan). The HFD set included a fourth control group fed LFD. The treatment lasted 6 weeks. κ-Carrageenan in the drinking water exacerbated colitis in HFD-fed mice, showing increased colon inflammation reported as occult blood in stool, higher levels of MPO and increased TLR4 and TNF-α gene expression. Carrageenan in the drinking water led to microbial shifts in groups fed both diets (mice given κ-carrageenan in the feed were excluded from this evaluation), showing distinct clustering and different diversity between the groups, and with within-diet differences in β-diversity. The authors reported that mice given HFD and carrageenan in the drinking water showed increased relative abundance of several bacteria associated with inflammatory processes, *Bacteroides acidifaciens, Alistipes finegoldii* and *Burkholderiales bacterium,* while the abundance of *Akkermansia muciniphila* increased in the treatment group fed LFD. The author indicated the need for additional research to evaluate the effects of carrageenan on the microbiota and gut environment. They also highlighted that the discrepancies between their results and findings reported by others might have been influenced by differences in the carrageenan used.

Wu *et al.* (2021) evaluated the potential involvement of λ-carrageenan (unclear if the compound was foodgrade) in *Citrobacter rodentium*-induced colitis. *C. rodentium* infection has been used as a model to study host-pathogen interaction in the gut, including the inflammatory response in bacteria-induced colitis (Bouladoux, Harrison and Belkaid, 2017; Collins *et al.*, 2014). This study consisted of three stages, all using C57BL/6 mice, either conventional or germ-free. In the first stage, animals were gavaged daily with three λ-CGN concentrations (1.7, 8.3 or 41.7 mg/kg) for 90 days, followed by a 7-day washout period before half of the animals were infected (gavaged) with *C. rodentium* (10^9 colony-forming units [CFU]/mouse). Although it was not clear if experimental doses referred to the concentration of the additive in the vehicle (gavage volume not provided), the authors indicated that the mid and high doses were equivalent to human daily exposure (Fernández-Ferreiro *et al.*, 2015; Tobacman, 2001), while the low dose has been reported to induce colonic inflammation in IL-10 deficient mice (Bhattacharyya *et al.*, 2013). The outcomes were evaluated 7 days post-infection. The volumes gavaged were not specified by the authors. Animals given λ-CGN alone and not infected with C. rodentium did not showed signs of inflammation. On the contrary, animals infected developed colitis and increased pro-inflammatory cytokine expression in the colon, which increased in severity when treated with λ-CGN in a dose–response fashion. The next experiments were conducted following the same periods described above but in germ-free mice. These animals were either (1) gavaged with the high dose of λ-CGN and infected with *C. rodentium* at the end of treatment, where treatment and control groups did not differ or (2) transplanted with faecal material from the group treated with the high dose of λ-CGN and then infected with *C. rodentium*. In the latter, alterations observed in the conventional mice (e.g. mucus layer, faecal LPS and SCFA) were reproduced after the FMT. These events led the authors to conclude that the microbiota was involved in the exacerbation of colitis. The microbiota was only evaluated in conventional mice treated with the high dose of λ-CGN and those receiving faecal material from this treatment group, as well as their respective controls. None of these animals were infected with *C. rodentium*. Regarding diversity, some indices were statistically significantly different (Shannon), while others (Chao1) and microbiota parameters (like total bacterial load, operational taxonomic unit [OTU], and abundance-based coverage estimator) remained unaltered. Regarding composition, λ-CGN groups showed a higher relative abundance of Proteobacteria and reduced Firmicutes and Verrucomicrobia at the phylum level. At lower taxa levels, treatment groups showed higher relative abundance of *Akkermansia, Bacteroides fragilis, Ruminococcus gnavus, Desulfovibrio, Anaerotrucus, Bilophila wadsworthia* and *Clostridium leptum*, and decreased abundance of *Bacteroides thetaiotaomicron, Faecalibacterium, Bifidobacterium, Blautia* and *Roseburia*. In addition, faecal LPS increased in carrageenan (CGN) groups, while the faecal SCFA content (e.g. acetic, butyric acids) decreased. Transplanted mice in the treatment group and infected with *C. rodentium* showed increased epithelial permeability and a thinner mucus layer than the control group. In the absence of abnormalities of goblet cells, the authors explained that the reduction in the depth of the mucin layers was due to the proliferation of mucin-degrading bacteria (e.g. *Akkermansia*), as observed in the

genomics evaluation. Bacteria were found closer to the epithelium in λ-CGN groups. Correlation analysis was also conducted between microbial compounds (faecal LPS and SCFA) and indicators of epithelial integrity. Lipopolysaccharide (LPS) showed a positive correlation with intestinal integrity but negative for mucus thickness, ratio of goblet cells in mucosa and distance of bacteria from epithelial cells. The authors suggested that λ-CGN per se does not directly influence the inflammatory response. Instead, it alters the intestinal environment, which favours inflammation by negatively affecting the microbiota composition. For clarification purposes, colitis was only observed in animal groups infected with *C. rodentium*, but not in those treated with λ-CGN and not infected, and microbiota was only investigated in non-infected mice.

In a later study, the same research group conducted a similar study but focusing instead on κ-carrageenan (Wu *et al.*, 2022). Animals used, dosing, experimental periods, protocols for faecal transplant and infection with *C. rodentium* were the same as described by (Wu *et al.*, 2021). In this study, faecal transplant was carried out from all dose groups (high, medium and low) and not only from the high κ-CNG dose as in Wu *et al.* (2021). The average molecular weight (MW) of κ-CNG – obtained from a supplier of chemical reagents (Sigma) and repurified in the lab to eliminate low MW components – was 198 kDa, with 20.15 percent sulfate content. Doses used were equivalent to reported daily exposures: intakes of 240 mg/5.8 kg infant/day (Tobacman, 2001) (high κ-CNG dose in Wu's study: 41.7 mg/kg/day); ophthalmic application of 500 mg/60 kg/day in adults (Fernández-Ferreiro *et al.*, 2015) (medium κ-CNG dose in Wu's study: 8.3 mg/kg/day); and the dose (50 µg/30 g/day) used in another mouse study (Bhattacharyya *et al.*, 2013) (low κ-CNG dose in Wu's study: 1.7 mg/kg/day). Experiments using samples from the three dose groups showed a dose–response relationship, where the low dose did not differ from controls in most tests. In conventional mice, all doses κ-CGN led to microbial shifts, with increased richness, while other α-diversity indices remained unaltered. In conventional and transplanted animals, the microbial composition from the high-dose groups clustered together and showed clear separation from the control in the principal coordinate analysis. These two groups had an increased relative abundance of Bacteroidetes, Ruminococcaceae_unclassified and *Bacteroides* and decreased Proteobacteria, *Akkermansia, Bifidobacterium,* Lachnospiraceae*, Faecalibacterium, Mucispirillum*. Firmicutes remained unaltered. Faecal SCFA were also reduced in the high dose κ-CNG treatment groups, mainly butyric and valeric acids. The high dose κ-CNG aggravated *C. rodentium*-induced colitis in both conventional and transplanted mice (epithelial damage, increased lipocalin-2 levels and inflammatory cytokines TNF-α and IL-6). In the absence of alteration of Muc2 expression and goblet cells, the authors speculated that proliferation of mucus-degrading bacteria (e.g. *Bacteroides*) was responsible for the observed thinning of the mucus layer. Based on this hypothesis, the researchers evaluated the abundance of genes related to carbohydrate utilization in the high dose κ-CNG groups by metagenomics analysis. The increased genes encoding mucosal polysaccharide binding proteins and mucin degrading enzymes correlated with the increased abundance of several Bacteroides species, i.e. *B. nordii, B. thetaiotaomicron,* and *B. intestinihominis*.

The decrease in other genes related to the use of cellulose, starch and mannan also correlated with reduced abundance of microbial groups containing those genes, i.e. Lachnospiraceae and *Ruminococcus torques*. In this study, an additional group of conventional mice, which received a probiotic mixture (10^9 CFU *Bifidobacterium longum* and *Faecalibacterium prausnitzii*) for 23 days after treatment with the high dose κ-CNG and before colonization with *C. rodentium*), showed partial recovery of the gut microbiota and the gut barrier function.

Sun *et al.* (2019) evaluated degraded κ-carrageenan oligosaccharides on the human faecal microbiota *in vitro* and resulting supernatants on HT29 cell culture. Gastric simulation was used to hydrolyse food grade κ-carrageenan for 3 or 6 h (KO3 or KO6 groups, respectively). The resulting oligossacharides (<3 kDa, 1 percent w/v) were added to fermenters inoculated with pooled faecal microbiota from 4 individuals and monitored at 6 different time points (0, 6, 12, 24, 48 and 72h). The oligosaccharides were further degraded over time in the presence of the microbiota. Filtered supernatants from 0, 6, 48 and 72-hour samples were added to HT29 cell cultures at three concentration levels (50, 100 and 200 µl/ml) and treated for 24 h. The KO3 or K06 were not toxic to cells but stimulated the production of the two pro-inflammatory cytokines evaluated (IL-1β and TNF-α), sIgA and mucin 2 in a dose–response fashion. Based on these results, the authors suggested the proinflammatory activity of κ-carrageenan oligosaccharides. The gut microbiota treated with KO3 and KO6 for 72 h resulted in reduced α-diversity and altered composition at the phylum, family and genus levels. Compared to the control, both treatment groups increased the relative abundance of Prevotellaceae, Veillonellaceae and Bifidobacteriaceae, while they reduced Enterobacteriaceae, Desulfovibrionaceae, Ruminococcaceae, Lachnospiraceae, Bacteroidaceae and Porphyromonadaceae. At the genus level, some changes were common in the two treatments (increases of *Prevotella, Megamonas* and *Bifidobacterium* and decreases of *Parabacteroides, Escherichia-Shigella* and *Desulfovibrio*) while others were treatment-dependent, e.g. KO3 increased the abundances of *Streptococcus* and *Lactobacillus*, while *Megaspharea* increased with KO6. Both treatments also increased the production of SCFA, mainly due to the contribution of acetic acid.

XANTHAN GUM

Annex III.13. contains a summary of the study evaluating xantham gum.

Sun *et al.* (2022) evaluated the impact of xanthan gum (XM) and low molecular weight XM (LMW-XG, fermented in-house) on an undefined mouse strain. Mice were gavaged daily for 28 days with 0.1 mg XM or LW-XG. Mainly LMW-XG led to changes in the caecal microbiota (increased α-diversity and relative abundance of Firmicutes and decrease in Bacteroidetes), and increased total SCFAs, acetate, propionate and butyrate. The two compounds did not induce toxicity in Caco-2 cells. The only host parameter evaluated was body weight, where LMW-XG led to more weight gain than the other experimental groups. Based on the findings, the authors suggested that LMW-XG is more susceptible to fermentation by gut microbiota members than the intact form of xanthan gum.

CURDLAN

Annex III.15. contains a summary of the study evaluating curdlan.

Rahman *et al.* (2021) evaluated the effects of curdlan in a mouse model of colitis (C57BL/6 treated with DSS). Female mice were given 1 mg/day curdlan by oral gavage for 14 days, followed by a 7-day treatment with water or DSS in the drinking water to induce colitis, and two additional days in the absence of treatment. In the host, curdlan improved some signs of inflammation in the colitis model, i.e. ulceration and crypt loss. The additive did not improve many other parameters altered by DSS. In addition, curdlan modulated the macrophage innate response. Regarding the microbiota, this study targeted colonic bacterial and fungal communities. DSS was the main driver for the alterations of the microbial populations, reducing bacterial α-diversity. The effects on the fungal community were less pronounced. Curdlan slightly improved microbial diversity, while it decreased fungal richness. In addition, the additive led to some compositional changes independent of the DSS treatment. It increased the abundance of Bifidobacterium (*B. choerinum*) and Lachnospiraceae and decreased *Blautia*. The authors focused most of their attention on Bifidobacteria due to reports on the beneficial effects of this microbial group in the maintenance of intestinal homeostasis, and *B. choerinum* in particular due its probiotic potential and its ability to degrade starch (Jung *et al.*, 2018). In a separate *in vitro* study using the i-Screen platform (developed by the Netherlands Organisation for Applied Scientific Research institute [TNO, 2023]), the research group evaluated the effects of curdlan (1, 2 or 4 mg curdlan/ml) on pooled faecal microbiota from six healthy human donors. The experiment was run for 24 hours. The effects observed were dose dependent, which included increases in α-diversity and distinct β-diversity among groups. Regarding microbiota composition, the relative abundance of Lachnospiraceae and *Bifidobacterium* increased while *Bacteroides* decreased. The authors indicated that although curdlan increased the abundance of bifidobacteria in both *in vivo* and *in vitro* studies, the species involved were different in the human and mouse microbiotas.

MALTODEXTRIN

Annex III.13. contains a summary of the study evaluating maltodextrin.

Laudisi *et al.* (2019) evaluated the impact of maltodextrin (MDX) in a rodent model of intestinal inflammation (Balb/c mice treated with dextran sodium sulfate [DSS]). In a preliminary investigation, mice were treated with different doses of MDX (1, 3 or 5 percent), 5 percent propylene glycol or 5 g/L animal gelatin, provided in the drinking water for 45 days. None of the compounds led to clinical or histological alterations in healthy animals. However, only the high doses MDX exacerbated intestinal inflammation in the colitis model. These effects were also observed in a second model of colitis (induced by indomethacin) and were independent from the mucosa-associated microbiota, which was not altered by 5 percent MDX. Additional experiments were conducted to investigate further the inflammation induced by the high dose MDX, revealing changes in the mucus barrier due to the activation

of endoplasmic reticulum stress. Tight junctions were not affected. The researchers also investigated the effects of 5 percent MDX in healthy mice after an extended exposure of 10 weeks. Mice developed low-grade intestinal inflammation and higher fasting blood glucose levels than controls. Furthermore, there was a reduction in the mucus protein Mucin-2. The microbiota was not evaluated in this experiment.

COLOURS

TITANIUM DIOXIDE

Annex III.17. contains a summary of the studies evaluating titanium dioxide.

Waller, Chen and Walker (2017) evaluated the impact of food- and industrial-grade titanium dioxide (TiO_2) on the faecal microbiota from one healthy vegetarian female donor in a colon bioreactor model for 5 days. The authors selected these two material grades because previous research groups reported differences in size distribution, morphology and surface composition between food and industrial TiO_2 particles, which can influence their fate and toxicity. Food-grade TiO_2 particles had a mean diameter of 122 ± 48 nm with the surface coated with inorganic phosphate,[27] while the industrial grade (P25) had a nominal size of 21 nm and no surface coating. Further particle characterization indicated that food-grade particles were more stable and less prone to aggregation than industrial-grade TiO_2. The dose was based on the estimated daily intake for adults ~0.3–0.7 mg/kg bw (Weir *et al.*, 2012). Shifts in the dominating phyla Proteobacteria and Firmicutes observed in the control group were less evident in the treatment groups, especially in the food-grade TiO_2 group, where Proteobacteria remained the most abundant group at the end of the 5-day study. Findings from additional tests (hydrophobicity and electrophoretic mobility) led the authors to suggest that food grade TiO_2 might exert a limited effect on microbial stability and biofilm formation. In addition, the researchers also indicated that particles with differing physico-chemical features might lead to different microbial response and should be considered when designing toxicity studies and evaluating exposure and risk.

Dudefoi *et al.* (2017) conducted an *in vitro* study using a chemostat bioreactor to evaluate the impact of TiO_2 on a standardized stool-derived microbial ecosystem therapeutics (MET-1) (Petrof *et al.*, 2013). This microbial community consists of 33 bacteria strains obtained from the faecal material of a healthy donor. The closest species matches, analysed by 16S rRNA gene sequencing, are *Acidaminococcus intestini, Akkermansia muciniphila, Bacteroides ovatus, Bifidobacterium adolescentis, Bifidobacterium longum, Blautia stercoris, Clostridium cocleatum, Collinsella aerofaciens, Dorea longicatena, Escherichia coli, Butyricicoccus pullicaecorum, Eubacterium eligens, Eubacterium limosum, Eubacterium rectale, Eubacterium ventriosum, Faecalibacterium prausnitzii, Lachnospira pectinoschiza,*

[27] Phosphate is added to the surface of TiO_2 to improve particle stability during food preparation and consumption. See Yang *et al.* (2014).

Lactobacillus casei, Lactobacillus paracasei, Parabacteroides distasonis, Enterobacter aerogenes, Roseburia faecis, Roseburia intestinalis, Ruminococcus obeum, Blautia luti, Ruminococcus torques, and *Streptococcus mitis*. Two food-grade TiO_2 samples from two different vendors were used in this study: E171-1 (17 percent nanoparticles) and E171-6a (21 percent nanoparticles), with different surface chemistry. Each additive was tested at two doses, low (100 ppm) and high (250 ppm) for 48 hours. The authors indicated that these doses are equivalent to the TiO_2 concentration in the intestine after the ingestion of 1 or 2 candy pieces. The effects of TiO_2 were very limited. Only the high concentration of E171-1 decreased the abundance of *Bacteroides ovatus*. Little or no effect was observed on bacteria respiration and fatty acid composition. The authors concluded that although their findings did not indicate a significant alteration of the microbiota, additional studies are needed to evaluate cumulative and chronic exposure to TiO_2, including *in vivo* validation of *in vitro* results.

Chen *et al.* (2019a) gavaged male Sprague-Dawley rats with 2, 10 or 50 mg/kg bw/day TiO_2 (average diameter 29 ± 9 nm) for 30 days. The authors used estimated dietary exposures in children (Weir *et al.*, 2012) to establish the low-end experimental doses. Based on the physicochemical evaluation of the particles in artificial gastric and intestinal juices, the authors suggested the possible tendency of TiO_2 particles to aggregate in the gastrointestinal tract. The faecal microbiota and metabolome were monitored on dosing days 7, 14, and 28. The microbiota remained practically unaltered, with no observed changes in total observed species, α- or β-diversities and SCFA production. With the exception of increases in the abundance of *Lactobacillus gasseri* in the high-dose group, there were no other time trends observed between days 14 and 28 for the other lower doses.

Serum LPS in the high-dose group differed from that in the control group.There were no differences in body weight between the groups, but some alterations in the colonic epithelium were observed in the highdose group. Of all biomarkers of oxidative stress and pro-inflammatory cytokines, only malondialdehyde, superoxide dismutase and IL-6 increased slightly with no clear dose-dependency. Also, the non-targeted faecal metabolome showed 25 metabolites differing between the high-dose group and the control, several related to amino acid pathways. The authors concluded that oral exposure to TiO_2 induced alteration in the gut microbiota and gut-associated metabolism and proposed a mechanism for the toxicity of TiO_2, in which microbial disturbances and microbial LPS led to oxidative stress and an inflammatory response.

This study can be used to illustrate the discrepancies between the content of the abstract and the results described in the manuscript. Despite the number of null effects observed after TiO_2 exposure, including an unaltered microbiota, and the limited effects in the rats (primarily at higher doses), the authors described a dysbiotic microbiota potentially acting as the initiator of a sequence of effects, including oxidative stress and inflammatory response in the intestine. In addition, there were some discrepancies between the text and figures. Similar conclusions apply to the following study.

The same research group conducted a similar study using the same model and conditions, except for the longer exposure period (90 days) and the focus on hepatic metabolomics (Chen *et al.*, 2019b). In this study, all parameters were evaluated only at the end of the study (no mid-time point monitoring). Concerning the faecal microbiota, diversity increased in the treatment groups. While there was no change in the relative abundance of the different phyla, the Firmicutes:Bacteroidetes ratio decreased in the mid- and high-dose groups. Several Firmicute members were affected, with increases in the abundance of *Lactobacillus reuteri* and decreases in *Romboutsia*, both in the medium dose only. Faecal LPS increased while faecal SCFA remained unaltered. Some hepatic markers were different from the control at medium and high doses. In addition, the researchers reported some signs of histopathological alterations in liver samples, suggesting the induction of a slight hepatic toxicity at high doses. Of the 263 hepatic metabolites identified, 29 were altered, most related to energy and oxidative metabolic pathways. In addition, the researchers reported oxidative stress and proinflammatory activity (increased IL-1α, IL-4 and TNF) after exposure to the high dose TiO_2.

In another study, three TiO_2 particle sizes in the nano range (10, 50 and 100 nm) were given to weaned C57BL/6J mice in the feed at a concentration of 0.1 percent by weight for 3 months (Mu *et al.*, 2019). To establish the dose, the authors referred to the upper limit for titanium dioxide (1 percent in food) established by the United States FDA (Title 21 of Code of Federal Regulations, § 73.575). The effects in the faecal microbiota were limited (no changes in total bacteria abundance or α-diversity). The groups treated with the two smaller particle sizes increased the abundance of Bacteroidetes and decreased Actinobacteria. At the genus level, the treatment with

the two smallest particle sizes decreased the abundance of *Bifidobacterium* and *Lactobacillus*, including *L. Johnsonii*. The largest particles also shifted the microbial composition but led to a different pattern than with the smaller TiO_2 particle sizes. Mice receiving 10 and 50 nm size particles had lower body and colon weights (no differences in food intake), with no alterations in the inflammation biomarker lipocalin-2. Such changes were not observed in mice treated with broad-spectrum antibiotics targeting gram-negative bacteria, which led the authors to suggest that intestinal inflammation might be caused by a dysbiotic microbiota. The authors indicated the possibility of immune imbalance after observing a decreased in CD4+ T cells, Treg cells and macrophages in mesenteric lymph nodes in treatment groups. Based on differences in body weight and colon length with respect to the control group, the authors indicated that dietary exposure to TiO_2 nanoparticles could aggravate DSS-induced intestinal toxicity and inflammatory response. Of note, no histopathological or cytokine measurements were conducted in this study.

Mao *et al.* (2019) gavaged pregnant Sprague-Dawley rats with 0.5 percent TiO_2 (~21 nm) for 12 days (from gestational day [GD] 5 to 18). The faecal microbiota was evaluated at day 0, GD10 (mid-term pregnancy) and GD 17. Although the treatment did not change α-diversity, it decreased the abundance of Clostridiales (GD10) and Dehalobacteriaceae (GD 17). Pregnant animals presented elevated fasting glucose at mid-term pregnancy. This result – along with increases in the predicted gene function related to type 2 diabetes and taurine and hypotaurine metabolism at mid-term pregnancy – led the authors to speculate that although TiO_2 exposure was not sufficient to induce gestational diabetes, it might contribute to adverse effects in pregnant rats and their offspring. Pups were not evaluated in this study.

The next three studies were conducted by the same research group, who evaluated the potential impact of TiO_2 NP (average diameter: 21 nm) given by oral gavage on the gut microbiota and gut-brain axis in adult mice (Zhang *et al.*, 2020), pregnant mice (Su *et al.*, 2021) and offspring of mothers exposed to the nanoparticles (Yang *et al.*, 2022b). In all three cases, the 150 mg/kg dose was calculated using the upper limit set in the Chinese Standard for Food Additives (GB2760-2015), based on a standard 60 kg person and considering the uncertainty factor for animal-to-human extrapolation (9.1). Of note, it was not clear if the dose was express as mg/kg vehicle or mg/kg bw. The volume of the gavaged solution was not specified.

In the first study, adult C57BL/6J mice were given 150 mg/kg TiO_2 NP (average diameter: 21 nm) via gavage for 30 days (Zhang *et al.*, 2020). The treatment affected α- (reduction) and β-diversities and shifted the microbial composition. Titanium dioxide did not cause inflammation or histopathological changes in the brain and small intestine and did not alter the gene expression levels of tested enteric peptides. The effects were limited to abnormal locomotor activities (open field test) and enteric neuronal activities. The authors acknowledged several limitations, including using one single dose, not using a more realistic dose, not covering non-bacterial components of the microbiome, and not being able to prove causality between microbial alterations and neurological impairments.

Su *et al.* (2021) gavaged pregnant C57BL/6J mice daily with 150 mg/kg TiO_2 NP (average diameter: 21 nm) between gestational days (GD) 8 and 21. The study evaluated the impact of TiO_2 on the gut-brain axis of the dams and offspring at different time points. The NPs did not affect maternal behaviour or the faecal microbiota (GD21). In the offspring, no alterations were observed at post-delivery day (PD) 21 (weaning). However, several of the evaluated parameters differed from the controls at PD49. Such changes included locomotor activities, learning and memory ability, increased anxiety-like behaviours, and histopathological alterations in the cerebral cortex and intestine (altered of lost villi, reduced goblet cells and increased mast cells). The expression of enteric neuronal markers, gut-derived neurotransmitters and gut-brain peptides was not affected. However, alterations of the enteric immune response (increased sIgA and diamine oxidase) were observed in most checkpoints. Also, the faecal microbiota composition differed from the control only at PD49, including declines in the relative abundance or Bacteroidota (or Bacteroidetes) and Cyanobacteria and increases in Campylobacterota. Based on the findings, the authors suggested the potential late effects of gestational exposure to TiO_2 NPs in the gut microbiota, which might lead to neurobehavioral impairments in adulthood. The authors acknowledged the same limitations described by Zhang *et al.* (2020).

The same research group conducted a follow-up study under the same experimental conditions, but on this occasion, the effects of TiO_2 NP treatment were evaluated in the mothers at a later time point, on PD60 (Yang *et al.*, 2022b). Contrary to the null effects reported for the dams between pregnancy and weaning (Su *et al.*, 2021), in this study, the authors observed alterations of the brain integrity (hippocampus and cerebral cortex), gut-brain axis (decreased expression of enteric neuronal receptors, gut-derived neurotransmitters and gutbrain peptides), neurobehavioral impairment, and also alterations of the small intestine (integrity, barrier function and decreased digestive enzymes). Although the exposure of mice during pregnancy did not affect α-diversity, the gut microbiota of treated animals clustered differently from the controls, with several affected microbial groups, including Bacilli, Clostridia, Verrucomicrobiae, and α-Proteobacteria, and decreases in the abundance of Verrucomicrobiota and Desulfobacterota. Based on the findings, the authors indicated that disrupting the microbiota-gut-brain axis might be linked to neurobehavioral impairments. However, they acknowledged that additional research is needed to demonstrate this connection. Although this was a crosssectional study (parameters evaluated at a single time point, PD60), the researchers suggested that alterations were persistent based on the assumption that such alterations could have started developing earlier. Not monitoring effects at several check time points was noted as a limitation.

Li *et al.* (2019) exposed C57BL/6 mice to three nano-sized TiO_2 particles (average diameter: 25, 50 and 80 nm) via gavage at a daily dose of 1 mg/kg bw for 7 days. The researchers focused on the 25 nm particles as they were the only ones found in blood and intestinal tissues. The authors observed alterations of the intestinal barrier (altered mucus layer and reduced expression levels of tight-junction biomarkers)

in the treatment group as well as distinct gut microbiota compositional changes. The researchers highlighted the reduction in the abundance of *Bifidobacterium*, *Dorea*, *Sutterella*, *Rikinella* and *Akkermansia*. Further experiments excluded the evaluation of the microbiota and targeted *Bifidobacterium* only. A faecal transplant from treatment donor mice to antibiotic-treated recipients reproduced some of the effects previously observed in the TiO_2 group, i.e. decreases in the thickness of the mucus layer. At the same time, the abundance of *Bifidobacterium* remained low. Although the gut microbiota was not evaluated in transplanted mice, the authors concluded that gut dysbiosis induced by titanium dioxide nanoparticles could be the cause of the mucus layer disturbance. Inulin supplementation in the drinking water before and during TiO_2 exposure minimized the effects of the compound. The authors indicated that a limitation of their study was the lack of functional assessment of the gut barrier, which would have demonstrated the potential of TiO_2 NP to alter intestinal permeability.

Pinget *et al.* (2019) treated C57BL/6J mice groups with three different doses (2, 10, 50 mg/kg bw/day) of foodgrade TiO_2 (average diameter: 202 nm) in the drinking water for 3 weeks and monitored the impact on intestinal homeostasis and the faecal and small intestine microbiota by 16S rRNA sequencing. The effects observed were dose dependent, with no or limited impact at the lowest dose. The treatments did not affect the diversity and composition of the small intestine microbiota. The α-diversity of the faecal microbiota was not altered by TiO_2 and the β-diversity analysis showed clustering of treatment groups separate from the control, which might indicate a minor impact of titanium dioxide on the microbial population. Changes in the relative abundance were observed in only a few genera. All doses increased the abundance of *Lactobacillus* and *Allobaculum*, while only the high dose increased *Parabacteroides*. The high dose decreased the production of SCFA and induced biofilm formation *in vitro*. In the mice, the dose-dependent effects included alteration in the expression of biomarkers of epithelial function (decreased *Muc2*, increased antimicrobial peptide β-defensin; other antimicrobial peptides and junction markers were not altered) and colonic immune and pro-inflammatory activity (increases in some immune cell populations and expression of pro-inflammatory cytokines). Based on these results, the authors concluded that food-grade TiO_2 is not inert and can disturb gut homeostasis, more significantly at the highest dose tested. They also suggested that reduced microbial production of SCFA, the biofilm formation and the pro-inflammatory activity after TiO_2 exposure could predispose the host to disorders such as inflammatory bowel disease and colorectal cancer.

Cao *et al.* (2020) evaluated the effects of 0.1 percent food-grade TiO_2 (E171, 112 ± 34 nm, 44 percent < 100 nm) and TiO_2 nanoparticles (NPs, 33 ± 14 nm, 100 percent < 100 nm) in C57BL/6 mice fed a low-fat (LFD) or high-fat diet (HFD), respectively. TiO_2 was provided in the rodent chow during 8 weeks at a 0.1 percent w/w (~150 mg/kg bw/day). Although many of the observed changes were due to the diet, TiO_2 NPs significantly altered several faecal microbiota members and host biomarkers, especially in the HFD group. Some significant differences were observed for E171 but to a lesser extent than the NPs. Within their respective diet groups, the two

TiO_2 did not differ from their control, except for TiO_2 NPs that reduced the relative abundance of *Bifidobacterium* and *Allobacullum* in the LFD group. After additional microbiota evaluations, the researchers indicated a possible synergistic effect of HFD and TiO_2 NP. Certain caecal SCFAs decreased depending on the treatment group or diet. For example, butyric acid was affected by all treatment groups. In addition, E171 reduced valeric and isovaleric in mice fed LFD, and acetic acid independent from the diet. In the host, TiO_2 NP altered a limited number of plasma hepatic and renal biomarkers, induced colonic inflammation, and altered protein expression (protein and fat digestion and absorption pathways), all of these with a stronger response in the HFD group. The pro-inflammatory activity of TiO_2 NP observed in HFD-fed mice was reproduced by MFT (faecal material from all HFD groups and LFD control given to antibiotic-treated mice). Based on these findings, the authors concluded that obese mice were more susceptible to the effects TiO_2 NP. The authors also monitored the presence of titanium in the faeces from treated mice and 20 human volunteers. Titanium levels in humans ranged from 0.02 to 3.57 µg/mg dry faeces (average: 0.93 µg/mg) and in mice from 5.37 to 14.37 µg/mg (average 8.79 µg/mg). The authors highlighted the relevance of the experimental dose (0.1 percent w/w), considering that the maximum level of titanium dioxide permitted in food in the United States is 1 percent.

Zhao *et al.* (2021) investigated the potential role of TiO_2 nanoparticles in metabolic syndrome (MeS), focusing on their impact on faecal microbiota, as well as the integrity and immune activity of both the liver and colon. Kunming mice given 30 percent fructose in the drinking water were gavaged with 20 mg/kg bw/day TiO_2 NPs (average diameter: 25.2 nm) for 8 weeks. In addition to the control group (no TiO_2 NP, no fructose), the study included another group that consumed fructose only. Generally, TiO_2 NPs aggravated several of the alterations induced by fructose. These included augmented hepatic pro-inflammatory activity and oxidative stress, colonic barrier alteration (epithelial structure, expression tight junction genes) and increased pro-inflammatory environment. Regarding the microbiota, the effects of the TiO_2 NPs group, compared to its fructose control, were limited to some specific changes. These included a more pronounced decrease in the relative abundance of Bacteroidetes and increased Firmicutes and Proteobacteria. At lower taxa levels, TiO_2 increased the abundance of Desulfovibrionaceae and Clostridia. LPS levels in faeces and serum were also higher in the TiO_2 group. Several of the physiological alterations observed in the TiO_2 group were reproduced after faecal transplant from donors of each group to antibiotic-treated mice. These included hepatic disturbances (hepatic biochemistry, inflammation, tissue damage), increased LPS levels and colonic pro-inflammatory cytokine TNF-α. Based on the findings, the authors suggested the potential participation of TiO_2 NP-induced microbial dysbiosis in liver and colon inflammation, which might increase the susceptibility to MeS. In this study, the mice exposed to fructose and TiO_2 NP and supplemented with *Lactobacillus rhamnosus* (108 CFU by oral gavage) did not develop – or developed to a much lesser extent – the alterations reported above.

Yan *et al.* (2022) evaluated the effects of food-grade micro-TiO_2 (average diameter: 0.25 µm) and TiO_2 NP (average diameter: 20 nm) given to freshly weaned ICR mice by gavage at two doses (10 or 40 mg/kg bw/day) for 28 days. The doses were determined based on reported estimates of daily oral intake of TiO_2 for children and the conversion factor for drug doses between experimental animals and humans. Different parameters were monitored at several time points. The evaluation of microbial β-diversity showed that the different treatments shifted the microbiota. The microbiota fluctuated along the 28 days of study (NOTE: Despite the authors highlighting several phyla and genera affected [e.g. decreased *Lactobacillus* and *Bifidobacterium* and increased *Prevotella*], there were no clear observable trends). The effect in the host generally depended on the dose and particle size. Although effects were reported for both micro- and nanoparticles, toxic effects were more relevant after exposure to smaller particle sizes. In the mice, TiO_2 disrupted the mucosa structure, altered gut barrier markers and led to increased pro-inflammatory activity. Faecal and serum LPS were also elevated in treatment groups. Similar findings were observed in mice transplanted with faecal material (in enemas) from high-dose-treated donors (both nano and micro TiO_2). Of note, recipients were not germ-free or treated with antibiotics. The metabolome analysis also showed differences between groups (micro and nano TiO_2) affecting the levels of several co-metabolites related to the energy and fat metabolic pathways. Titanium levels in colonic tissue did not differ between treatment and control, indicating no absorption or accumulation after a 28-day oral intake of TiO_2. The authors suggested that microbiota disruption might drive the observed effects. However, they stated the need for additional research to evaluate the translatability of results from animal to humans.

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Zhu *et al.* (2022) used a mouse model of human atherosclerosis disease – apolipoprotein E-deficient ($APOE^{-/-}$) – to evaluate the effects of 40 mg/kg bw/day food-grade (E171) TiO_2. Mice were fed either normal rodent feed (NCD) or high choline Western diet (HCD) and received TiO_2 treatment by gavage for 4 months. The treatment exacerbated the effects developed by mice fed HCD. These included the promotion of atherosclerosis progression and atherosclerosis lesions. The treatment also increased trimethylamine-N-oxide (TMAO, a risk factor for atherosclerosis), its precursor trimethylamine (TMA) and microbial TMA lyases, which are involved in the conversion of choline to TMA. There was also an enrichment of microbiota members carrying TMA lyases, including *Clostridium* XIVa and *Eubacterium*. These genera were positively correlated with detected faecal TMA lyases. The relative abundance of *Prevotella* and Lachnospiraceae also increased, while *Akkermansia muciniphila* decreased. After depleting the microbiota with antibiotics, the E171 groups did not differ from their controls, leading the authors to suggest that the microbiota might be involved in the aggravation of atherosclerosis features caused by the HCD.

Yang *et al.* (2022a) evaluated the effects of 100 mg/kg bw/day TiO_2 NP (average diameter: ~10–30 nm) and 5 or 50 mg/kg bw/day bisphenol A (BPA) or their combined exposure to TiO_2 NP and bisphenol A (BPA) administered via gavage in weaned C57BL/6J mice for 13 weeks. The scientists based the TiO_2 dose on estimated sweet consumption by teenagers (Khan *et al.*, 2019) and the no-observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL) for BPA (Shelby, 2008). The effects observed for TiO_2 NP and BPA were generally antagonistic. TiO_2 NP treatment alone or in combination with BPA, but not BPA alone, reduced the faecal microbiota α-diversity. TiO_2 alone led to a decrease in the relative abundance of Firmicutes and an increase of Bacteroidetes, while such effects tended to be reversed in the presence of BPA in a dose-dependent manner. At lower taxa levels, changes in some genera were more or less pronounced or even divergent after exposure to BPA or TiO_2 alone than when combined (e.g. TM7, *Lactobacillus, Oscillospira* and *Odoribacter*). The co-exposure reduced total caecal SCFA, attributed to TiO_2, was mainly due to decreases in butyric and propionic acids. Groups exposed to TiO_2, either alone or combined with BPA, led to structural alterations of the colon and a pro-inflammatory response. The combined exposure also led to changes in the faecal metabolome, specifically affecting compounds related to amino acids, carbohydrate and purine metabolism. The authors acknowledged the complexity of the TiO_2 and BPA interaction and the need for additional research to elucidate mechanisms involved in the combined effects.

Lin *et al.* (2023) conducted a subchronic (90 days) oral toxicity study of TiO_2 NP (average diameter: ~40 nm) in Sprague-Dawley rats following the Organization for Economic Cooperation and Development (OECD) test guideline 408. The experimental doses (10, 100 and 1 000 mg/kg bw/day) were administered via

gavage. The middle dose was based on the maximum exposure of TiO_2 in children (0.16–1.04 mg/kg bw/day) reported by EFSA after the safety assessment of the additive E171 (EFSA, 2016a), multiplied by a factor of 100. This study included two more groups of rats exposed for (1) 45 days (mid-term) or (2) 90 days, followed by a 28-day clearance period. No abnormal general signs or adverse effects were observed after assessing the hematology, clinical biochemistry and numerous parameters related to the function and structure of multiple organs. The authors reported a limited number of statistical differences. However, they indicated that biochemistry values fell within normal ranges (compared to their historical data). Clinical differences were isolated and intermittent and structural lesions were sporadic and spontaneous, leading the authors to conclude that the findings were biologically or toxicologically irrelevant. The evaluation of titanium content in the different tissues revealed that there was no systemic distribution of this element. TiO_2 NPs had a limited impact on the microbiota. The text compound did not alter α- and β-diversities or the microbiota composition at the phylum level. Only the relative abundance of a few genera increased in the high-dose groups, *Bacteroides* and *Eubacterium* in males and *Oscillibacter* in females. Based on these results, the authors reported the high dose (1 000 mg/kg bw/day) as the NOAEL for TiO_2. In this study, particles were shown to aggregate in gastrointestinal juices, with reported hydrodynamic sizes of over 400 nm. The authors suggested that such aggregation could have changed the bioavailability and kinetics of the TiO_2 NPs, which was a plausible reason behind the limited observed biological effects. The research group did not evaluate oxidative stress or inflammatory responses, which they considered a study limitation.

Agans *et al.* (2019) evaluated the impact of 100 mg/day TiO_2 NP (average diameter: ~25 nm) and Ag NP (average diameter: ~30–50 nm) on a human microbiota using a human gut simulator (HGS) model. The model consisted of three fermentation vessels mimicking the three colon regions (proximal, transverse and distal) containing a medium similar to that found in an individual consuming a Western diet and inoculated with colonic microbiota from three healthy volunteers. The 7-day treatment started after the microbiota stabilized, followed by another 7-day clearance period. Both NP types decreased the microbial cell density, especially in the Ag NP group. Changes in both groups went back to baseline during the clearance period although at a difference pace (faster in the TiO_2 group). Titanium dioxide did not alter the diversity and composition of the microbiota, their predicted microbial functional capacity or SCFA production. These parameters were not evaluated in the Ag NP group. The authors explained that TiO_2 NP aggregation in the model medium (hydrodynamic size > 3 000 nm) could be the reason for the limited effects in the microbiota. The size of Ag NP could be seen unchanged and inside bacterial cells. The authors highlighted the limitations of the *in vitro* study, including absence of host and host-microbiota interactions, and the reliance only on a predictive metagenomics-based model to evaluate functional microbial capacity.

SILVER

Annex III.18. contains a summary of the studies evaluating silver.

Bredeck *et al.* (2021) evaluated the effect of nanomaterials used in food and consumer product applications Ag, TiO_2, SiO_2 and CeO_2 (not used in food) in C57BL/6J mice. The test compounds were non-food grade (to facilitate comparison with existing studies) and were contained in feed pellets. The particles were evaluated in two different studies: In the first study, female mice were exposed to 1 percent CeO_2 NP (average diameter: ~35 nm) and SiO2 NP (average diameter: ~13 nm) for 21 days. In the second study, 1 percent TiO_2 NP (average diameter: ~26 nm) and Ag NP (average diameter: ~40 nm, with polyvinylpyrrolidone (PVP) as a dispersant to prevent aggregation) were given to male and female mice for 28 days. The selection of doses (expressed as 1 percent w/w feed) was based on the maximum permitted level of the additives in food (1 percent) set by the United States FDA. The estimated daily intake was about 2 000 mg/kg bw for the 1 percent doses, and 400 mg/kg bw for 0.2 percent Ag NP. The host did not present macroscopic lesions and the treatment did not affect body weight. Treatments did not affect α- or β-diversities, with only limited effects on the microbiota composition. These included a reduction in the relative abundance of Actinobacteria in the SiO_2 group and increased *Roseburia* in female mice and Tenericutes in males of the Ag group. Some other treatment-independent but gender-dependent changes were also observed in the study. According to the authors, the results suggested that oral exposure to the nanomaterials under the studied conditions did not pose a major health hazard, although individual susceptibilities should be further studied. Since different studies evaluating nanomaterials reported inconsistent microbiota effects, the authors highlighted the need to standardize microbiome study designs, to consider gender as a variable as well as to define the microbiota composition before the treatment.

Another study evaluated the subacute oral toxicity of two forms of Ag, either nanoparticulate (average diameter: 14 nm, PVP-stabilized suspension) or ionic (silver acetate) in Wistar rats (Hadrup *et al.*, 2012). The treatment was administered by gavage for 28 days at the following doses: 2.25, 4.5 or 9.0 mg/kg bw/day for Ag NP and 14 mg/kg bw/day for Ag acetate (equivalent to the high Ag NP dose). The caecal microbiota, which was limited to the evaluation of the abundance of phyla Firmicutes and Bacteroidetes, was not disturbed by any of the treatments. Silver resistance genes were also monitored but the treatments did not pose sufficient selection pressure to up-regulate them. Biochemistry, several organ biomarkers and endpoints were evaluated in the rats. No observed adverse effects were reported for Ag NP, and the NOAEL was set as 9 mg/kg bw/day. However, the equivalent dose of Ag acetate resulted in alterations to a few markers, including decreased body weight, increased plasma alkaline phosphatase (ALP), reduced plasma urea, and reduced absolute and relative thymus weights. Moreover, Ag acetate was found at higher concentrations in plasma and organs compared to Ag NP. The authors highlighted the importance of careful interpretation when evaluating alterations of single markers of low specificity if they are not placed in context, along with other

related findings. This is the case with ALP, a biomarker not specific to liver function, as it is also present in other organs. Therefore, the elevated ALP levels are relevant when complemented with findings indicative of liver, kidney, bone or intestinal dysfunction and/or histopathological change.

Williams *et al.* (2015) evaluated the impact of different nanoparticulate Ag sizes (average diameter: 10, 75 and 110 nm, citrate-stabilized suspension) at various doses (9, 18 and 36 mg/kg bw/day) and Ag acetate (100, 200 and 400 mg/kg bw/day) administered to male and female Sprague-Dawley rats by gavage for 13 weeks. The ileum was the targeted intestinal section because Ag NPs are absorbed in this region and taken up by Peyer's patches (Hadrup and Lam, 2014). Animals treated with the high dose Ag acetate were moribund or developed severe gastroenteritis at mid-dose. Other observed effects were dose- and size-dependent (more evident at the lowest dose and smallest particle size) and influenced by gender. Such effects included alterations of targeted mucosa (ileum)-associated microbiota members (Firmicutes, Bacteroidetes, *Bacteroides, Lactobacillus, Bifidobacterium* and Enterobacteriaceae) and downregulated expression of marker genes related to the intestinal functional immunity. Such expression was not observed in the high-dose Ag NP and Ag acetate groups, probably indicating that nanoparticle interactions are more relevant than Ag ions. The authors concluded that the potential health impact derived from the observed disturbances caused by Ag NP is unknown and would require additional research.

Wilding *et al.* (2016) evaluated two sizes of Ag NP (average diameter: 20 or 110 nm) stabilized with either PVP or citrate. This study included a positive control given Ag ions (Ag acetate). C57BL/6NCrl mice were gavaged with each test compound at a dose of 10 mg/kg bw/day for 28 days. The dose was equivalent to 2 000 times the United States Environmental Protection Agency's oral reference dose for colloidal silver (0.005 mg/kg bw/day) (EPA, 1988). Building on previous *in vitro* findings showing the antimicrobial properties of Ag NP, the study aimed to determine whether these effects are reproducible *in vivo*. However, the diversity and composition of the caecal microbial community in all four treatment groups did not differ from the controls. The researchers discussed several possibilities for the discrepancy between the antimicrobial activity observed *in vivo* and *in vitro* (other studies). These included differences in Ag NP concentration between the two studies, the lack of host modulation of *in vitro* systems, differences in the physicochemical properties of Ag NP particles and how they are affected by the environmental conditions along the gastrointestinal tract (precipitation or aggregation reduce free Ag ions bioavailability).

van den Brule *et al.* (2016) gave rodent chow supplemented with Ag NP (average diameter: ~55 nm, PVPstabilized) to C57BL mice at doses of ~ 0.011, 0.114 and 1.140 mg/kg bw/day Ag NP, (0.009, 0.071 or 0.679 mg/kg bw/day after measuring feed consumption). The doses were based on estimates of Ag NP intake in adult humans (70–90 µg/day, ~0.011 mg/kg bw/day) (Wijnhoven *et al.*, 2009) and multiplied by a factor of 10 because the daily intake was likely to be underestimated.

The experiments followed the OECD guideline for testing chemicals 407 (repeated dose 28-day oral study in rodents). The treatment had no apparent effect in the mouse (body weight, intestinal damage or structural alterations, C-Reactive protein). However, it led to dose-dependent changes in the microbiota. This included reduction of diversity evenness (not richness) and distinct β-diversity between treatment and control groups. The microbiota composition was affected by Ag NP at phylum (decreased relative abundance of Bacteroidetes and increased Firmicutes) and lower taxa levels (decreased Odoribacteraceae, Bacteroidaceae, S24-7 family and increased Lactobacillaceae, Lachnospiraceae). This study was replicated at months 4 and 8 using the same Ag NP-supplemented feed (aged feed), resulting in a reduction (4 months) or no significant effects (8 months) on the microbial population. To help explain this finding, the researchers monitored Ag sulfidation in feed pellets, using freshly prepared Ag NP-supplemented pellets and air as controls. They observed an age-dependent increase in the Ag sulfidation, accompanied by a reduction in the release of Ag ions, which is indicative of a decreased bioavailability. The authors emphasized the importance of evaluating Ag NP using realistic scenarios (e.g. feed vs gavage) and monitoring their bioavailability (Ag+ release) and degree of sulfidation. They also highlighted the need to develop approaches to improve the translatability of observations from rodent models to a human exposure scenario.

Cattò *et al.* (2019) conducted a 24-hour *in vitro* study (fermentation vessel with medium mimicking high fat/high protein diet) to evaluate the impact of 1 µg/ml Ag NP (average diameter: ~14 nm citrate stabilized) on the diversity, composition and function of human faecal microbiota. The probiotic *Bacillus subtillis* was also evaluated alone or in combination with Ag NP. The short exposure did not affect the diversity, core microbiota composition, or SCFA production. Also, no cytotoxic or genotoxic effects were observed after transferring cellfree media to Caco-2 cells and incubating for 24 hours. The only significant observations in the Ag NP group were a decrease in the targeted bacterial *Faecalibacterium prausnitzii* and *Clostridium coccoides/Eubacterium rectales* and changes in the predicted microbial function of four gene categories (decreased cell motility, translation, transport and catabolism, and increase in xenobiotics degradation and metabolism). The latter was not observed in the *B. subtilis* groups (alone or combined with Ag NP), which the authors interpreted as a possible protective activity of the probiotic.

Cueva *et al.* (2019) evaluated two types of Ag NPs (average diameter: ~4–6 nm, PEG-stabilized or average diameter: ~3–5 nm, GSH-stabilized) *in vitro* in a static fermentation model mimicking the conditions of the large intestine or Ag NP-GSH using a dynamic simulator of the gastrointestinal tract (Simgi®). This computer-controlled model consisted of five interconnected vessels simulating the stomach, small intestine, and ascending, transverse, and descending colon, where the content moves from one compartment to the next by peristaltic movement. Experimental doses were 11 µg/mL Ag NP-PEG or 7.6 µg/mL Ag NP-GSH. The authors highlighted the food-context relevance of the selected particles and concentrations as they are used to control the growth of some microorganisms involved in wine making, helping reduce other additives (e.g. sulphites).

Both model systems were inoculated with human faecal microbiota from healthy individuals. The exposure experiments were run for 48 hours, and in the Simgi® model, a single dose exposure was followed by an 8-day washout period (samples were collected on days 1, 2, 3, 4, 5 and 8). The microbiota was evaluated by targeting specific microbial groups by plate counting and qPCR (total aerobes, total anaerobes, Enterobacteriaceae, *Clostridium* spp., lactic acid bacteria and *Enterococcus* spp. Although some disturbances were observed (gastrointestinal segment- and donor-dependent), the researchers concluded that there were no significant changes in microbial composition or their metabolic activity (proteolytic activity). However, changes in particle structures were observed after exposure to gastrointestinal fluids.

OTHER COLOURS

Annex III.16. contains a summary of the study evaluating other colours.

He *et al.* (2021) evaluated the impact of four azo-colorants in a series of experiments investigating their role in the development of colitis in R23FR mice. Other mice were used to further assess or confirm some study outcomes, including FR, *IL-22*$^{-/-}$, CD45.1, *Rag1*$^{-/-}$, *Ifng*$^{-/-}$, germ-free *Rag1*$^{-/-}$, germ-free R23FR, *Ifng*$^{-/-}$ *Rag1*$^{-/-}$. All mice had the C57BL/6 background. The compounds tested were Allura Red AC (Red 40, E-129), Erythrosine (Red 3, E-127), Sunset yellow FCF (Yellow 6, E-110) and Brilliant Blue FCF (Blue 1, E-133). From the chemical supplier site, only Brilliant Blue was reported to be suitable for use in food and beverages. The colours were provided in drinking water (0.025 percent w/v, 0.25 g/L) or rodent chow (0.25 g/kg, Allura Red only) for 3 weeks with 7-day clearance periods between the treatment weeks.

Mice overexpressing the cytokine IL-23 developed colitis in the Allura Red and Sunset Yellow groups. The colitis onset was dependent on the microbial reduction of azo dyes, particularly on the product metabolite 1-amino-2-naphthol-6-sulfonate sodium salt (ANSA-Na). The researchers identified the ability of commensal bacteria *Bacteroides ovatus* and *Enterococcus faecalis* to metabolize Allura Red and Sunset Yellow. They concluded that these colorants are risk factors for experimental IBD in conditions of immune dysregulation. The researchers also reported several limitations of their study. The outcomes were observed in the mouse model, requiring additional research to assess if similar effects happen in humans. Further research should focus on elucidating the mechanisms through which IL-23 alters the immune response to Allure Red, shifting from tolerance to colitis, and on understanding how colour metabolites induce the immune response.

PRESERVATIVES

Annex III.14. contains a summary of the studies evaluating preservatives.

Hrncirova *et al.* (2019) evaluated the susceptibility of bacterial strains isolated from the stools of three healthy adult donors to three preservatives (sodium benzoate, sodium nitrite and potassium sorbate) and their combinations. The bacteria identified were *Escherichia coli, Enterococcus faecalis, Lactobacillus paracasei, Bifidobacterium longun, Bacteroides coprocola, Helicobacter hepaticus, Bacteroides thethaiotaomicron* and *Clostridium tyrobutyricum*. Serial dilutions of the compounds with concentrations ranging from 1 µg to 100 mg/ml were tested for 6–10 h for aerobic strains and 2–3 days for anaerobic strains. IC_{50} values showed differences in susceptibility among the different strains, with *Bacteroides coprocola* as the most sensitive, especially to nitrite and all nitrite combinations ($IC_{50} \leq 0.1$ µg/ml), and *Enterococcus faecalis* the most resistant to all additives and their combinations ($IC_{50} \geq 10$ mg/ml). In general, sodium nitrite and its combinations had the most potent effects, with the combination of benzoate, nitrite and sorbate showing the highest degree of synergistic effect. The scientists identified several limitations of their study. These included (1) the limited number of preservatives evaluated, (2) the ratio of additive combinations led to effects that could differ when the preservatives are combined in different proportions, and (3) the evaluation focused only on cultivable bacterial strains. In addition, the authors highlighted the need for more holistic approaches to evaluate the additives, specifically mentioning chronic exposure of the gut microbiome to food additives using suitable *in vivo* models and omics analyses.

The impact of the same preservatives on the gut microbiota was evaluated on C57BL/6 mice (Nagpal, Indugu and Singh, 2021). The test compounds were provided in the feed *ad libitum* for 12 weeks at the following concentrations: 0.1 percent benzoic acid (BA), 0.3 percent potassium sorbate (PS) and 0.05 percent sodium nitrite (SN), which corresponded to daily intakes of 0.019, 0.049, and 0.007 mg/kg bw, respectively (calculated by the researchers based on feed consumption).

This study focused primarily on the evaluation of the faecal microbiota. The analysis of β-diversity showed distinct microbial signatures in the three treatment groups, while PS reduced α-diversity. Regarding the microbiota composition, all treatment groups reduced Proteobacteria, Erysipelotrichae and *Sarcina*, and increases in Actinobacteria, *Lactobacillus* and *Blautia*. Some other changes were dependent on the tested compound. For example, BA and PS increased the abundance of Bacteroidetes, *Parabacteroides* and *Lactobacillus*. BA increased *Bacteroides* and *Ruminococcus* and reduced the abundance of *Turicibacter*. SN increased Verrucomicrobia, *Turicibacter* and *Akkermansia*. Based on these results, the authors indicated that the preservatives did not induce gut dysbiosis or have a negative impact on beneficial bacteria. In the host, the study only evaluated the expression of tight-junction genes, showing a reduction in some of them after the treatment with the three preservatives. However, the researchers suggested additional studies to confirm and evaluate the biological meaning of their findings, as well as the impact of the preservatives on the interactions between mucosal-associated microbiota and epithelial integrity and function. They also indicated some limitations of their study, including the use of male mice only, which did not allow the evaluation of gender-specific effects.

CHAPTER 5
DISCUSSION

In recent years, the microbiome has gained recognition as a fundamental element of ecosystems, which can influence the well-being and functioning of the niche it inhabits. Research has proven the participation of the gut microbiome in different physiological processes, such as digestion or maintenance of the intestinal barrier. However, much research attributing the microbiome roles in health and disease is based on associations, where the causal relationship lacks demonstration or clear underlying mechanisms. This situation has created a grey area susceptible to speculation. When considering the role of the gut microbiome in health or disease, it is important to keep in mind that (1) microbiome-host interactions are numerous and complex, (2) information obtained with omic technologies can be challenging to interpret, and (3) oversimplification of the research context can exclude multiple key elements relevant to the overall microbiome-host ecosystem. Based on all the points above, evaluating the science addressing if and how food additives potentially affect the gut microbiome and subsequent health outcomes is essential before drawing conclusions about their overall impact. Therefore, this discussion will consider the following points:

- study design, analytical methodologies and influential research components in research outcomes;
- factors affecting scientific quality and rigour;
- result interpretation;
- scientific limitations, knowledge gaps, and research needs;
- areas for improvement; and
- considerations for risk assessment.

MODELS

Selecting a suitable model for studying the gut microbiome is a critical step that can significantly influence the validity, applicability and translatability of study results. The choice of model depends on the research question and the specific objectives of the study. Critical criteria include the model's similarity to human physiology (biological relevance), its manipulability, availability, reproducibility (including validation and standardization), maintenance requirements, cost, and ethical considerations.

IN VIVO

Model species and genetic background

Animal models allow the evaluation of microbiome-host interactions under controlled conditions and help control and minimize some of the confounding effects observed in human studies (Kostic, Howitt and Garrett, 2013; Pham and Mohajeri, 2018). Different models can serve different research purposes and contexts. For example, they can be used to investigate the effects of dietary chemicals (e.g. food additives) in healthy and vulnerable populations or the mechanisms underlying how the gut microbiome influences health outcomes, such as the onset or progression of diseases, following exposure to chemicals.

The exposure of the gut microbiome to food additives has been investigated in:

> healthy animals;

> models genetically predisposed to certain disorders or models of disease, which is often induced chemically;

> genetically modified animals (i.e. knockout) to mimic certain genetic conditions or to evaluate mechanisms; and

> animals with depleted microbiota after antibiotic treatment or born and bred microbiota-free animals, which have also been used to assess causal relationships between the gut microbiome and health outcomes.

Most of the studies included in this review were carried out *in vivo*, mainly in rodent models, of which 84 percent were conducted in mice and 16 percent in rats. Inbred C57BL/6, or mice with this genetic background, have been the most frequently chosen strain. C57BL/6 has been reported to have a more stable gut microbiota than other common laboratory mice, e.g. BALB/c, therefore offering a more resilient microbial community and a more stable symbiosis between the host and the microbiota (Guo *et al.*, 2022). Other mice strains were less commonly used, including CD-1, Swiss Webster, Kunming, BALB/c, ICR, SAMP1/YitFc or AKR/J. Both outbred Sprague-Dawley and Wistar rats are also used in several of the studies reported in this review.

Inbred strains (e.g. C57BL/6 or BALB/c mice) are genetically similar, reducing the effect of genetic variability (confounding factor) on research outcomes and facilitating more consistent and reproducible data (Hugenholtz and de Vos, 2018). On the contrary, outbred strains (Swiss Webster, ICR, CD1 mice and Sprague Dawley and Wistar rats) are non-homogeneous populations with high genotypic and phenotypic variance, being more representative of human populations. Such genetic similarities or dissimilarities can be a factor influencing intra-individual microbial variation.

Germ-free laboratory animals or animals with depleted microbiota, most commonly mice and rats, are used to evaluate mechanisms and causal relationships of microbiome-host interactions. The strains most commonly used in the studies were C57BL/6 or Swiss Webster. This topic is further discussed in the section Cause-effect: associations and causality.

Mahalak *et al.* (2020) conducted the only non-human primate study on a tufted capuchin monkey (*Cebus apella*). This monkey species is broadly used in biomedical research as it shares several commonalities with humans, e.g. biochemistry, immunology, neurology and anatomy. However, Firrman *et al.* (2019) reported that the main microbial phyla of *C. apella* are Firmicutes and Proteobacteria, differing more from humans than other monkey species such as the Rhesus monkey (*R. macaque*), which also has a high representation of Bacteroidetes.

The evaluation of food additives on the gut microbiota and its potential contribution to different disorders has been assessed using rodent models only. Here are two main approaches used in these studies:

> Diet- or chemically-induced models: In this approach, researchers induce the disease in rodents using specific diets (HFD-induced obesity) or chemicals (DSS-induced colitis). The animals typically used in such studies are genetically predisposed to the conditions.

> Genetically modified rodents: These animals often lack or overexpress genes known or thought to be involved in the disorder. These animals are used as surrogates of vulnerable populations or to investigate mechanisms underlying causal relationships.

Obesity is typically induced by feeding rodents with a predisposition to this disorder high-caloric diets. C57BL/6 mouse is a popular model in dietary intervention studies as it develops an obese phenotype and obesity-related disorders (e.g. type-2 diabetes) when fed a high-fat diet (HFD) (Hugenholtz and de Vos, 2018; Wong *et al.*, 2016). In this context, HFD-fed C57BL/6 mice were used as a model to evaluate sucralose (Wang *et al.*, 2018; Xi *et al.*, 2020), saccharin (Suez *et al.*, 2014), stevia (Becker *et al.*, 2020), erythritol (Han, Kwon and Choi, 2020; Han *et al.*, 2020), xylitol (Uebanso *et al.*, 2017a), polysorbate 80 (P80), carboxymethyl cellulose (CMC) (Chassaing *et al.*, 2017) and κ-carrageenan (Mi *et al.*, 2020). Sprague-Dawley and Wistar rat strains are known for their susceptibility to diet-induced obesity and insulin resistance (Buettner, Schölmerich and Bollheimer, 2007). The Sprague Dawley (SD) rat is the best-characterized diet-induced obese model (Lutz, 2020). Both rat strains fed high-caloric diets were chosen to evaluate the potential of several food additives to induce microbiome-mediated metabolic alterations, specifically, aspartame (Nettleton *et al.*, 2020; Palmnas *et al.*, 2014) and rebaudioside A (Nettleton *et al.*, 2019) in Sprague-Dawley and sucralose and steviol glycosides in Wistar rats (Sanchez-Tapia *et al.*, 2020).

There is also an interest in evaluating food additives in the context of inflammatory bowel disease and cancer. Several research groups have investigated P80 and CMC in immune-deficient mice that develop spontaneous colitis, like IL-10$^{-/-}$ (Chassaing *et al.*, 2015; Rousta *et al.*, 2021). This deficient mouse is the most-studied colitis model (Johansson and Hansson, 2016). Mouse models of dextran sodium sulfate (DSS)-induced colitis or ileitis have also been used to evaluate the impact of sucralose (Guo *et al.*, 2021), titanium dioxide (Mu *et al.*, 2019) and curdlan (Rahman *et al.*, 2021) on the susceptibility to intestinal inflammation. In addition, as intestinal

inflammation has been associated with an increased risk of colorectal cancer, a mouse model of colitis-induced colorectal cancer (C57BL/6 treated with DSS and azoxymethane) has been used to evaluate the impact of P80 and CMC (Viennois *et al.*, 2017) and sucralose (Li *et al.*, 2020a). As mutations of the adenomatous polyposis coli (APC) gene – a key tumour suppressor gene – have been linked to an increased risk of colon cancer (Aoki and Taketo, 2007), Viennois and Chassaing (2021) chose the mouse APC^{min} model to evaluate the impact of P80 and CMC in cancer initiation and progression. This mouse strain develops spontaneous intestinal neoplasias, which makes them susceptible to cancer (Moser, Pitot and Dove, 1990).

Apolipoprotein E-deficient ($APOE^{-/-}$) mouse, one of the first and best-characterized models of atherosclerosis disease in humans (Golforoush, Yellon and Davidson, 2020; Oppi, Lüscher and Stein, 2019), was chosen to evaluate the exposure to titanium dioxide (Zhu *et al.*, 2022).

Several reviews are available for more insights into animal models specific to the microbiome study (Douglas, 2019; Hugenholtz and de Vos, 2018) or models of disease, including rodent models of immune-mediated diseases (Hansen and Hansen, 2021), obesity and type 2 diabetes (Lutz, 2020), metabolic syndrome (Wong *et al.*, 2016) and atherosclerosis (Golforoush, Yellon and Davidson, 2020; Oppi, Lüscher and Stein, 2019)

Gender

Gender is one of the factors that contribute to shaping the microbiome. It is not only influenced by hormonal status but also by gender-specific immune activities. Such differential modulation of the gut microbiome can further contribute to the differences in the immune system between males and females (Fransen *et al.*, 2017).

All interventional and observational human studies recruited both males and females (Ahmad, Friel and Mackay, 2020a; Beards, Tuohy and Gibson, 2010; Chassaing *et al.*, 2021; Frankenfeld *et al.*, 2015; Laforest-Lapointe *et al.*, 2021; Ramne *et al.*, 2021; Serrano *et al.*, 2021; Suez *et al.*, 2022; Suez *et al.*, 2014). The only exception was Thomson *et al.* (2019), who excluded females to avoid the potential influence of menstrual cycle changes in insulin sensitivity following short-term exposure to sucralose. Although these studies included males and females, the studies did not report gender differences.

The majority of animal studies were conducted only on males. Some studies included both genders but it was not always clear if researchers evaluated the gender influence in the study outcome as it was not mentioned or referenced in the results or discussion. However, some research groups reported gender-dependent outcomes. Bian *et al.* (2017a) observed sex-specific differences in microbiota composition and faecal metabolome of mice given acesulfame-K. Becker *et al.* (2020) also reported sex as a relevant driver for the differences between-sample diversity and composition in mice given stevia or saccharin and fed HFD. Based on these results, the authors highlighted the need to consider both genders in animal studies evaluating the microbiota.

Also, in the context of a high fat-sucrose diet, male and female offspring (mothers receiving aspartame and stevia) had different microbial composition. In this study, aspartame altered insulin sensitivity of male offspring only. CMC and P80 exposure in animal models have led to some degree of gender-dependent effects on cancer development (Viennois and Chassaing, 2021), microbiome and behaviour (Holder *et al.*, 2019; Jin *et al.*, 2021). Bredeck *et al.* (2021) and Williams *et al.* (2015) reported gender differences in the microbial community structure of mice after treatment with silver nanoparticles. Like other research groups, (Bredeck *et al.*, 2021) also highlighted the importance of studying the microbiota in both genders.

Not all authors specified the gender of animals used in the studies (Dudefoi *et al.*, 2017; He *et al.*, 2021; Laudisi *et al.*, 2019; Li *et al.*, 2014; Li *et al.*, 2020a; Martínez-Carrillo *et al.*, 2019; Serrano *et al.*, 2021; Shang *et al.*, 2017; Wang *et al.*, 2018; Xi *et al.*, 2020).

Age and early exposure

In addition to animal species, genetic background and gender, age is also relevant when studying the gut microbiome because the microbial community structure changes during the individual's lifespan (Martino *et al.*, 2022). Therefore, the age of subjects participating in a study can influence the interactions between additives and the gut microbiome. Furthermore, to better understand how food additives might influence disease and reflect real-life situations more accurately, studies need to consider the age groups most susceptible to the disease.

Most of the rodent studies included in this review were conducted on young animals, typically 3–8 weeks old and no older than 12–14 weeks. It was interesting to observe that animal age was not specified in many publications (n=15). Based on these figures, treatments start at ages when the gut microbiome has not reached stability. In a survey to assess the age range of rodent models used in biomedical research, researchers appear to have varying opinions on the age at which rodents reach adulthood, with a survey indicating that scientists' estimates ranged from 6 to 20 weeks (Jackson *et al.*, 2017). Most responders answered between 6–10 weeks, when mice become sexually mature. However, mice are considered fully developed between 3 and 6 months of age (Flurkey, M. Currer and Harrison, 2007), but when does the microbial community of mice reach stability? If a study is designed to investigate the effects of a dietary treatment when the gut microbiome is still developing, could age introduce additional variability, therefore confounding the interpretation of the results?

In the human context, the first 1 000 days of life are considered critical for child growth and development (Robertson *et al.*, 2019). The gut microbiome starts developing at birth, reaches its maturity in adolescence, remains practically stable during adulthood, and becomes compositionally unstable and less diverse in the elderly (Lynch and Pedersen, 2016). How the gut microbiome develops at early stages can influence its community structure and function later in life, and it may predispose the individual to diseases such as metabolic and immune-mediated disorders (Rautava, 2021). For this reason, there is a special interest in the effect of dietary substances (e.g. food additives, antibiotics) in the microbiome of young individuals, through direct exposure to food or indirectly from mothers during gestation and lactation. This subject has only been covered by five animal studies evaluating a combination of acesulfame-K and sucralose (Olivier-Van Stichelen, Rother and Hanover, 2019), aspartame or stevia (Nettleton *et al.*, 2020), sucralose (Dai *et al.*, 2020), polysorbate 80 (Jin *et al.*, 2021) and titanium dioxide (Su *et al.*, 2021). In addition, one human observational study monitored the microbiota and urine metabolome of children during their first year of life after maternal consumption of artificially sweetened beverages during pregnancy (Laforest-Lapointe *et al.*, 2021). The effects of these additives on the microbiota and the host are discussed later in the section *Effects of food additives on the gut microbiota and the host*.

IN VITRO

Although *in vitro* systems are limited in capturing bidirectional microbiome-host interactions, they offer several advantages, including highly controllable environments, affordability, reproducibility and less ethical burden (Pham and Mohajeri, 2018). *In vitro* systems are useful for determining direct interactions between the microbial community and the test compound, as well as intra-community interactions in response to chemical exposure under some physiological conditions. More specifically, they have been used to evaluate shifts in the microbial composition and activity (e.g. production of SCFA, vitamins) in the presence of certain compounds (e.g. food additives, prebiotics or antibiotics), microorganisms (e.g. pathogens, probiotics) or in response to changes in environmental conditions or stressors (e.g. pH, pO_2).

These systems have also been useful in investigating microbial activation or inhibition of metabolic pathways, production of microbial metabolites, or microbial transformation of non-nutritional compounds (e.g. pharmaceuticals, pollutants) (Nissen, Casciano and Gianotti, 2020). *In vitro* systems encounter several challenges. For example, no universal medium allows all microbiota members to grow (Biagini *et al.*, 2023). Also, establishing environmental conditions accurately representing each intestinal section proves difficult due to inter-individual variations (e.g. colonic pH). The pH factor can either inhibit or promote the growth of specific microbial groups (Biagini *et al.*, 2023). *In vitro* systems do not provide information on microbiota-host interactions due to the absence of anatomical structures and the full array of physiological features that regulate microbiota activity, including immune responses. Consequently, translating *in vitro* findings into the human context becomes a challenging endeavour.

Different *in vitro* systems have been used to study the impact of food additives on the gut microbiome, from simple plate culture to complex and dynamic gastrointestinal simulator systems. In some instances, *in vitro* assays have been used to complement *in vivo* studies to provide additional information on the interaction between the additive and specific microbiota members. Often, these studies target specific microbial species and are used to:

- evaluate the bactericidal or bacteriostatic activity of the additive or ability of bacteria to grow in the presence of, for example, sweeteners (Li *et al.*, 2014; Mahalak *et al.*, 2020; Olivier-Van Stichelen, Rother and Hanover, 2019; Rodriguez-Palacios *et al.*, 2018b; Sunderhauf *et al.*, 2020; Wang *et al.*, 2018), emulsifiers (Elmén *et al.*, 2020), or preservatives (Hrncirova *et al.*, 2019);
- characterize bacteria's utilization of food additive, e.g. sugar alcohols (Hattori *et al.*, 2021; Sato *et al.*, 2017; Xiang *et al.*, 2021);
- identify and characterize the species present in treatment samples (Martínez-Carrillo *et al.*, 2019); and
- investigate positive microbial interactions like cross-feeding, typically monitored in co-cultures of specific bacteria. Different gut bacteria have diverse enzymatic repertoire, and in positive microbial interactions, the products resulting from the degradation of a compound by one bacterium can be used as a source of energy or further metabolized by another bacteria (Canon *et al.*, 2020; Das *et al.*, 2018). For example, a cross-feeding relationship was observed in the use of xylitol among *Lactobacillus reuteri, Bacteroides fragilis and Escherichia coli* (Xiang *et al.*, 2021). Also, κ-carrageenan oligosaccharides were more effectively degraded in co-culture of *Bacteroides xylanisolvens* and *Escherichia coli* isolates from faecal samples, than in single culture of *B. xylanisolvens*, which seemed to be the primary degrader (Yin *et al.*, 2021).

Although single microbial cultures can provide information on their interactions with food additives, these observations need to be interpreted with caution because the interaction or effect may not be the same in the presence of the entire microbial community or in the intestinal environment.

The effects of food additives on human or rodent faecal microbiota were evaluated in fermentation systems of diverse complexity, containing different media compositions and simulating different gastrointestinal conditions. The most basic formats, such as media broth culture and static batch fermentation, only allow for short-term studies as media cannot be supplemented or refreshed. For example, Brain Heart Infusion broth was used to evaluate the impact of five emulsifiers on the composition, SCFA production and levels of virulence factors in human faecal microbiota (Elmén *et al.*, 2020). Gerasimidis *et al.* (2020) used a batch fermenter to evaluate the composition and metabolic activity of human faecal microbiota in the presence of sucralose, stevia and commercial aspartame (Canderel), carboxymethyl cellulose, polysorbate 80, κ-carrageenan, sodium sulfite, sodium benzoate or cinnamaldehyde. Batch fermentation was also employed to evaluate the ability of human faecal microbiota to degrade high- and low-molecular-weight κ-carrageenan (Yin *et al.*, 2021), to evaluate the impact of emulsifiers (CMC, P80, soy lecithin, sophorolipids and rhamnolipids) in the composition and function of human faecal microbiota (Miclotte *et al.*, 2020), and to monitor the effects of silver nanoparticles (Cattò *et al.*, 2019).

Continuous systems are more versatile, often computer controlled, and allow the feeding of fresh media to the system, which enables longer study periods. They are available as single or multiple chambers connected in sequence. Single chambers like the chemostat have been employed to evaluate different food-grade titanium dioxide preparations on a defined intestinal microbial community (Dudefoi *et al.*, 2017). A single colon reactor was also used to compare the impact of food- and industrial grade titanium dioxide on human faecal microbiota (Waller, Chen and Walker, 2017). Mahalak *et al.* (2020) assessed the commercial product Splenda Naturals plus Stevia (erythritol and rebaudioside D) and erythritol in a continuous system inoculated with faecal microbiota from one person. The colonic simulator GIS1 (GIS Systems, 2023) was used to investigate the effects of several commercial preparations (sodium cyclamate, sucralose, saccharin and steviol) (Vamanu *et al.*, 2019) and stevioside (Gatea, Sârbu and Vamanu, 2021) while Naimi *et al.* (2021) evaluated twenty different emulsifiers in MiniBioReactor arrays.

More complex systems can connect several vessels mimicking environmental conditions of different gastrointestinal sections, including peristaltic movements (e.g. SHIME®[28] [Van de Wiele *et al.*, 2015]; TIM[29] [TNO, 2013]; SIMGI®[30] [CIAL, 2023]) or even a mucin surface (mucosal SHIME® or M-SHIME®) (Pham and Mohajeri, 2018). Several of these continuous models have been used to evaluate the effect of sweeteners on the composition and activity (e.g. production of microbial metabolites) of human faecal or intestinal samples. A three-vessel system (CDMN) simulating the ascending, transversal and descending colonic sections, including mucin-covered beads, was used to evaluate xylitol (Xiang *et al.*, 2021).

28 The Simulator of the Human Intestinal Microbial Ecosystem (SHIME®).

29 Gastrointestinal model (TIM).

30 Dynamic Gastrointestinal Simulator (SIMGI®).

Suez *et al.* (2014) treated mice microbiota with saccharin in chambers but did not provide any details about the system used. Agans *et al.* (2019) evaluated the impact of titanium dioxide on human distal gut microbiota inoculated into a colonic simulator consisting of three vessels replicating conditions of the three colonic sections. The SIMGI® system composed of 5 interconnected compartments simulating the conditions of the stomach, small intestine, and the three colon sections (ascending, transverse and descendent) was the choice of (Cueva *et al.*, 2019) to evaluate the potential effects of two different forms of silver nanoparticles on human faecal microbiota.

When cultivating gut microbiota in these *in vitro* models, a critical question is how the freshly obtained faecal microbiota from donors (or pooled donors) compares with the microbiota after reaching stability in the bioreactor. Chassaing *et al.* (2017) observed a rapid change in bacterial composition after inoculating the M-SHIME model (9 vessels mimicking the conditions of stomach, small intestine and colon) with human faecal microbiota, with a reduction of about 50 percent α-diversity after reaching stability (evaluated by 16S rRNA gene sequencing). This observation illustrates the need to monitor the stability of the microbial community and characterize the microbiota baseline at time 0 of the experimental phase of the *in vitro* study. Another aspect to consider is related to the origin of the microbiota sample (typically faecal) and the physico-chemical conditions of the vessels. Reports indicate that faecal and distal colon microbiotas are closer in composition than the microbiotas of proximal gastrointestinal regions (Donaldson, Lee and Mazmanian, 2016; Gu *et al.*, 2013; Lkhagva *et al.*, 2021; Shalon *et al.*, 2023). For this reason, it is likely that, for example, faecal microbiota inoculated in simulators of the proximal colon is affected differently than when inoculated in vessels mimicking the distal colon. Therefore, follow-up questions are: How relevant are these microbial changes, and how accurate are the outcomes? Additionally, what are the implications for the translatability of results to humans?

In vitro models keep evolving to incorporate more physiologically relevant components and improved control systems. Several reviews provide additional details and compare advantages and drawbacks of each model (Nissen, Casciano and Gianotti, 2020; Pearce *et al.*, 2018; Pham and Mohajeri, 2018; Roupar *et al.*, 2021).

Cell lines are also used in microbiota studies, often combined with other *in vitro* formats. There are different intestinal cell lines available which have been employed in gut microbiome studies: colonic (e.g. Caco-2, HT-29, T84) and small intestine (IEC-6, IEC-18, IPEC-J2, IPEC-1) (Pearce *et al.*, 2018). They are used, for example, to investigate the effects of bacterial products (e.g. butyrate from culture supernatants or bioreactor media) on cell function and integrity and provide mechanistic insights on microbe-host interaction. For example, Dai *et al.* (2020), who evaluated sucralose, treated human colorectal cancer cell lines (HCT8 and HCT116) with *Clostridium butyricum* supernatants to evaluate its anti-inflammatory capacity. Sun *et al.* (2019) investigated the effects of κ-carrageenan oligosaccharides on human faecal microbiota and SCFA production in a fermentation vessel. The inflammatory potential of the resulting supernatant was evaluated in HT29 cell lines.

Ex-vivo models are a step between *in vivo* and *in vitro* systems and consist of tissues or organoids, which can be embedded in a chip (gut-on-a-chip, organ-on-a-chip). Although promising, these systems have not been employed yet to evaluate the food additive-gut microbiome interactions.

Many research groups evaluating food additives in surrogate *in vitro* and *in vivo* models discussed the relevance of human studies to validate their findings and further assess causal relationships between diet-induced microbiome changes and health outcomes in humans. However, there are limitations and challenges in conducting human studies. These, especially epidemiological studies, are strongly confounded by lifestyle and behavioural factors, which challenge and limit the interpretation of findings (Wade and Hall, 2020). Although interventional studies can limit the effect of some confounders and are run under controlled exposure conditions, they are costly and time-consuming (Wade and Hall, 2020).

HUMAN STUDIES

This review includes only a few studies involving human subjects. All of them were conducted to evaluate non-nutritional sweeteners, except for one interventional study assessing the effects of carboxymethyl cellulose. The study designs and methodological approaches were diverse. Out of the seven interventional trials (Table 6), five were randomized, double-blinded, and evaluated the effects of saccharin (Serrano *et al.*, 2021), maltitol (Beards, Tuohy and Gibson, 2010), aspartame and sucralose given sequentially (Ahmad, Friel and Mackay, 2020a), four commercial products (saccharin, sucralose aspartame and stevia) (Suez *et al.*, 2022) and carboxymethyl cellulose (Chassaing *et al.*, 2021). A non-randomized interventional study evaluated commercial saccharin (Suez *et al.*, 2014). A short-term (4 days) cross-sectional study monitored changes in the faecal microbiota after the consumption of products containing acesulfame-K and aspartame (Frankenfeld *et al.*, 2015), and two large observational studies, involving several generations, evaluated the consumption of artificially sweetened beverages (Laforest-Lapointe *et al.*, 2021; Ramne *et al.*, 2021).

TABLE 6. **INTERVENTIONAL STUDIES EVALUATING THE IMPACT OF FOOD ADDITIVES ON THE HUMAN FAECAL MICROBIOME**

DURATION	DOSE	DURATION	GENDER (AGE)	PARTICIPANTS, NUMBER OF VOLUNTEERS PER GROUP	REFERENCE
Saccharin*	5 mg/kg bw/day	6 days	M, F (adults, 28–36 years old)	7 select volunteers received treatment (no controls)	(Suez *et al.*, 2014)
Aspartame> sucralose, Sucralose> aspartame	ASP: 40 mg/kg bw/day SUC: 9 mg/kg bw/day	2 x 2 weeks separated by 4-week washout	M, F (adults, 18–45 years old)	17 total volunteers split randomly into 2 treatment groups (n=8-9). No control group.	(Ahmad, Friel and Mackay, 2020a)
Saccharin	400 mg/day	2 weeks	M, F (adults, 18–45 years old)	46 total volunteers split randomly into 3 treatment groups (n=10-13) and control (n=11)	(Serrano *et al.*, 2021)
Sucralose	780 mg/day	7 days	M (adults, 18–50 years old)	34 total volunteers split randomly into treatment and control groups (n=17)	(Thomson *et al.*, 2019)
Maltitol	22.8 – 46.6 g/day	2 weeks	M, F (adults, 20–40 years old)	40 total volunteers split randomly into 3 treatment groups and control (n= n.s. ~10?)	(Beards, Tuohy and Gibson, 2010)
Saccharin* Sucralose* Aspartame* Stevia*	180 mg/day 102 mg/day 240 mg/day 180 mg/day	2 weeks	M, F (adults, 18–70 years old)	120 total volunteers split randomly into 4 treatment groups and control groups (n=20)	(Suez *et al.*, 2022)
Carboxymethyl cellulose	15 g/day	11 days	M, F (adults, 18–60 years old)	16 total volunteers split randomly into treatment group (n=7) and control (n=9)	(Chassaing *et al.*, 2021)

* Commercial products; ASP: aspartame; SUC: sucralose; M: male; F: female; n.s.: not specified.

Sources: See References

TEST COMPOUNDS, DOSES, ADMINISTRATION METHOD AND EXPOSURE TIMES

TEST COMPOUNDS

Selecting a suitable test substance for evaluating dietary exposure can have relevant implications for study outcomes. The specifications, quality and source of test substances have not always been considered, but they are key to mirroring appropriate or realistic exposure scenarios. Therefore, it is important to dedicate some space to this topic.

a. Compound grade (food, industrial, and reagent) and specifications.

Food grade is a quality attribute. It refers to substances deemed suitable for human consumption and are manufactured according to the specifications defined, for example, in monographs produced by JECFA. Since these compounds are made for oral consumption, assessing the impact of food-grade substances on the gut microbiome provides a more accurate and realistic representation of potential effects in real-life dietary situations.

Non-food grade compounds may contain impurities, which can be harmful to the host or cause disruption of the gut microbiome, influencing the research outcomes, and leading to misinterpretation and inappropriate conclusions. Consequently, these substances can hinder the practical application of findings to real-world dietary contexts and safety evaluations. For these reasons, scientists should exert care when selecting test substances to ensure the relevance of their research.

The specifications and grade of test substances are often not reported in the manuscripts included in this review. Moreover, some manuscripts mention chemical providers but not catalogue numbers, preventing the manuscript reader from identifying the type of compound used in the study and its suitability for dietary studies.

Some compound types are often characterized, but their food-grade attribute is not always reported, such as titanium dioxide and silver. In the case of nanoparticles like titanium dioxide, a typical characterization of the particle includes, for example, its crystal form, size distribution, average size, fraction of the particle size distribution below 100 nm, and hydrodynamic size in water or simulated gastrointestinal conditions. In foodgrade titanium dioxide, around 10–40 percent (Geiss *et al.*, 2021; Geiss *et al.*, 2020) of particles fall in the nanoscale (diameter < 100 nm). Surprisingly, in several studies, even when the authors recognize that most food-grade titanium dioxide particles are larger than 100 nm, they only include particles in the nano scale, averaging 20–30 nm. Some studies observed inverse size-dependent effects when comparing the effects of food- and industrial-grade particles (Cao *et al.*, 2020; Waller, Chen and Walker, 2017; Yan *et al.*, 2022). In its most recent TiO_2 evaluation, JECFA concluded that these studies on non-representative materials (100 percent NPs) were not relevant to the safety assessment of food additive TiO_2 (INS 171) (FAO and WHO, 2023b).

Similarly, several studies evaluated silver particles with different properties, including, for example, size (average diameter: 3-110 nm) and coating agents used as particle stabilizers (citrate, polyvinylpyrrolidone, polyethylene glycol, glutathione). Like titanium dioxide, the characterization of silver particles is usually reported in the scientific manuscript. However, these studies do not specify whether such particles are foodgrade, therefore posing questions about the extent to which the findings from these studies mirror the effects of actual dietary exposures. The initial physicochemical properties of silver nanoparticles (e.g. size, coating properties) can determine further changes promoted by the different conditions along the gastrointestinal tract (e.g. pH, interaction with food components), and these can affect their bioavailability and interaction with the gut microbiome and mucus (Bi *et al.*, 2020).

In the studies involving carrageenan, it was unknown if the compounds used were food-grade. Some research groups produced the test compounds in their laboratory. For example, carrageenan has been extracted from red algae or purchased as a reagent and repurified in the laboratory (Mi *et al.*, 2020; Wu *et al.*, 2021; Yin *et al.*, 2021). Although they are purified, there was no indication of whether the resulting substances met the specifications described in the relevant food additive monograph (e.g. JECFA) or regulatory requirements.

There is a controversy surrounding the safety of the carrageenan. This controversy is based on the nomenclature confusion between the high-molecular weight food-grade additive (average weight ~100–652 kDa [FAO and WHO, 2015; Uno *et al.*, 2001]) and industrial lower molecular weight (LMW) derivatives (~10–40 kDa) obtained by acid hydrolysis (aka poligeenan and degraded poligeenan – earlier also known as degraded carrageenan) (Liu *et al.*, 2021; McKim *et al.*, 2019). Oral administration of poligeenan has induced conditions like intestinal inflammation or cancer in animal models (McKim *et al.*, 2019). In fact, Munyaka *et al.* (2016) used a high dose of poligeenan (referred to by the authors as "carrageenan gum") to induce colitis in a piglet model of inflammatory bowel disease and evaluated the impacts on the mucosa-associated microbiota (this study has not been included with our review). Similar situations have been observed in the studies evaluated here, with several authors reporting on "carrageenan" or "degraded carrageenan" when, in fact, they are using poligeenan (Yin *et al.*, 2021). Situations like these can be avoided by educating scientists to focus not only on results but also on the overall context of research (in this case, the dietary and food safety context) and the properties of the test compound (food-grade, food additive specifications).

b. Use of commercial formulations.

Food additives sold as ingredients to the food industry or as preparations to the consumer can contain more than one substance. For example, commercial carrageenans, even when identified as pure κ-, λ- or ι-carrageenan by the producer, can contain more than one polymer type (FAO and WHO, 2015). The preparation can also include other co-formulants, such as compounds to retain the additive properties (e.g. salts to maintain gelling properties of carrageenan [FAO and WHO, 2015]), bulking ingredients (e.g. maltodextrin, glucose in sweeteners) or a mix of compounds belonging to the same additive class (e.g. mixes of non-nutritional sweeteners). However, the product label does not always show the exact product composition or component proportions. This can make it difficult to define proper controls to account for potential effects derived from the co-formulant. Several studies evaluated the impact of commercial sweeteners on the gut microbiota, containing one or combination of some of the following: saccharin, sucralose, steviol glycosides, aspartame or sodium cyclamate (Falcon *et al.*, 2020; Gerasimidis *et al.*, 2020; Mahalak *et al.*, 2020; Rodriguez-Palacios *et al.*, 2018b; Sanchez-Tapia *et al.*, 2020; Suez *et al.*, 2022; Suez *et al.*, 2014). Suez *et al.* (2022) investigated four commercial formulations specifying the NNSs (saccharin, sucralose, steviol glycosides and aspartame), the bulking agent and the proportion between them. However, the actual product name was not provided, preventing other research groups from reproducing the investigation or further studying those products.

Combined exposure has gained attention within the risk assessor community because, in real-life situations, foods can contain more than one food additive or regulated substances with the potential for synergistic or antagonistic effects. In addition to food additive combinations, one of the studies investigated the impact of titanium dioxide and bisphenol A (a controversial substance used to produce plastics, including food contact materials) (Yang *et al.*, 2022a).

DOSES

Experimental doses are chosen based on the research question and purpose of the study. The safety assessment of dietary compounds is relevant when the experimental dose mimics realistic exposure scenarios. Estimating the population´s exposure to food additives is somehow challenging because of difficulties in identifying how much is added to foods. In some cases, ingredient lists mention the general functional class (e.g. acidifiers) instead of the name of the individual additives, preventing the identification of the specific additive added to the product. Also, the amount of the additive used in ingredients, foods and beverages is not required or reported by the manufacturer. The *Codex General Standard for Food Additives* (FAO and WHO, 1995) or national regulations specify the maximum amount permitted for certain additives in different food categories, typically reported as mg of additive for kg or L of product. Some additives with no safety concerns do not have specific limits. However, they should be used according to good manufacturing practices, meaning that the manufacturer should add the minimum amount of additive to achieve the desired functional effect.

In this review, the acceptable daily intake (ADI) has been used as a reference value to assess whether the experimental dose chosen by the research group falls within what is considered safe and relevant from a dietary exposure perspective. For additives with no ADIs, consumption estimates by the population were used as reference, where available.

Additive ADIs, as defined by Codex Alimentarius, the United States FDA, Health Canada, or EFSA, are the most common reference values researchers use to determine experimental doses. In addition, some studies also considered available consumption estimates or compared doses with typical additive content in food products. For instance, some studies compared the concentration of sweeteners investigated to the equivalent number of soda cans (Ahmad, Friel and Mackay, 2020a) or titanium dioxide content in gums or candies (Dudefoi *et al.*, 2017). Some other studies based their experimental doses on the maximum levels of the additive permitted in certain foods. For example, the maximum level of TiO_2 permitted in foods is 1 percent, according to the United States FDA (Bredeck *et al.*, 2021).

There were differences in how doses were reported in animal studies. Several research groups reported daily intakes per kg body weight (mg/kg bw/day). Some other scientists reported doses as the additive concentration in the vehicle (mg/kg or mg/L). In these cases, the estimated daily intake was calculated based on the information in the manuscripts (animal weight, food or liquid intake) or existing conversion tables (FAO and WHO, 2009b). In a limited number of studies, the additive unit was mg/kg, but it was unclear if this was the concentration of the additive in the vehicle, or the daily intake per kg body weight.

In principle, daily intakes per body weight facilitate the comparison of doses between studies, existing ADIs, and human exposure estimates. However, the reported daily intake was, in some cases, theoretical and, in other instances,

realistic after considering changes in body weight and actual consumption of the additive-containing vehicle (drinking water or feed). The following are examples of the differences in the information used to calculate such daily intakes:

> Some provided daily intakes without indicating if they based the values on theoretical or observed body weight changes and food and liquid consumption.

> Some indicated that animal weight and food and liquid consumption were monitored, but it was unclear if this data was used to adjust additive doses to maintain the additive daily intake constant throughout the study duration.

> In a limited number of studies, the authors explicitly indicated monitoring animal weight, food and water consumption, which were used to adjust doses and maintain the additive daily intake constant (e.g. (Becker *et al.*, 2020)). Food and water consumption were often not provided, but some included them as graphs or tables.

Not only changes in body weight but also food and liquid intake should be monitored and used to improve the accuracy of the food additive exposure and identify potential issues that can otherwise go unnoticed. This is particularly relevant when the test substance can dramatically change the palatability of the food or water and consumption behaviour. For example, Suez *et al.* (2014) treated mice with drinking water containing 10 percent commercial saccharin (5 percent saccharin + 95 percent glucose), sucralose (5 percent Sucralose), or aspartame (4 percent aspartame), 10 percent glucose, 10 percent sucrose or no sweetener (water control group). Notable differences between the groups were observed in liquid and food consumption patterns throughout the 80-hour monitoring period. All treated mice consumed more liquid. For example, the saccharin group consumed more additive-containing water than the glucose control group and approximately ten times more than the water control group. Based on feed and water intake graphs provided by the researchers in the supplementary information (collected over 4 days of the 13-week study), our estimation for the daily saccharin consumption was ~5000 mg/kg bw (calculated based on liquid consumption ~20 ml/day, 20 g mice), which is 1 000 times higher than its ADI. Animals also consumed less food than their water control group. There are several implications related to this exposure: Firstly, the treatment groups consumed more and different amounts of sweetener (and bulking agent) than expected and reported. Consequently, treatment and control groups would no longer provide accurate comparisons. For example, the saccharin group would no longer match its glucose control. Secondly, the high glucose intake (bulking agent in saccharin commercial preparation) and the reduction in food consumption could potentially result in metabolic alterations. However, in this study, disruptions in glucose homeostasis were attributed solely to the sweeteners. In summary, such observations would have remained unnoticed if the researchers had not provided detailed consumption data.

Similar observations, high fluid consumption and reduced food intake, were reported after long exposure to aspartame (Palmnas *et al.*, 2014) and two commercial non-nutritional sweeteners, Splenda® and Svetia® (Martínez-Carrillo *et al.*, 2019).

Therefore, to ensure the accuracy of experimental doses and to conduct realistic assessments of substances provided *ad libitum*, fluid and food intake should be monitored regularly, and doses adjusted according to changes in body weight and the consumption of the additive-containing feed or water. This information and how consumption is calculated should be clearly stated in manuscripts.

Generally, a wide range of doses, above and below the ADI, were used in animal studies. As expected, the doses used in the human interventional studies were at or below the ADI of the tested additive or, for additives lacking ADI, closer to reported consumption estimates. Several epidemiological human studies have indicated that daily consumption of no- or low-caloric sweeteners across geographical regions is lower than their corresponding ADIs (Barraj, Bi and Tran, 2021; Lenighan *et al.*, 2023; Martyn *et al.*, 2018; Tennant, 2019). Although some studies report intakes above the ADI, these could result from overestimations due to methodological limitations (Martyn *et al.*, 2018).

Food additive doses used in gut microbiome studies

For acesulfame K, all animal studies tested doses at the JECFA ADI (15 mg/kg bw/day) or above, ranging from 15–150 mg/kg bw/day (Bian *et al.*, 2017a; Hanawa *et al.*, 2021; Uebanso *et al.*, 2017b). Frankenfeld *et al.* (2015) estimated the human consumption of acesulfame-K to range between 1.7 and 33.2 mg/day based on the responses to the questionnaire used in the cross-sectional study.

In the case of aspartame, all doses used in animal studies (5–7 mg/kg bw/day) were lower than the JECFA ADI for this compound (40 mg/kg bw/day) (Nettleton *et al.*, 2020; Palmnas *et al.*, 2014). Two human interventional studies evaluated aspartame at doses corresponding to 14 percent of the Canadian/JECFA ADI (Ahmad, Friel and Mackay, 2020a) and 8 percent of the United States FDA ADI (Suez *et al.*, 2022). In the cross-sectional study by Frankenfeld *et al.* (2015), the estimated daily consumption of aspartame, based on a 4-day food record completed by the participants, ranged between 5.3 and 112 mg/day.

Saccharin was given at doses matching the JECFA ADI (5 mg/kg bw/day) in mice and human studies (Becker *et al.*, 2020; Suez *et al.*, 2014; Sunderhauf *et al.*, 2020), slightly above the ADI (6–7 mg/kg bw/day) in a human interventional study (Serrano *et al.*, 2021) or below the ADI in a mouse model (3 mg/kg bw/day) (Labrecque *et al.*, 2015) and a human interventional study (20 percent US FDA ADI) (Suez *et al.*, 2022). Several studies also used doses several times higher than the JECFA ADI for saccharin (Bian *et al.*, 2017c; Serrano *et al.*, 2021; Suez *et al.*, 2014).

Most studies on sucralose tested the sweetener with doses at or below the JECFA ADI (Abou-Donia *et al.*, 2008; Bian *et al.*, 2017b; Dai *et al.*, 2020; Gerasimidis *et al.*, 2020; Rodriguez-Palacios *et al.*, 2018b; Shi *et al.*, 2021; Thomson *et al.*, 2019; Uebanso *et al.*, 2017b; Wang *et al.*, 2018; Xi *et al.*, 2020). A human interventional study evaluated sucralose at a dose corresponding to 20 percent of the Canadian ADI (9 mg/kg bw/day), which is lower than the JECFA ADI (Ahmad, Friel and Mackay, 2020a).

In a more recent human interventional study, the daily dose corresponded to 34 percent of the US FDA ADI (5 mg/kg bw/day) (Suez *et al.*, 2022). The other studies evaluated doses at least ten times higher than the JECFA ADI for sucralose (Guo *et al.*, 2021; Li *et al.*, 2020a; Sanchez-Tapia *et al.*, 2020).

Steviol glycosides doses were tested around or below the JECFA ADI (4 mg/kg bw/day) (Becker *et al.*, 2020; Gatea, Sârbu and Vamanu, 2021; Gerasimidis *et al.*, 2020; Li *et al.*, 2014; Nettleton *et al.*, 2020; Nettleton *et al.*, 2019) (Suez *et al.*, 2022) and also above (Li *et al.*, 2014; Mehmood *et al.*, 2020; Sanchez-Tapia *et al.*, 2020; Xi *et al.*, 2020).

The only study investigating neotame used a dose of 0.75 mg/kg bw/day, below the JECFA ADI (2 mg/kg bw/day) (Chi *et al.*, 2018)

The dose units reported in the different studies involving sugar alcohols were very variable, probably due to the lack of ADI for these compounds. They are typically reported as the concentration in percentage values, ranging between 0.1–10 percent xylitol (equivalent to about 1–10 g/kg bw/day) (Tamura, Hoshi and Hori, 2013; Xiang *et al.*, 2021; Zuo *et al.*, 2021) or 5–10 percent sorbitol (Hattori *et al.*, 2021). Uebanso *et al.* (2017a) tested lower doses of xylitol (40-200 mg/kg bw/day).

Determining daily consumption of non-nutritional sweeteners was challenging for some commercial preparations, where the proportion of the sweetener or mix of sweeteners was not reported (Falcon *et al.*, 2020; Martínez-Carrillo *et al.*, 2019).

This review includes several emulsifiers, stabilizers and thickeners. Chassaing *et al.* (2015) carried out an initial animal investigation involving P80 and CMC at a concentration of 1 percent in drinking water. Although the study tested a range of doses, starting as low as 0.1 percent, the subsequent analysis focused primarily on the 1 percent concentration due to its more pronounced effects. This study was a reference for other researchers that also used the 1 percent CMC or P80 dosage (Chassaing *et al.*, 2017; Furuhashi *et al.*, 2020; Holder *et al.*, 2019; Jin *et al.*, 2021; Li *et al.*, 2020b; Rousta *et al.*, 2021; Singh, Wheildon and Ishikawa, 2016; Viennois *et al.*, 2020; Viennois and Chassaing, 2021; Viennois *et al.*, 2017). Food and water consumption was not monitored or reported in most of these studies. A dose of 1 percent corresponds approximately to a daily intake of 1 000– 1 200 mg/kg bw (considering a mouse of 20–30 g and 2–3 ml daily water consumption). While the ADI for CMC has not been established, this estimate is higher than the JECFA ADI for P80 (25 mg/kg/bw/day). Only one interventional study evaluated the effects of a daily dose of 15 g CMC (214 mg/kg bw/day in a 70 kg individual) (Chassaing *et al.*, 2021). These doses of CMC and P80 are also higher than daily intake estimates (< 100 mg/kg bw/day) (EFSA, 2018b; Shah *et al.*, 2017; Vin *et al.*, 2013).

Doses tested to evaluate monoglycerides of fatty acids (glycerol monolaurate, glycerol monocaprylate) ranged from 150 to 1 600 mg/kg food (equivalent to ~23–240 mg/kg bw/day considering a mouse of 20 g consuming 3 g food/day) (Jiang *et al.*, 2018; Mo *et al.*, 2019; Zhang, Feng and Zhao, 2021; Zhao *et al.*, 2019; Zhao *et al.*, 2020; Zhao *et al.*, 2022). Such doses align with estimated daily intakes (1 300 mg/kg bw/day, high-level in infants) (EFSA, 2021). The natural emulsifier soy lecithin was also evaluated at a dose of 10 percent in feed (15 g/kg bw/day), which is higher than the estimated daily intake (< 200 mg/kg bw/day) (EFSA, 2017).

A refined estimate exposure assessment indicated that the daily carrageenan intake in adults ranges from 22.0 to 88.9 mg/kg bw (EFSA, 2018a). The doses of the different types of carrageenan used in experimental studies ranged from 2 mg/kg bw/kg (Shang *et al.*, 2017) to ~5 000 mg/kg bw/day (Mi *et al.*, 2020; Yin *et al.*, 2021) (estimated by us based on 2 ml daily consumption by a 20 g mouse). Water or feed intake was not monitored or reported in these studies. Two other studies provided doses in mg/kg (1.7, 8.3 and 41.7), which were given to the mice by gavage (volume gavaged not provided) (Wu *et al.*, 2021; Wu *et al.*, 2022). In these studies, the methodology section did not clarify if the units referred to daily intakes per kg of body weight or concentration of the additive in the preparation given to mice. Only in the discussion were these doses referred to as daily exposure per kg of body weight when compared to existing exposure in humans (Fernández-Ferreiro *et al.*, 2015; Tobacman, 2001) or doses used in other rodent studies (Bhattacharyya *et al.*, 2013). These carrageenan studies illustrate the need for clear reporting of experimental doses (in the methodology section of the publication).

It is also important that the context of reference studies used to select doses or discuss results is relevant to the dietary exposure. For example, Fernández-Ferreiro *et al.* (2015) used a non-oral route of exposure (ophthalmic) as a reference to select their experimental dose.

Doses of titanium dioxide ranged from 1 to 1 000 mg/kg bw/day, with the majority tested at levels below 100 mg/kg bw/day. About 45 percent of the studies included doses in the range of 10–50 mg/kg bw/day, and about 55 percent of the doses were higher than these. The estimated exposure of children to titanium dioxide from food sources has been reported to range from 1 to 3 mg/kg bw/day (Weir *et al.*, 2012).

Estimates of silver dietary exposure are in the single-digit µg/kg bw for children and adults (Bi *et al.*, 2020; EFSA, 2016b). However, daily doses tested in the studies included in this review were in the mg/kg bw range. The only exception was the study by van den Brule *et al.* (2016), where the lowest dose tested was 9 µg/kg bw/day.

Single versus multiple doses

Evaluating multiple doses in a single study permits the evaluation of dose–response relationships (FAO and WHO, 2009a).[31] Dose–response curves help identify the threshold at which a response begins to occur. This information is essential for establishing safe exposure levels. Risk assessors can use these curves to determine, for example, the lowest dose at which adverse effects are observed (LOAEL)[32] (FAO and WHO, 2009a) or the dose where no adverse effects are observed (NOAEL)[33] (FAO and WHO, 2009a). This information is then used for setting health-based guidance values, such as the ADI. For the purpose of risk assessments, it is relevant to identify a range of concentrations with at least one dose showing no effects. The selection of doses should also consider real-world dietary scenarios in which exposure to the substance might occur. The identification of relevant experimental doses often requires preliminary pilot studies. For standard toxicological studies conducted according to Good Laboratory Practices or GLP, such as OECD guidelines, the minimal number of doses are recommended.

While most studies evaluated single doses, some investigated multiple concentrations, typically limited to two or three, using different dose ranges.[34] In general, studies reported dose-dependent effects.

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[31] Dose–response relationship. Relationship between the amount of an agent administered to, taken up by or absorbed by an organism, system or (sub)population and the change developed in that organism, system or (sub)population in reaction to the agent. Related terms: Concentration–effect relationship, Dose–effect relationship.

[32] Lowest-observed-adverse-effect level (LOAEL). Lowest concentration or amount of a substance, found by experiment or observation, that causes an adverse alteration of morphology, functional capacity, growth, development or lifespan of the target organism distinguishable from normal (control) organisms of the same species and strain under the same defined conditions of exposure.

[33] No-observed-adverse-effect level (NOAEL). Greatest concentration or amount of a substance, found by experiment or observation, that causes no adverse alteration of morphology, functional capacity, growth, development or lifespan of the target organism distinguishable from those observed in normal (control) organisms of the same species and strain under the same defined conditions of exposure.

[34] Dose range: range of concentration between the highest and the lowest dose.

Within the sweetener class, Uebanso *et al.* (2017b), Abou-Donia *et al.* (2008) and Rodriguez-Palacios *et al.* (2018b) evaluated *in vivo* several doses of sucralose in ranges below the ADI. Li *et al.* (2014) treated mice with two doses of rebaudioside A (5 and 50 mg/kg bw/day), both higher than the JECFA ADI for steviol glycosides (4 mg/kg bw/day). None of the doses seem to have a relevant effect on the microbiota. The same study included an *in vitro* assay that tested multiple doses of the compound. Mehmood *et al.* (2020) evaluated the renoprotective effects of two high doses of stevia extracts (200 and 400 mg/kg bw/day) in mice with induced chronic kidney disease, of which the high dose showed modulatory effects. Three rodent studies investigated several xylitol doses. Doses 40 and 194 mg xylitol/kg bw/day did not affect the mouse lipid metabolism but led to dose-dependent disturbances in the faecal microbiota (Uebanso *et al.*, 2017a). In another study investigating three xylitol doses (0.9, 3.15 and 9.90 g/kg bw/day), only the high xylitol dose altered the microbiota (Zuo *et al.*, 2021). Also, 2.17 and 5.52 g xylitol/kg bw/day did not change the overall structure of the microbiome (Xiang *et al.*, 2021). Olivier-Van Stichelen, Rother and Hanover (2019) evaluated two doses of a combination of sucralose and acesulfame K, corresponding to their individual ADIs and twice the ADI. Pups born to mothers exposed to the higher dose (ADI2x) showed more evident metabolic changes.

Chassaing *et al.* (2015) investigated CMC and P80 at three different concentrations of 0.1, 0.5 and 1 percent in drinking water. After observing a dose–response relationship *in vivo*, they selected the higher concentration for use in subsequent studies. The same research group evaluated *in vitro* the impact of multiple CMC and P80 doses (also in the range 0.1–1 percent) on LPS and flagellin production, with no clear lineal dose-dependency in the P80 treatment groups (Chassaing *et al.*, 2017).

Wu *et al.* (2021) and Wu *et al.* (2022) observed alterations of the SCFA production, structure and virulence of the faecal microbiota, and physiological parameters at the highest evaluated dose of κ and λ-carrageenan (1.7, 8.3 and 41.7 mg/kg).

Two studies evaluating glycerol monolaurate at two ranges of doses, 150–300 and 456 mg/kg (Zhao *et al.*, 2019) and 400, 800 and 1600 mg/kg (Mo *et al.*, 2019) reported favourable effects at the high dose. These included the microbiota-dependent attenuation of metabolic alterations induced by an HFD (Zhao *et al.*, 2019) and the promotion of beneficial gut bacteria (Mo *et al.*, 2019).

Several animal studies evaluating titanium dioxide were conducted using multiple doses ranging from 2 to 1 000 mg/kg bw/day. Of note: When evaluating nanoparticles, it is necessary to consider the size since different sizes can lead to distinctly different outcomes at a given concentration. Three studies monitored the same three concentrations, 2, 10 and 50 mg/kg (Chen *et al.*, 2019a; Chen *et al.*, 2019b; Pinget *et al.*, 2019). Chen *et al.* (2019b) observed a dose-dependent increase in gut microbiota diversity, with hepatotoxicity at the highest concentration of titanium dioxide (particle size ~29 nm). The research group observed similar dose dependency (gut dysbiosis and intestinal inflammation) using the same particle size and concentrations (Chen *et al.*, 2019a). Pinget *et al.* (2019) observed disturbances

of the colonic microbiota of the colon and gut homeostasis at the highest dose of food-grade titanium dioxide. Yan *et al.* (2022) reported the influence of particle size (micro and nano titanium dioxide), concentration (10 and 40 mg/kg bw/day) and their combination on the gut microbiota, production of microbiota-host metabolites and the intestinal barrier. The highest concentration of titanium dioxide nanoparticles evaluated by Lin *et al.* (2023) (10, 100 and 1000 mg/kg bw/day) was reported as NOAEL. An *in vitro* study revealed limited effects of 100 and 250 ppm food-grade titanium dioxide in the human gut microbiota (Dudefoi *et al.*, 2017).

The microbiota was not affected by any of the acetate-coated silver nanoparticles (2.25, 4.5 and 9 mg/kg bw/day) evaluated by (Hadrup *et al.*, 2012). However, toxicity was reported for silver ions but not silver nanoparticles at the highest concentration. Despite the absence of toxic effects to PVP-coated silver nanoparticles (0.009, 0.071 or 0.679 mg/kg bw/day), van den Brule *et al.* (2016) reported disruption of the gut microbiota diversity in a dose-dependent manner. Williams *et al.* (2015) observed dose- and size-dependent effects on the intestinal microbiota of acetate-coated silver nanoparticles tested at 100, 200 and 400 mg/kg bw/day.

ADMINISTRATION METHOD, VEHICLE AND MATERNAL EXPOSURE

When conducting animal studies to evaluate the effects of dietary substances on the gut microbiome, the method of administration (gavage vs. oral consumption, typically *ad libitum*) and the vehicle used to deliver the test compound (e.g. drinking water, oil, food) can influence the outcomes and interpretations of the study. These can be more or less representative of a realistic exposure scenario.[35]

Oral or gastric gavage: This method involves administering the dietary substance directly into the stomach using a syringe or gavage needle. This method ensures precise dosing and immediate exposure. However, gavage can be stressful for animals, potentially leading to altered physiological responses due to stress, therefore confounding the effects. Microaspiration has also been suggested to occur in as many as one third of mice dosed by oral gavage, resulting in detection of dose material outside the gastrointestinal tract (Craig and Elliott, 1999). In addition, gavage does not model natural dietary exposure (Turner *et al.*, 2011; Vandenberg *et al.*, 2014). Administering the full dose of the test compound in a single daily bolus is not representative of typical food or fluid intake of small amounts consumed several times a day. Additionally, the rapid introduction of a substance into the stomach, avoiding mouth interactions, might not accurately reflect the natural process of digestion and absorption that occurs with regular feeding.

Ad libitum exposure: The additive is provided in the food or drinking water, which the study subjects can access freely at any time throughout the day. It results in a more natural exposure to the test compound and resembles the normal consumption in humans.

[35] By realistic exposure, we mean (a) natural route of exposure (here, voluntary *ad libitum* oral consumption), (b) amounts of the additive consumed by individuals and used in foods or beverages, and (c) foods or food matrices and beverages typically containing the additive.

This method is less stressful for animals compared to gavage. However, there are challenges in controlling the actual dose each animal receives, as it depends on the food or water consumed by each individual. Moreover, changes in taste or smell due to the addition of the test substance might affect consumption. Examples and implications have been discussed at the beginning of the section "Doses" section.

It is important to consider that the vehicle (e.g. water, saline buffer, oil, food matrix) used to deliver the additive and the manufacturing process can influence the physicochemical properties of the test substances, how it interacts with other matrix components and their bioavailability. For example, interactions with the matrix can aggregate titanium dioxide nanoparticles or induce conformational changes in the case of carrageenans, therefore modifying their bioavailability and how they interact with the intestinal biology (Liu *et al.*, 2021; Winkler *et al.*, 2018). Compared to gavage, Bredeck *et al.* (2021) discussed the importance of the feed matrix as it can influence retention time in the stomach and dissolution degree of the text compound (engineered nanoparticles in this example) in the acidic gastric environment.

For a more approximate evaluation of the additive exposure, the form of administration and vehicle selected should represent realistic applications of the additive in foods or beverages. In the animal studies included here, the preferred vehicle depended on the type of additive. Sweeteners were more frequently provided *ad libitum* in the drinking water (n=27) or the food (n=7). Only five studies chose gavage as administration method.

P80 and CMC were mostly provided in the drinking water (n=9), while only two studies delivered the compounds via gavage. Chassaing *et al.* (2015) observed similar phenotypes (pro-inflammatory effects and metabolic alterations) when comparing vehicles (food vs drinking water). The only human interventional study evaluating CMC used food as the vehicle (Chassaing *et al.*, 2021). However, other emulsifiers like monoglycerides of fatty acids and lecithin were provided in the rodent chow (n=6) or in a combined exposure of lecithin in the feed followed by one final dose by gavage (Robert *et al.*, 2021).

Titanium dioxide was administered to animals mostly via gavage (n=12), and less frequently in the feed (n=3) or drinking water (1). Silver nanoparticles were given primarily by gavage (n=3) and in the feed (n=2).

The polysaccharides curdlan and xanthan gum were given by gavage only. Mi *et al.* (2020) compared the influence of two forms of administration, drinking water vs feed (high- or low-fat diet), in the effects of κ-carrageenan. In animals fed HFD, the inflammatory effects and microbial changes observed when the additive was provided in tap water (0.5 percent) were not visible when it was supplemented in the animal feed at a higher concentration (5 percent) (no colitis observed in any of the groups under a low-fat diet). The selection of the vehicle to deliver carrageenan is a relevant consideration since it can influence the bioavailability, toxic potential and functional properties of the food additive (Liu *et al.*, 2021). In aqueous solution and in the absence of cations or binding proteins, carrageenan molecules are disorganized and likely to interact with other dietary or membrane proteins of intestinal cells (Weiner, 2014). Liu *et al.* (2021) argued that the delivery mode in many *in vivo* studies may not be representative of real food scenarios.

A special "form of administration" is via maternal exposure during gestation and lactation. In order for maternal exposure to occur, the additives have to be absorbed, cross the placenta, or be released into the milk, which – depending on the food additive – may result in minimal to no detectable additive concentration. In addition, housing and litter handling can influence milk intake. Litter size influences food intake, where pups from small litters consume more than those of higher numbers (Lutz, 2020).

Understanding the toxicokinetics of the additive and measuring its presence in relevant samples (e.g. milk, placenta) can provide insights into a possible direct exposure and intake estimation. Sylvetsky *et al.* (2015) detected saccharin, sucralose, and acesulfame-K, but not aspartame, in the breast milk of 65 percent of participating women (n=20) using LC-MS. Saccharine has been detected in neonate serum at levels lower than 160 ng/mL (Cohen-Addad *et al.*, 1986). Saccharin and acesulfame-K were present in human cord sera in single digit ng/mL (Cohen-Addad *et al.*, 1986; Halasa *et al.*, 2021; Sturtevant, 1985), and acesulfame-K, saccharin, steviol glucuronide and sucralose were found in amniotic fluid at levels lower than 100 ng/mL (Halasa *et al.*, 2021). Rother *et al.* (2018) also found sucralose and acesulfame K in the milk of lactating mothers after soda consumption. Another study found acesulfame-potassium, saccharin, cyclamate, and sucralose in plasma and breast milk (except for sucralose) of lactating mothers (n=49) who had consumed a beverage containing the NNS (Stampe *et al.*, 2022). Further research is needed to evaluate the impact of human lactation or transplacental exposure to sweeteners on the offspring's microbiota and immune and metabolic health and how this exposure compares to microbiota transfer from NNS-consuming mothers to offspring before weaning.

Only one study monitored the presence of food additives in biological samples from mothers and offspring (Olivier-Van Stichelen, Rother and Hanover, 2019). The researchers detected sucralose and acesulfame-K in milk from lactating mouse dams (sweeteners administered combined at 1 or 2 times their US FDA ADI in the feed), but at lower levels than in blood and faeces. In the lactating pups, very low or no sucralose was detected in faecal samples, while acesulfame-K was found in urine only.

EXPOSURE PERIODS

Given the frequent use of many food additives in food products and beverages, it would be appropriate to say that humans are exposed to these compounds chronically. Therefore, studies evaluating the safety of these substances should be designed considering long treatment periods. In animal studies, treatment periods were very variable. Regarding non-nutritional sweeteners, exposure periods ranged from 2 weeks to 6 months (2–3 weeks: 5 studies; 4–8 weeks: 13 studies; 9 weeks–6 months: 15 studies). For non-sweeteners, treatment periods ranged from 5 days to 22 weeks (< 3 weeks: 8 studies; 4–8 weeks: 21 studies; 9 weeks–22 months: 22 studies).

Exposure periods in interventional human trials are usually shorter than for animals: aspartame, acesulfame-K (Frankenfeld *et al.*, 2015), sucralose (Thomson *et al.*, 2019) and saccharine (Suez *et al.*, 2014) were evaluated for one week or less. Ahmad, Friel and Mackay (2020a) and Serrano *et al.* (2021) investigated aspartame, sucralose and saccharin for two weeks. These two research groups identified these short periods as limitations of their studies. They indicated that two weeks are not sufficient to evaluate chronic exposures and might not have been long enough to induce changes in the microbiota and physiological parameters. Maltitol was trialled for six weeks, the longest human study we reported for sweeteners (Beards, Tuohy and Gibson, 2010). The only non-sweetener interventional study in human volunteers investigated CMC for two weeks (Chassaing *et al.*, 2021).

Almost all experimental studies ended at the end of the treatment. Only a small number of humans studies *in vivo* and *in vitro* research included a clearance period after the treatment to assess the persistence of observations or the capacity of the microbiota and the host parameters to recover.

CONFOUNDING FACTORS AND SOURCES OF VARIABILITY

Confounders are variables that influence the outcome of a study in addition to the main variable being tested (often called the treatment) (van Stralen *et al.*, 2010). These confounding variables can make it seem like the treatment has an effect when it really doesn't, or they can mask the true effect of the treatment. In animal research, non-experimental variables or factors can inadvertently confound the study outcomes, therefore impacting the results' validity and research reproducibility (Baker and Lipman, 2015; Colby, 2020; Ericsson and Franklin, 2021). For these reasons, confounding factors need careful consideration and a plan to control or minimize their potential impact (Rodriguez-Palacios, Basson and Cominelli, 2021). Unfortunately, scientists often do not recognize many of these factors, which can go unreported.

The influence of confounding factors in animal research has been widely reviewed elsewhere (Baker and Lipman, 2015; Colby, 2020). Briefly, confounding factors can be intrinsic to the animal (e.g. genetics, age, sex, immune status, nutritional status, circadian Rhythms, endocrine factors) or extrinsic (e.g. physical and chemical factors, microbial agents and stressors). Ericsson and Franklin (2021) discussed confounding factors affecting the gut microbiome of mice and provided considerations for best

practices to minimize or control some of the factors. The following are factors that can influence the results of a study: colony characteristics, which are dependent on the supplier of laboratory animals; diet composition and lot-to-lot differences; potential microbial and chemical contamination of water, feed and during storage; effects of co-housing (animal density per cage); animal behaviour (e.g. coprophagy); husbandry (e.g. type of bedding and caging) or stressors factors such as animal handling, e.g. during gavage (Allen-Blevins *et al.*, 2017). Moreover, there are also interactions between several confounding factors like sex x diet x genetic background (Bolnick *et al.*, 2014; Ericsson and Franklin, 2021; Org *et al.*, 2016) or between bedding and caging type (Ericsson *et al.*, 2018). Interestingly, in this last example, the effects of these variables were associated with the intestinal microbiota while were undetected in the faecal microbial community. To account for variability due to caging, Kim *et al.* (2017) recommended that, for each condition, animals should be distributed in different cages.

Human research, especially epidemiological studies, are also confounded by numerous factors, including diet, lifestyle (e.g. exercise, travel), environment and physiological characteristics (Jokela *et al.*, 2023; Wade and Hall, 2020), which limits the determination of causal evidence (Wade and Hall, 2020). Confounders can lead to inconsistent results across studies and hinder the efforts to understand the role of gut microbiota in health and disease. Vujkovic-Cvijin *et al.* (2020) investigated these factors and recommended host variables that should be monitored in human microbiota studies to help improve robustness and reproducibility as well as identify microbiota members associated with human disease more accurately.

Different epidemiological studies have found discrepancies in the association between diet soft drink intake and metabolic diseases. Palmnas *et al.* (2014) indicated that the difficulties in controlling confounding variables might explain such discrepancies, for example, differences in consumption patterns between obese and diabetic individuals and non-diabetics. In their study, they chose lean and diet-induced obese animal models to help control these variables.

In the analysis of the publications included in this review, it is noteworthy that only a few research groups demonstrated a commitment to minimizing some confounding variables and have taken and reported explicit measures to mitigate the influence of some of them. These efforts have been concentrated mainly on the management of animal handling and husbandry practices, as well as control of diet. Some examples are provided in the next sections.

As will be discussed more extensively below (see sections *Microbiota samples and sampling* and *Microbiome analysis*), methodological or **technical factors** can also impact the accuracy of biological outcomes. These can include the faecal transplant procedure itself, quality of the test compound (e.g. non-food grade), sampling, (e.g. time of stools collection, time gap between collection and processing, storage), inclusion of matching controls, or analytical factors (e.g. sequencing). To illustrate these sources of variations with an example, Jokela *et al.* (2023), who studied the sources of gut microbiota variation in a large longitudinal infant cohort in Finland, indicated that the effect of technical variables on microbiota composition explained about 15 percent of the cumulative variance in infants and up to 13 percent in adults.

ANIMAL HANDLING AND HUSBANDRY

Animal handling and husbandry practices also impact the dynamics of the microbial community, and measures should be implemented to minimize their influence on the research outcome (Kostic, Howitt and Garrett, 2013; Turner, 2018).

Acclimation of animals upon arrival in the research facility is one of the factors frequently described and controlled by the research groups. The acclimation period is usually 1 week, but some scientists extend it to 2 or up to 4–5 weeks (Serrano *et al.*, 2021). Animals need to adapt to the new environmental conditions and a new diet. If the microbiota is not stable before initiating the study, it will likely influence the study outcome. Becker *et al.* (2020) indicated that the two-week period used to get mice used to the facilities and a low-fat diet might not have been sufficient to stabilize their gut microbiome. This lack of stabilization could explain differences in the microbiota of the control group before and after the 10-week experimental period.

Co-caging and animal density have also been identified to potentially influence the study outcomes (Kim *et al.*, 2017). However, this information was not always reported in the studies included in this review. Some research groups explicitly indicated measures to minimize the effect of co-caging. For example, Nettleton *et al.* (2020) limited the litter size to ten offspring to minimize confounding due to variable litter size. Some scientists chose to house mice individually to minimize cage-to-cage variability (Rodriguez-Palacios *et al.*, 2018b).

Soiled bedding has also been shown to introduce bias in microbiome research (Rodriguez-Palacios *et al.*, 2018a). Rodriguez-Palacios *et al.* (2018b) implemented several measures to control the "cyclical bedding-dependent bias", including using HEPA-filtered pressurized standard dorms to keep low cage humidity and replacing cages periodically and at the same time. Coprophagic behaviour is a common source of horizontal microbiome transfer in mice (Kostic, Howitt and Garrett, 2013). Chassaing *et al.* (2015) implemented measures to avoid this possibility. However, it is challenging to fully control coprophagia. For example, Nettleton *et al.* (2020) did not rule out microbiota transfer from rat dams to offspring in their study with aspartame and stevia.

DIET AS CONFOUNDING FACTOR

To address potential diet-related confounding factors, researchers should carefully plan their experiments. Below are examples of this type of confounders and the measures taken to reduce or manage their impact.

The **introduction of microbes in the diet and drinking water** can influence the gut microbiome composition. It can be controlled by feeding animals with irradiated or autoclaved chow and water and replacing them regularly to prevent microbial overgrowth (Rodriguez-Palacios *et al.*, 2018b). Mi *et al.* (2020) provided animals access to tap water in their study evaluating κ-carrageenan, but the research team did not address its potential impact on the study outcomes. Falcon *et al.* (2020) used low-fat yoghurt as a vehicle to compare the effects of a commercial NNS and sucrose.

Yoghurt is produced using bacteria cultures such as Bifidobacteria or Lactobacilli species, some known as probiotics, which remain live in the consumed product (if not heattreated). These bacteria cultures could have potentially influenced the microbiota evaluated in the study. Evidence shows that dairy products can modulate the gut microbiota (Aslam *et al.*, 2020). Unfortunately, this study had no proper negative control to assess the yoghurt effect alone on the microbiota.

Lot-to-lot variability. The ingredient composition can change slightly between production lots. This possibility was addressed by Becker *et al.* (2020), who used the same lot throughout the study. It helped them exclude lot variability as a potential contributing factor to the observed differences in microbiota composition before and after treatment.

Food composition. Several studies evaluated the impact of food additives on the gut microbiome and the host in the context of obesity. Animals are fed a high-caloric diet, typically rich in high fat or a combination of high fat and high sugar. The composition and proportion of fat (and sugar, when included) in the product among these studies vary, which makes it difficult to compare results. In addition, high-fat diets and different fat profiles are known to influence the microbial community structure, physiological activities (gut permeability) and metabolic outcomes (Lam *et al.*, 2015). These diets can confound the results and need to be controlled to ensure that the effects observed are due to the treatment, not the diet. This can be conducted, for example, by including suitable controls. For example, Becker *et al.* (2020) and Sanchez-Tapia *et al.* (2020) indicated that the HFD had more influence on the outcomes than the treatment. However, the diet effect is not always controlled. For example, Suez *et al.* (2014) studied the impact of pure saccharin in mice fed HFD but lacked a control group fed normal chow.

In studies involving different diets, the food **composition and ingredient proportions** have to be modified to accommodate the introduction of the test substance or other ingredients. The implications of such changes are often not discussed by the research groups. Basal diets are not necessarily the same across the groups, as observed in the feed composition reported in some studies (Nettleton *et al.*, 2019; Tamura, Hoshi and Hori, 2013). As mentioned above, several studies compared, for example, high- and low-fat diets. In addition, high or low-fat diets differ in composition across studies. Preparing such diets requires substituting food ingredients (Han, Kwon and Choi, 2020; Han *et al.*, 2020). For example, Wang *et al.* (2018) recognized the possible confounding effect of the diets used in their study, high-fat diet and normal chow, due to differences in their fibre content. Some scientists have tried to minimize the effect of these confounders. For example, Robert *et al.* (2021) used the same amount of lipid-free diet base and lipid blends when preparing the treatment diets, supplemented with 10 percent soybean lecithin or 1, 3 or 10 percent rapeseed lecithin. They avoided introducing new ingredients or nutrients while maintaining a balanced diet in terms of nutrients and caloric input. While these adjustments may not impact the host significantly, given the gut microbiome's sensitivity to dietary shifts, their potential to impact the gut microbiota should be further explored.

Adding a test substance to food, especially if it does not have nutritional value and is used at high concentrations, can lead to a **nutritionally or calorically imbalanced diet**. The animals can increase food intake to compensate for the caloric or nutritional deficiency (Weiner, 2014), and potentially increase the exposure to the test substance. Some OECD guidelines of chronic toxicity (e.g. 452 and 453) limit the highest dose of test material to 5 percent in the diet or drinking water to prevent its interference with normal nutrition (OECD, 2018a, 2018b).

The addition of the test substance in the food or drinking water can lead to changes in palatability, for example, when evaluating sweeteners. It can modify the animal's **eating or drinking behaviour**. Such behavioural changes can be especially relevant when the additive is used at high doses, which could explain the observed alterations in food and liquid consumption in mice given 10 percent commercial non-nutritional sweetener (containing 5 percent saccharin, 5 percent sucralose or 4 percent aspartame) in the drinking water (Suez *et al.*, 2014). In this case, the most extreme example occurred in the saccharine group, where animals consumed about 20 ml water/day (typical daily consumption is 2–3 ml), probably due to the need of the animal to eliminate the high sweetness intensity of the water, and consequently leading to decrease food consumption due to satiety induced by the high liquid consumption (10 percent saccharin product contains 95 percent glucose) or to compensate for the calories obtained from the glucose-saccharin solution.

Also, the authors should carefully consider the suitability of the diet composition when this is not included as an experimental variable in the study. For example, LabDiet rodent chow #5021 (Lab Diet, 2023), a high-energy formulation suitable for high-reproducing mice and postpartum use, was given to young mice starting at 4 or 5-weeks of age (Viennois *et al.*, 2020). The authors did not discuss the potential influence of this diet on the study outcome.

The human diet varies widely among and within individuals and populations, and changes constantly over time. In addition, participants in human studies or microbiota donors for *in vitro* studies or faecal transplant studies can follow different diets (e.g. omnivores, vegan, vegetarians), as seen in the studies by Chassaing *et al.* (2021), Elmén *et al.* (2020) and Miclotte *et al.* (2020). Dietary preferences can determine differences in the microbial community structure among individuals, influencing how each responds to treatments. Such variability confounds human studies (Vo, Lynch and Roberts, 2019). Chassaing *et al.* (2021) recognized the difficulties in studying the impact of individual dietary substances on the gut microbiome due to variations in the quantity, quality and composition of food consumed by different individuals as well as differences in the composition of their respective microbiomes.

Fillers, also food additives, in commercial products (e.g. sweeteners) can potentially lead to microbial changes and physiological responses. They can act as confounders, making it difficult to evaluate the health impact of commercial sweeteners on the human population (Rodriguez-Palacios, Basson and Cominelli, 2021). In animal studies, the effect of fillers can be controlled, for example, by introducing additional control groups.

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Diets should be standardized to improve reproducibility of results of microbiome studies in laboratory animals. This could include, for example, a basal standard rodent chow and a high-fat diet. Although some efforts have been carried out to standardize diets, it remains challenging due to, for example, differences in macro and micronutrients in products from different vendors (Joshi and Fiorotto, 2021). In addition, guidelines should be developed to help gut microbiome researchers identify confounding factors and implement control measures to minimize their impact on study outcomes.

Confounding factors and measures to minimize their impact should be described in the methodology or supplemental section of papers. It should also include a reference to the commercial diet used in the study or, in the case of home-made diets, the description of the diet composition.

EXPERIMENTAL CONTROLS

Well-designed experimental controls are essential for obtaining quality data and drawing reliable conclusions. Controls act as a reference point, allowing researchers to distinguish between the actual effect of their experiment, natural variations in the system, and confounding factors that can influence the results. Without proper controls, changes in the microbiome or unexpected biological responses could remain masked, therefore limiting our ability to accurately interpret the experiment's outcome and understand its potential impact on health, disease, and overall well-being.

CONTROL GROUPS

Controls groups typically receive the same vehicle as the treatment group but excluding the test substance. However, the research may include additional control groups depending on the study purpose or the product being evaluated. For example, some studies evaluating commercial NNS formulations, which also include a filler, involve a control group with the filler only alongside a negative control group that remains unexposed to any of these compounds (Mahalak *et al.*, 2020; Suez *et al.*, 2022; Suez *et al.*, 2014). However, the identification of proper control groups is not always possible because the composition of commercial NNS or the proportion of the different substances is not always known (Abou-Donia *et al.*, 2008; Gerasimidis *et al.*, 2020; Mahalak *et al.*, 2020; Rodriguez-Palacios *et al.*, 2018b; Vamanu *et al.*, 2019). In these cases, observed effects cannot be clearly attributed to the additive of interest only but to the entire commercial product, requiring careful interpretation of outcomes.

Falcon *et al.* (2020) gave rats low-fat yoghurt containing a commercial sweetener (saccharin and sodium cyclamate) or sucrose. Although the authors reported no differences in microbiota between the two groups, the study lacked a negative control (yoghurt only). Also, as discussed above, some questions arise about the suitability of low-fat yoghurt as a vehicle for the sweetener, as it might have influenced the outcome.

Mahalak *et al.* (2020), who used a single monkey to evaluate a commercial stevia product (~1 percent rebaudioside D and erythritol), did not include control groups (negative or erythritol) but used the microbiota baseline as a reference control. Because of the lack of control groups, the researchers could not attribute the observed shifts in microbiota diversity to the specific additive, rebaudioside D or erythritol.

Models of disease – where the disorder is induced in healthy animals by dietary manipulation (e.g. high-fat diet in models of obesity), chemical treatment (e.g. DSS-induced colitis) or via infection with pathogens – these studies typically include a healthy or a lean non-obese group (fed standard rodent chow) as a reference control to assess the relative effect of the treatment or diet used to induce the condition. For example, studies conducted in the context of obesity to investigate the impact of food additives + high-fat diet often include two control groups, one fed standard chow (lean control group) and a second one fed a high-caloric diet (obese control group) (Becker *et al.*, 2020; Han *et al.*, 2020; Palmnas *et al.*, 2014; Sanchez-Tapia *et al.*, 2020; Xi *et al.*, 2020). However, Suez *et al.* (2014), who evaluated a commercial saccharine product containing sucrose as filler in animals fed HFD, included only one control group consisting of sucrose in animals fed HFD. However, this study lacked both HFD and lean controls. The human interventional trial, part of the same research (Suez *et al.*, 2014), also lacked negative controls.

Another situation observed relates to how the control group is used when evaluating samples for the different tests carried out in a study, in particular, when samples from the control group are used as references in the evaluation of some but not all of the

study parameters. For example, Nettleton *et al.* (2020), who studied aspartame and rebaudioside A in the context of obesity, excluded the lean reference group from the evaluation of several variables (e.g. microbiota, insulin tolerance, glucose tolerance in transplanted mice). In both studies, the omission limited a proper assessment and interpretation of the diet effect in the observed microbiota and glycaemic alterations.

MICROBIOTA BASELINES AND MICROBIAL STABILITY

The **baseline** microbiota is an essential piece of information for:

- Establishing a reference point: The baseline gut microbiota provides a reference against which the effects of the intervention can be measured. Without knowing the starting state, it would be challenging to attribute any changes observed to the intervention itself.
- Establishing microbiota homogeneity across groups in an animal study: The evaluation and monitoring of the baseline microbiota are critical to ensure the homogeneity of the microbial communities across experimental groups and to guarantee that they have reached stability after the acclimation period and before initiating the treatment. Non-homogeneous and unstable populations can make it difficult to interpret results. Moreover, assuming that the baseline microbiota is homogeneous and stable risks making accurate conclusions. To reduce the risk of non-homogeneity, many research groups randomly assigned animals to different control and exposure groups. This practice is also used to distribute human volunteers into different groups in interventional studies. This is a common practice to ensure that any variation in the baseline gut microbiome is evenly distributed among the groups. Another practice to ensure an homogeneous gut microbiome baseline is to mix mice during the acclimation period (van den Brule *et al.*, 2016).
- Evaluating the gut microbiome resilience: The baseline is fundamental to assess the capacity of the gut microbiome to revert or return to its baseline state after changes are induced by the treatment.
- Determining gut microbiota stability: In studies aimed to evaluate the effects of food additives and other exogenous compounds on the gut microbiome, it is crucial to ensure that the microbiota has reached stability before initiating the treatment. The gut microbiome stability is affected, for example, upon arrival of animals at the research facilities, after inoculating gut microbiota into *in vitro* systems, or after recipients receive faecal transplants. Microbial changes due to the lack of stability can influence the final outcome and interpretation of findings and study conclusions. An unstable gut microbiome will go unnoticed in the absence of a baseline evaluation at different time points. Becker *et al.* (2020) suggested that the observed differences in the microbiota composition of the control group before and after treatment might be attributed to a lack of microbial stability before the experimental treatment began. In the *in vitro* study conducted by Naimi *et al.* (2021), the faecal microbiota was monitored for

72 hours before initiating the treatment. By looking at the figures reported in the supplementary information (Figures S1 and S2), the number of OTUs seemed to reach stability 48-hour post-inoculation. However, the researchers normalized their data (microbiota parameters and pro-inflammatory potential) by using the 24-hour time point (non-stable microbiota) as a reference instead of the 72-hour point right before treatment. This approach questions the validity of findings and raises the question of whether the statistical differences found at the 24-hour point would remain significant if the researchers had used the 72-hour point, once the gut microbiota appeared stable. The lack of microbial stability before treatment can impact the validity, accuracy of results and data interpretation.

- Understanding individual variability: Inter-individual variability can influence how individuals respond to treatments. Knowing the baseline state helps interpret the results in the context of individual differences.
- Enhancing study design: Researchers can design better-controlled studies by understanding the baseline gut microbiota. For example, they might stratify subjects based on certain microbiota characteristics to ensure balanced groups, which can lead to more robust and interpretable results. For example, identify four microbiome clusters in the investigation of potential impacts of maternal consumption of artificially-sweetened beverages on the maturation of infant gut microbiome and BMI during the first year of life (Laforest-Lapointe *et al.*, 2021).
- Determining effects: Knowing the baseline enables researchers to determine if the treatment caused a change and to distinguish between general shifts in microbiota versus the effects of the intervention.
- Causal inferences: To make more robust causal' inferences about the relationship between an intervention and outcomes, it's essential to demonstrate that the intervention led to changes from an established baseline.

Although the baseline microbiota was often not monitored (or not reported), some research groups opted for including and reporting this option in their investigation. Bredeck *et al.* (2021) emphasized the need to incorporate both genders in research and characterize their basal microbiota composition. This approach helps interpret post-treatment results and prevent the wrong attribution of gender effects to the experimental chemical, especially when microbial populations already differ at the baseline. Serrano *et al.* (2021) determined pre-treatment baselines for all parameters studied for each individual (humans and mice), which allowed the determination of within-subject changes over time and between-group variations.

Determining baselines should not be limited to the gut microbiome but should also consider the host. Before beginning the randomized, double-blind intervention in humans, Thomson *et al.* (2019) conducted a baseline assessment. Based on their findings, the researchers suggested the importance of assessing metabolic differences before interventions as they may have a higher impact on the gut microbiota than the treatment itself.

MICROBIOTA SAMPLES AND SAMPLING

SAMPLE SIZE

Sample size is a fundamental aspect of experimental design that impacts the quality, reliability, and ethical considerations of the research. One of the primary reasons for determining an appropriate sample size is to ensure suitable statistical power of the research hypothesis, which refers to the probability of detecting a true effect if it exists. Factors such as expected effect size, variability, and the desired confidence level should be considered when determining sample size. Inadequate sample sizes can lead to low statistical power, making detecting real differences or true effects difficult. Because of ethical considerations, the number of animals should be kept to the minimum, while aiming for a sufficiently large sample size to achieve adequate statistical power to detect an effect size (typically 80 percent or higher). Such calculations should be made before initiating the study.

The researchers of most *in vivo* studies did not explain if or how they calculated the number of animals in the study. Li *et al.* (2020b) reported a sample size of 12 mice that provided a study power of 80 percent but did not specify the targeted effect. For a more accurate evaluation of the study's validity and the relevance of the reported power, further clarification from the authors regarding the targeted effect and its relationship to the sample size used for specific analyses would be essential. Unlike human interventional studies, sample size calculation in animal research is not a common practice (Muhlhausler, Bloomfield and Gillman, 2013). At least for standard toxicological studies conducted according to Good Laboratory Practices (i.e. OECD guidelines), minimal recommended animal number per dose group is described.

Several human studies calculated sample size typically aiming at 80 percent statistical power with 0.05 significance level. The specific targeted effect varied but was commonly based on glycaemic responses to NNS (Serrano *et al.*, 2021; Suez *et al.*, 2022; Thomson *et al.*, 2019). Chassaing *et al.* (2021) targeted differences in the distance of the nearest bacteria to the epithelium after CMC exposure. However, it was not clear if this research group calculated sample size *a priori* during the study design phase or *a posteriori* during data evaluation, as they reported that "with a sample size of 8 subjects per group and assuming a within group SD of 7.17 mm, we projected to have 90 percent and 80 percent power to detect a difference…"

Most *in vivo* studies evaluating the effect of food additives included between 5 and 10 animals per group (control and treatment). However, there are studies using as few animals as one monkey (Mahalak *et al.*, 2020) or those including over 30 mice in one of the experiments (Chassaing *et al.*, 2015). Unfortunately, several studies do not specify the total number of animals treated or included in control groups. In these cases, we had to refer to the charts reporting n for the variables plotted, which may or may not reflect the number of animals in each group.

Some complex studies investigated different experimental conditions and sometimes it was not clear how many animals were included in each group (control and treatments) (e.g. Dai *et al.*, 2020; Viennois *et al.*, 2017). In some of these studies,

understanding "n" can be confusing because the number of animals per treatment group and the number of samples per group for a given test dose may be different. These situations make the interpretation of results difficult and can challenge the validity of results, for example, (1) when the number of samples used to evaluate different parameters or analytes is lower than the number of animals in the treatment of control groups, and (2) when the number of samples varies among groups for a given test and among tests, often without justification (e.g. Suez *et al.*, 2014; Viennois and Chassaing, 2021; see supplementary table 1 from corrigendum Chassaing *et al.*, 2016 of Chassaing *et al.*, 2015). In these situations, it is not clear if differences in sample size are due to the need to reduce costs for the different analyses, the elimination of outlier data, animal casualties, or the selection of a subset of animal samples, which would have required clarification about selection process (i.e. random or following specific criteria). So, such variability in sample sizes makes it difficult for the reader to assess if samples from the same animal undergo all tests and if all observed alterations (e.g. flagellin levels, body weight, glucose tolerance, microbial alterations) belong to the same animal. The concerns arising from these situations would be related to their impact on the accuracy or validity of comparisons and correlations. Another example of an issue in reporting analytical sample size is when the number of samples analysed in the different tests are systematically given in ranges, e.g., n=5-8 (Viennois *et al.*, 2017).

Sample size in *in vitro* studies can have different components, including the number of microbiota donors and the number of simulations (replicates). Due to resource limitations and the complexity of some *in vitro* gastrointestinal systems, it is often challenging to perform parallel simulations, including replicates of the same experimental conditions (Chassaing *et al.*, 2017). The following are possible scenarios related to experimental sample size, which may have different implications for the interpretation of results:

- The study is conducted with faecal microbiota from a *single donor* in multiple runs or replicates (Chassaing *et al.*, 2017; Mahalak *et al.*, 2020; Naimi *et al.*, 2021; Waller, Chen and Walker, 2017). Due to inter-individual variability, the faecal microbiome from one individual may not represent a given population (e.g. healthy). Moreover, due to temporal microbiome fluctuations within an individual, the analytical findings of microbiome samples collected on one day might differ from those collected on a different day, even if they are from the same person (Chassaing *et al.*, 2017).
- The study evaluates faecal microbiota from *multiple donors* independently (Cueva *et al.*, 2019; Gerasimidis *et al.*, 2020; Yin *et al.*, 2021). This approach allowed the identification of intra- and inter-individual responses to different food additives.
- The study evaluates pooled faecal microbiota from multiple donors in single (Sun *et al.*, 2019) or multiple simulations (replicates) (Agans *et al.*, 2019; Cattò *et al.*, 2019; Gatea, Sârbu and Vamanu, 2021; Vamanu *et al.*, 2019).
- The study evaluates a synthetic bacteria consortium (MET-1) with no replicate simulation (Dudefoi *et al.*, 2017).

ORIGIN AND SOURCES OF MICROBIOME SAMPLES AND SAMPLING

As mentioned in the introduction, the different physiological activities and microenvironments along and across the gastrointestinal tract define the composition and function of the microbial populations. Therefore, microbiome information is specific to the physiological and environmental context of the sampling site.

The collection of microbiota and host samples from the same anatomical location enables the investigation of context-specific effects (e.g. histological damage) and the identification of possible correlations between microbiome alterations and local intestinal changes. In exposure studies, selecting the appropriate microbiota sample site will depend on (1) the research question; (2) the bioavailability and toxicokinetics of the test substance, where the substance compound is transformed, absorbed and metabolized and if resulting products are released back to the gut environment; and (3) the accessibility and likelihood of the compound to interact with the microbiota (e.g. whether the compound reaches the colon in an intact form or not). For instance, if researchers are investigating the utilization or biotransformation of a specific additive by gut microbes, they may need to sample from the location where this microbial activity is most likely to occur. For example, practically all acesulfame-K and about 85–95 percent saccharine (in humans) are quickly absorbed intact in the small intestine. Therefore, no or a small amount of the sweetener can reach the large intestine (Magnuson *et al.*, 2016). However, the absorption of sucralose is very limited, therefore reaching the colon (Magnuson *et al.*, 2016). Other sweeteners are metabolized at the intestinal level. Aspartame is digested in the intestine by host enzymes, and the resulting products (phenylalanine, aspartic acid and methanol) are absorbed in the small intestine (Magnuson *et al.*, 2016). Steviol glycosides reach the colon unmodified, where they are cleaved by colonic bacteria to glucose and steviol, which is absorbed by the intestine (Magnuson *et al.*, 2016).

The study by Hanawa *et al.* (2021) can be used to illustrate the implications of host and microbiota sampling sites on the interpretation of results and overall study conclusions. The research group investigated if acesulfame-K (high dose)-induced dysbiosis of the caecal microbiota was involved in upstream mucosal damage (identified by the authors as middle small intestine). They could not reproduce the intestinal damage in antibiotic-treated recipient mice after transplant with caecal microbiota from treated donors, concluding that dysbiosis did not cause histological alterations. Several questions arise from this study, including how valid it is to evaluate the influence of caecal microbiota in histological changes of earlier intestinal segments. Would it have been possibly more appropriate to investigate the microbial population from the location where the damage was observed? What is the validity of the results?

Faecal material is the most popular choice for microbiome analysis. This is because it's cost-effective and easy to collect. Unlike some other methods, it doesn't require invasive procedures. This makes faecal samples collection convenient and suitable for use in longitudinal studies. Scientific reports have highlighted differences between the microbiota found in faecal samples and the microbial communities residing within the lumen and mucus (mucosa-associated microbiota) of the gastrointestinal tract,

especially the proximal sections of the colon and small intestine (Donaldson, Lee and Mazmanian, 2016; Gu *et al.*, 2013; Lkhagva *et al.*, 2021; Shalon *et al.*, 2023). Such findings raise questions about the representativeness of the faecal microbiome when investigating effects observed in the small intestine. Representative microbiome samples are essential to properly assess the impact of food additives on the microbial community and microbiome-host interactions.

In the studies reviewed, stool samples were more frequently used to evaluate the impact of food additives on the gut (or, more accurately, faecal) microbiome. Only a limited number of studies evaluated luminal microbiota from the cecum (Hadrup and Lam, 2014; Hanawa *et al.*, 2021; Nettleton *et al.*, 2019; Rousta *et al.*, 2021; Shi *et al.*, 2021; Tamura, Hoshi and Hori, 2013; Wilding *et al.*, 2016), colon (Rahman *et al.*, 2021; Shang *et al.*, 2017) or small intestine (Martínez-Carrillo *et al.*, 2019). In addition, two studies evaluated the mucosal-associated microbiota, obtained from the mucus layer of the colon (Laudisi *et al.*, 2019) and small intestine (Williams *et al.*, 2015). Some studies evaluated the microbiota from multiple locations, including faeces and cecum (Nettleton *et al.*, 2019; Uebanso *et al.*, 2017b), faeces and small intestine (Pinget *et al.*, 2019), and ileum and cecum (Furuhashi *et al.*, 2020). All human trials evaluated only faecal microbiota. Although Chassaing *et al.* (2021) analysed biopsies from the distal colon to identify the degree of microbial encroachment after carboxymethyl cellulose treatment, the actual microbiota composition and metabolome were evaluated from stool samples.

Some studies used **synthetic bacteria consortia** to evaluate the impact of some food additives. The microbial ecosystem therapeutic-1 (MET-1) was used to investigate the impact of titanium dioxide *in vitro* (Dudefoi *et al.*, 2017). This microbial community consists of 33 different bacteria strains,[36] which originated from the stools of a healthy donor (Petrof *et al.*, 2013). This consortium has been used for therapeutical purposes, e.g. to treat *Clostridium difficile* infections. Chassaing *et al.* (2017) and Viennois *et al.* (2020) used a pathobiomefree microbiota Altered Schaedler Flora (ASF) in studies investigating CMC and P80. This consortium consists of eight bacterial strains, predominantly Firmicutes (*Clostridium* spp., *Lactobacillus intestinalis, Lactobacillus murinus, Eubacterium plexicaudatum*, Firmicutes bacterium), one Bacteroidetes (*Parabacteroides* sp.), and one species from the gastrointestinal mucus of laboratory rodents *Mucispirillum shaedleri* (Robertson *et al.*, 2005), belonging to the phylum Deferribacteres. Other phyla from the human microbiota are not represented in the ASF consortium, e.g. Actinobacteria, Verrucomicrobia, Proteobacteria. Viennois *et al.* (2020) used the ASF in a gnotobiotic model to evaluate the ability of the consortium to prevent the colonization of pathobionts after CMC and P80 treatment.

[36] Bacterial species present in the MET-1 consortium: *Acidaminococcus intestini, Akkermansia muciniphila, Bacteroides ovatus, Bifidobacterium adolescentis, Bifidobacterium adolescentis, Bifidobacterium longum, Blautia stercoris, Clostridium cocleatum, Collinsella aerofaciens, Dorea longicatena, Escherichia coli, Butyricicoccus pullicaecorum, Eubacterium eligens, Eubacterium limosum, Eubacterium rectale, Eubacterium ventriosum, Faecalibacterium prausnitzii, Lachnospira pectinoschiza, Lactobacillus casei, Lactobacillus paracasei, Parabacteroides distasonis, Enterobacter aerogenes, Roseburia faecis, Roseburia intestinalis, Ruminococcus obeum, Blautia luti, Ruminococcus torques, Streptococcus mitis*.

The ASF consortium has been previously used to study the gut microbiome community and physiological interactions between the microbes and the host (Wymore Brand *et al.*, 2015).

Synthetic consortia simplify complex communities, making it easier to study microbiome-host interactions, and are often developed for clinical applications (van Leeuwen *et al.*, 2023). However, the suitability of these communities to evaluate the safety impact of food additives (or exposure to any other chemical) on the human microbiota and host health remains unclear. The main challenge is related to the representativeness of the consortium. For example, they do not include all the relevant bacterial strains and non-bacterial members contributing to the overall community response. In addition, the activity of the select group of bacteria may not be able to perform all the necessary functions to process chemicals and interact with the host. This could potentially lead to underestimating or missing certain effects of chemical exposure. In addition, it is unclear to which extent it is possible to evaluate dysbiosis in these communities. The development of a consortium suitable for the safety assessment of chemicals or the applicability of existing consortia would require additional investigation and validation.

SAMPLING TIMELINE

There are two approaches to conducting time-related evaluations of the microbiome: cross-sectional and longitudinal. The selection of the most appropriate approach depends on research goals, available resources, and the specific research questions being addressed.

Cross-sectional studies are more time- and resource-efficient since they involve data collection at just one point in time. They allow a larger sample size, which can make studies statistically more powerful. This type of study is better suited for exploratory research and hypothesis generation. Since cross-sectional studies provide a snapshot of the microbiota at a single moment, it is difficult to determine if (1) observed microbiome changes are sporadic or reflect a true adverse effect, and (2) interactions between the microbiota and the host are causative, and if so, in what way.

Cross-sectional is the most common approach for *in vivo* studies evaluating food additives, with parameters typically assessed at the end of the study.

Longitudinal studies involve repeated sampling and measurements over time, allowing researchers to (Luna, Mansbach and Shaw, 2020; Xia, 2021):

> track how the microbiota changes within individuals or experimental groups over time;

> distinguish between (a) normal fluctuations in the microbial community structure or function of the microbiota and (b) deviations from normality;

> investigate the microbiome resilience (whether alterations are sustained over time or whether variables return to baseline after the treatment stops) and plasticity (the microbiome can also change to a different state due to adaptation). In the case of adaptation, it would be interesting to investigate if the new microbiome state is either normal or if there are reasons for concern;

> support causality assessment and its direction. Longitudinal studies facilitate the identification of a sequence of events and whether changes in the microbiome precede changes in the host or viceversa;

> monitor individual or population variability and how it evolves over time. This is valuable for identifying personalized microbiota changes and individual responses to treatments; and

> evaluate conditions and mechanisms in which the gut microbiota can potentially contribute to the onset and progression of disease.

Despite all the advantages of longitudinal studies, they are resource-intensive in terms of time and cost. These factors can limit, for example, the number of experimental subjects in the research and analytical tests. Ideally, all experimental parameters (e.g. microbiota composition, metabolome, cytokine levels) should be monitored at all selected time points. However, this is not always feasible for the reasons mentioned above. Typical time points for sampling include the baseline (right before treatment initiation), end of treatment, immediately before a change in the intervention (which serves as the baseline for new experimental condition) and at the end of such intervention.

The rest of this subsection will discuss some observations and concerns about sampling checkpoints and when experimental parameters are tested.

Several research groups evaluated some parameters only at mid-points and not at the end of treatment (or results are not reported). For example, Viennois *et al.* (2017) gave mice CMC or P80 for 91 days (13 weeks) before treating them to cause colitis-induced colorectal cancer. Most parameters (e.g. microbiota composition, flagellin C – FliC –, lipopolysaccharide – LPS –, lipocalin – Lcn2) were evaluated at day 63 of treatment (week 9), or earlier (days 21 or 28). Surprisingly, the authors did not report any information for the last day of treatment (day 91). Would this date have served as a more appropriate baseline than day 63 as a reference to evaluate the results obtained after inducing colorectal cancer (day 141)? The same group

conducted another study to evaluate the influence of a 15-week treatment (105 days) with CMC and P80 on the development of spontaneous intestinal adenomas in WT and susceptible mice (Viennois and Chassaing, 2021). While the markers Lcn2, LPS and FliC were evaluated only at days 0, 28 and 56, with no or limited significance, the rest of the parameters were assessed only at the end of treatment. Again, should it not have been more relevant to evaluate those markers at the same time later or at the end of treatment to align results with the rest of parameters evaluated?

In a human interventional trial with four different non-caloric sweeteners, Suez *et al.* (2022) monitored multiple parameters during the 7-day baseline and 14-day treatment. However, not all parameters were evaluated at the same key time points, e.g. last day of baseline, mid- and last day of treatment. For example, blood tests and plasma metabolomics were evaluated only at day 0 of baseline and at the mid-point of treatment, but not at the end of treatment. It is possible that the researchers had an explanation for this sampling design. Reporting the reasoning behind this selection would have been useful for the reader to understand this decision.

Sun *et al.* (2019) evaluated *in vitro* the capacity of human faecal microbiota to ferment κ-carrageenans oligosaccharides over time and the inflammatory potential or the different fermentation products. The researchers used independent vessels for each time point rather than sampling the same fermenter vessel at different time points. Continuous sampling within a single vessel allows for a better understanding of how variables change over time. By using independent vessels for each time point, the study does not capture the natural microbial evolution or the progression of fermentation. This lack of continuity can make it challenging to assess these changes. Furthermore, inter-vessel variability may confound the interpretation of results, making it difficult to determine whether observed differences are due to the progression of microbial composition and function or to simply vessel-specific effects.

One of the challenges of longitudinal studies is to distinguish a mere sporadic change in microbiome measurements from an alteration of concern, e.g. that associated with or leading to a negative effect on the host physiology. The characteristics of dysbiosis are not well defined. For example, a change of concern could remain stable over time and negatively impact the host physiology. The lack of definitions and guidance to interpret changes in the microbial population has led to different approaches in how researchers interpret findings. For example, Bian *et al.* (2017b) and Bian *et al.* (2017c) reported alterations of some bacteria taxa in mice happening only at 3 or at 6 months of saccharin or sucralose consumption. Other markers (faecal metabolome, functional gene enrichment based on 16S rRNA gene sequencing data, transcription of liver markers) were evaluated only at the end of the study (month 6). Based on such findings, the authors suggested that sucralose increases the risk of liver inflammation by disrupting the gut microbiota. In this case, it appeared clear that the abundance of some of the evaluated taxa fluctuated overtime, with no indication of stable change or signs of trends. In addition, it is not clear whether such changes are biologically relevant. Just to include another example (Yan *et al.*, 2022), despite the authors concluding that TiO_2 micro and nanoparticles disrupted the homeostasis of the gut microbiota, the careful evaluation of the information provided seemed to

show only limited changes overtime after weekly evaluations over a month, which excluded a baseline analysis.

CLEARANCE PERIODS

Clearance (recovery or washout) typically refers to a specific timeframe during which an individual or system is allowed to recover, heal, or return to its normal state after experiencing a particular condition, treatment or stressor. Recovery periods have been used, for example, between changes in the intervention (Ahmad, Friel and Mackay, 2020a) or to remove residual additive from stools before a faecal material transplant (Wu *et al.*, 2021). Also, washout periods are also used after treatment to monitor the capacity of the microbiome or the host to recover and return to baseline. In this sense and given that exposure to many food additives is chronic, a washout or recovery period is only employed to investigate the significance of any microbiota changes. This is a relevant aspect indicative of the organism or microbiome resilience and provides information on potential long-term or delayed effects of the substance evaluated.

Unfortunately, washout periods are seldom included in studies designed to evaluate the impact of food additives on the microbiome and health outcomes. Washout periods after treatment varied in length, ranging between 3 days in an *in vitro* model evaluating different emulsifiers (Naimi *et al.*, 2021) to about 13 weeks after a human interventional trial with CMC (Chassaing *et al.*, 2021). Serrano *et al.* (2021) included a 2-week washout period following a human trial with saccharin, and Lin *et al.* (2023) monitored parameters for 4 weeks after the end of the intervention with TiO_2 in rats. In both cases, no negative effects were observed at the end of treatment and no effects emerged post-treatment. Chassaing *et al.* (2021) followed up participants taking part in a 2-week intervention with CMC for about 13 weeks post-treatment. Although alterations observed in participants (n=7) returned to normality, some changes remained stable in two individuals (faecal LPS levels, β-diversity). After monitoring several emulsifiers tested *in vitro* during a 3-day washout, Naimi *et al.* (2021) reported that some of the tested substances led to "irreversible" changes in bacterial density, microbial diversity and LPS production. One of the questions arising from this study is whether a 3-day period is sufficient to evaluate the recovery of the microbial population. The length of the clearance period should be reasonably long enough to allow the different metrics to recover without incurring additional cost burden to the study. The speed of recovery may differ for the different parameters evaluated.

The gut microbiome might not always return to its original state (baseline) after treatment. There are several reasons for this:

- natural progression of the microbial population, e.g. in studies initiated at young ages;
- permanent (long-lasting) effects after treatment; and
- adaptation: the gut microbiome returns to a different state as a result of an adaptative process. In this scenario, it would be necessary to evaluate further if the new state is desirable or not.

In summary, to gain a deeper understanding of the changes in the microbiome over time, the effects of longterm exposure to food additives, and the potential influence of host-microbiome relationship in health outcomes, it is crucial to develop a sampling frequency plan that incorporates a baseline and a washout period. This would enable the researcher to determine whether the observed changes are temporary or permanent.

OTHER SAMPLE-RELATED CONSIDERATIONS

Careful attention must be given to the processes of sampling and handling samples to ensure the preservation and integrity of the gut microbiome and its associated metabolites within the sample. Controlling the following aspects can help minimize bias and improve the accuracy of results. Some of these practices include (Jones *et al.*, 2021; Tang *et al.*, 2020; Vandeputte *et al.*, 2017; Xu *et al.*, 2019):

- sample collection method;
- avoidance of cross-contamination from other samples and the environment;
- time and conditions from collection to storage and sample preparation; and
- sample homogenization to ensure uniformity.

MICROBIOME ANALYSIS

Different analytical approaches have been implemented to evaluate the impact of food additives on the microbiome. Most studies investigated primarily the diversity and taxonomical composition of the microbiota, while the microbial function was less frequently targeted. This evaluation was primarily conducted using omics methods, often combined with classical microbiological techniques to further characterize the species relevant to the study or assess their sensitivities to the additive being evaluated.

GENOMIC EVALUATION

The 16S rRNA gene sequencing has been the most frequent analytical approach to evaluate the microbial community structure. Most of the studies targeted the regions V3–V4 or V4 of the 16S rRNA gene (see Annex III. Summary tables), often using universal primers. For example, practically all the studies amplifying and sequencing the V4 region used primers 515F and 806R. Other regions of the 16S rRNA gene have also been targeted (V1–V2, V1–V3, V2, V2–V3, V3, V4–V5), but much less frequently. Although commonly targeted regions across studies could, in principle, be indicative of some degree of standardization, the fact is that there are many other factors that can influence the divergence of results. These include, for example, the capabilities of the sequencer used, sequencing strategies and the choice of bioinformatic processing pipelines and analytical tools (Abellan-Schneyder *et al.*, 2021). Although most of the taxonomic assignments have been based on operational taxonomic units (OTUs), a few studies opted to use methods based

on amplicon sequence variants (ASVs) (Bredeck *et al.*, 2021; Laforest-Lapointe *et al.*, 2021; Nettleton *et al.*, 2019; Rahman *et al.*, 2021; Xi *et al.*, 2020; Yan *et al.*, 2022). ASVs have been regarded as being more precise in the taxonomic assignment (Abellan-Schneyder *et al.*, 2021; Chetty and Blekhman, 2024). Although both approaches can lead to different results, there are methods to reconcile the differences between both (Chiarello *et al.*, 2022).

Regarding the analysis of other microbiota members, fungi were only evaluated in very few studies, all targeting different regions: 18S rRNA gene (Mi *et al.*, 2020) and the ITS regions,[37] ITS1 (Rahman *et al.*, 2021) and ITS2 (Xiang *et al.*, 2021). It has been previously reported that there are amplification differences and sequencing biases in the analysis of ITS regions when compared to the 18S rRNA gene marker, therefore influencing the mycobiome characterization (Frau *et al.*, 2019; Thielemann *et al.*, 2022). Like bacteria, Archaea is evaluated by sequencing the 16S rRNA gene. This microbiome group was investigated only in one study by sequencing the V4 region of the 16S rRNA gene and using an Archaea-specific set of primers (Mi *et al.*, 2020).

The analysis of the 16S rRNA gene is also used to assess the effect of the food additives on microbial diversity. Several metrics and indexes evaluate α-diversity (e.g. Shannon, Simpson, Fisher, Chao1, ACE) and β-diversity (e.g. Jaccard distance, weighted or unweighted weighted UniFrac, Bray-Curtis dissimilarity). Different research groups utilized different approaches to assess diversity, often including more than one index for α-diversity. These indexes differ in how much weight they give to the different components of α-diversity, i.e. richness and evenness. Kers and Saccenti (2022) observed that different alpha and beta diversity metrics resulted in different study power and recommended that the selection of the suitable index should be conducted *a priori* as part of the statistical design of the study. This should avoid issues related to p-hacking, or in other words, selecting *a posteriori* the index that results in a statistically significant result (p-value > α).

Microbial functional profiles can be predicted from 16S rRNA gene sequencing data by using different inference tools (e.g. PICRUSt2, Tax4Fun) and reference genome databases (e.g. KEGG, Integrated Microbial Genomes and Microbiomes [IMG/M] database). There are limitations to this approach in producing functional profiles (Martinez-Guryn, Leone and Chang, 2019). They seem to lack the sensitivity required to distinguish functional changes in the microbiome that are relevant to health (Matchado *et al.*, 2024). Therefore, the information provided by this analysis should be interpreted carefully.

Sequencing the 16S rRNA amplicon has been shown to have limited resolution beyond the genus level, for which shotgun metagenomics is more suitable (Costea *et al.*, 2017; Laforest-Lapointe *et al.*, 2021; Lloyd-Price *et al.*, 2017). Despite its higher resolution and ability to identify not just bacterial species but also other members

[37] Internal transcriber spaces (ITS): ITS1 is located between 18S and 5.8S rRNA genes, while ITS2 is between 5.8S and 28S rRNA genes.

of the microbiota, such as viruses, archaea, and eukaryotes, shotgun metagenomic sequencing has not been used as frequently as 16S rRNA gene sequencing to assess the effects of food additives on the gut microbiome. A few studies used shotgun metagenomics in addition to 16S gene sequencing (Chassaing *et al.*, 2021; Rodriguez-Palacios *et al.*, 2018b; Sanchez-Tapia *et al.*, 2020; Suez *et al.*, 2014) or used it as a stand-alone in the absence of the 16S analysis (Rousta *et al.*, 2021; Suez *et al.*, 2022). Although shotgun metagenomics has the potential to investigate all kingdoms present in the microbiome, there are still technical challenges. Because of these issues, Rodriguez-Palacios *et al.* (2018b) focused on analysing the bacterial population while only screening for viruses. The analysis of the virome is challenging. It requires greater sequencing depth in shotgun metagenomics analysis than bacteria and the sequence homology to current databases is very low (~10 percent) (Aggarwala, Liang and Bushman, 2017).

Although amplicon sequence has become the most common approach to study the microbiome, other DNA-based analytical approaches have often been used to provide complementary information. For example, several studies evaluated the total bacterial load or abundance of special bacteria groups or species using quantitative PCR (qPCR).

GENE EXPRESSION

Targeted quantitative Reverse Transcription PCR (q-RT PCR) techniques were primarily used to study the expression of specific genes in host tissues, such as the intestine and liver. These methods helped monitor several markers of, for example, intestinal integrity and function, inflammatory responses, and liver health. Some of the microbiome-related markers were also monitored in some studies. These include Toll-like receptors 4 (TLR4) and 5 (TLR5), which bind the structural bacterial components lipopolysaccharide (LPS) and flagellin, respectively (Guo *et al.*, 2021; Li *et al.*, 2020a); and G protein-coupled receptors, which bind microbial metabolites SCFAs (Mi *et al.*, 2020; Williams *et al.*, 2015). Host transcriptomics (RNA sequencing), which can provide insights into the functional interactions between host genes and the microbiome (Chetty and Blekhman, 2024), was used less frequently (Laudisi *et al.*, 2019; Viennois *et al.*, 2020; Yin *et al.*, 2021). Metatranscriptomic approach to monitor the function of the gut microbiome was conducted only in the *in vitro* studies evaluating xylitol (Xiang *et al.*, 2021) and different emulsifiers (Chassaing *et al.*, 2017; Naimi *et al.*, 2021). Such studies were only evaluated *in vitro* because metatranscriptomics is challenging to carry out *in vivo* due to the limited microbial biomass and the interference of animal or human transcripts (Martinez-Guryn, Leone and Chang, 2019).

Several reviews have covered more specific information about the gut microbiome and gene expression, transcriptomics and metatranscriptomic analysis, which also address limitations and challenges (Nichols and Davenport, 2021; Ojala, Kankuri and Kankainen, 2023; Shakya, Lo and Chain, 2019).

PROTEOMICS AND METABOLOMICS

Proteomic analyses are conducted mainly in host samples (e.g. intestinal tissue, liver, blood) using a variety of techniques, including LC-MS, gel electrophoresis, immunoblotting, immunohistochemistry. Similar to transcriptomics, the main targets were related to intestinal function and integrity, inflammatory response, or receptors of LPS, flagellin or microbial metabolites like SCFAs.

The metabolome was evaluated using diverse detection methodologies, applying targeted or untargeted approaches, most often using mass spectrometry (MS) or nuclear magnetic resonance (NMR). The analysis of the microbial metabolome focused primarily on faecal or intestinal SCFAs, of which total SCFA, acetate, propionate and butyrate were the most common targets. Untargeted metabolomic profiling was conducted in different types of samples, e.g. stools, blood, and urine. Studies using this approach were, for example, those evaluating the effects of saccharin, acesulfame-K, sucralose, neotame, xylitol, carboxymethyl cellulose, or monoglycerides of fatty acids (Bian *et al.*, 2017a, b; Bian *et al.*, 2017c; Chassaing *et al.*, 2021; Chi *et al.*, 2018; Olivier-Van Stichelen, Rother and Hanover, 2019; Suez *et al.*, 2022; Zhao *et al.*, 2020; Zhao *et al.*, 2022). Changes in microbial metabolites or host-gut microbiome co-metabolites identified in these studies included, for example, SCFAs, daidzein, genistein, secondary bile acids, N-formylmethionine, indole derivatives, hippurate, or TMAO.

In addition to microbial metabolites produced by the gut microbiome and those resulting from the biotransformation of host or dietary chemicals, structural components such as lipopolysaccharide (LPS) and flagellin have also been the targets of several studies (e.g. Chassaing *et al.*, 2015; Elmén *et al.*, 2020; Naimi *et al.*, 2021; Singh, Wheildon and Ishikawa, 2016; Viennois *et al.*, 2017; Wu *et al.*, 2021). In several instances, LPS or flagellin were evaluated using biological assays, i.e. cell cultures expressing receptors for these microbial compounds.

There are several challenges in the study and understanding of gut microbial metabolites and their biological relevance. These include the heterogeneity of this group of compounds, which are intermingled with host metabolites, some co-produced by the microbes and the host, the absence of comprehensive metabolite information in existing databases, and the difficulty in translating research findings into clinical practice (Li, Liang and Qiao, 2022; Yan *et al.*, 2016).

For further reading, numerous scientific publications offer comprehensive overviews of metabolomic approaches (Bauermeister *et al.*, 2022; Smirnov *et al.*, 2016; Vernocchi, Del Chierico and Putignani, 2016; Xu *et al.*, 2019).

DATA ANALYSIS AND STATISTICS IN MICROBIOME STUDIES

Microbiome research has unique data analysis challenges due to the inherent properties of microbiome data, including zero inflation (phenomenon where a large proportion of the counts in a dataset are zeros, leading to no detection of taxa), overdispersion (the variance is often greater than expected), high dimensionality (microbiome contains numerous taxa), and sample heterogeneity (samples can vary significantly due to individual differences, environmental factors, and other sources of variation). Therefore, microbiome data require dedicated statistical treatments to interpret data accurately (Lutz *et al.*, 2022).

While this section briefly touches on aspects of data analysis and statistical approaches utilized in microbiome studies, a comprehensive evaluation of these methods is beyond its scope. Nonetheless, the importance of this topic calls for further investigation as their selection and application impact the quality of data and research outcomes.

The studies included in this review employed diverse bioinformatic pipelines, contributing to variability in the results, making any comparison of the results challenging. The coordinators of a multi-laboratory study identified bioinformatics as

one of the main sources of variability along with the type and origin of samples, DNA extraction and sample handling (Sinha *et al.*, 2017). Recent reviews have described different bioinformatic pipelines and machine learning tools and approaches for use for metagenomics and multi-omics integration (Arıkan and Muth, 2023; Chetty and Blekhman, 2024; Graw *et al.*, 2021; Marcos-Zambrano *et al.*, 2023).

Relevant to investigating the impact of food additives on the gut microbiome and health outcomes are the *differential abundance analysis*, which aims at detecting differentially abundant taxa across phenotype groups (e.g. healthy vs. diseased), and *integrative analysis*, which is applied with the purpose to identify associations between the microbiome and covariates (e.g. metabolites or food additives) (Lutz *et al.*, 2022).

Statistical methodologies should be carefully selected and applied, and data should be properly interpreted to accurately identify relevant microbial patterns and their associations with health outcomes. Numerous established and emerging methodologies are available for processing and analysing omics data and for integrating data from multi-omic approaches. However, the variety of data analysis options and statistical approaches, in addition to the limited guidance and consensus on the best practices, pose significant challenges for scientists in selecting the most appropriate methods to ensure robust and meaningful research outcomes (Khomich *et al.*, 2021).

OTHER ANALYTICAL ASPECTS

In addition to the analytical approaches cited above, the location of the microbiota was also evaluated in histological samples using fluorescent *in situ* hybridization (FISH). For example, this technique was employed, in conjunction with staining of the mucus layer, to evaluate the approximation of bacteria to the epithelial layer after treating mice with CMC, P80, κ-carrageenan or the commercial sweetener Splenda (Chassaing *et al.*, 2021; Chassaing *et al.*, 2015; Chassaing *et al.*, 2017; Rodriguez-Palacios *et al.*, 2018b; Viennois *et al.*, 2020; Wu *et al.*, 2022). This feature, regarded as encroachment, has been associated with intestinal inflammation in IBD and metabolic syndrome (Chassaing *et al.*, 2015; Viennois *et al.*, 2020). The morphological evaluation of the gastrointestinal tract, including the mucus layer (housing the mucosa-associated microbiota) and the digesta (containing most of the microbial biomass) is challenging due to the difficulties in preserving all components during histological preparations and the limitations of available visualization techniques (Tropini *et al.*, 2017). Therefore, it is essential to consider these limitations when conducting microscopic analysis and interpreting resulting images.

Bacterial culture methods have been used to further isolate and characterize bacteria involved in specific study outcomes. These methods allowed scientists to evaluate the bactericidal or bacteriostatic activity of a food additive, e.g. glycerol monolaurate and several non-nutritive sweeteners (Elmén *et al.*, 2020; Sunderhauf *et al.*, 2020; Wang *et al.*, 2018). Furthermore, these methods enabled monitoring of the bacteria's potential to utilize or biotransform the food additive in pure cultures, e.g. Red 40 (He *et al.*, 2021) or co-culture. Bacterial co-culture allowed the evaluation of cross-feeding activity in the utilization of xylitol (Xiang *et al.*, 2021).

FOOD ADDITIVE ACCESSIBILITY, BIOAVAILABILITY AND TOXICOKINETICS

A complete understanding of the interactions between food additives, the gut microbiome and the host, should also consider that, in real-life exposure scenarios, additives are contained in a food along with other food components. In addition, food components can interact with each other after undergoing different food manufacturing conditions and processes. The matrix effect is described as the *integrated physicochemical domain that contains and/or interacts with nutrients providing unique functionalities and behaviours from those exhibited in isolation* (Krishnan *et al.*, 2022, p. 43). The food matrix determines the compound's bioaccessibility (fraction of an ingested compound – e.g. nutrient, bioactive – which is released or liberated from the food matrix in the gastrointestinal tract) and bioavailability (fraction of a given compound or its metabolite that reaches the systemic circulation) (Aguilera, 2019). The product formulation and the different processes involved in food manufacturing can lead to various food matrix categories, which can impact the bioavailability and bioaccessibility of food components in multiple ways (Aguilera, 2019).

The food matrix can protect food components from early degradation or alter their release rate in the digestive tract. Those compounds embedded in a food matrix may be released more slowly or at different stages of digestion, affecting when and where they are available for microbial interaction (Aguilera, 2019).

All these factors can potentially contribute to the modification of the chemical structure and the bioaccessibility of the molecule. For these reasons, it is important to consider the types of food or beverage in which the food additive is permitted. While different methods are available to determine bioavailability and bioaccessibility (Sensoy, 2014), such features have been rarely addressed or referred to in the studies mentioned here. For example, Cao *et al.* (2020) suggested the influence matrix effect (fatty diet) on the fate of titanium dioxide along the gastrointestinal tract compared to a low-fat diet.

Bioaccessible and bioavailable compounds will be absorbed in the intestine, reaching the blood where they will be distributed, further metabolized in the liver and excreted in the urine (ADME). As part of the chemical toxicokinetic, it is possible that after biotransformation in the liver, the compound is secreted back to the gut where it can further interact with the gut microbiome. Non-absorbed compounds will remain in the intestinal tract, where they can interact with the microbial community before they are eliminated in the faeces.

The conditions within the gut, including pH, enzymatic activity, and the presence of other microbial metabolites, can also impact the fate of food additives. For example, aspartame is rapidly and completely hydrolysed in the gastrointestinal tract into phenylalanine, aspartic acid and methanol (EFSA, 2013), which limits the possibilities of the additive to interact with the microbiota. Under gastrointestinal conditions, low gastric pH affects the surface charge of titanium dioxide, facilitating its agglomeration,

as reported in studies characterizing the properties of this compound in gastric simulations. Such agglomeration can impact bioavailability by decreasing particle internalization by epithelial cells (Agans *et al.*, 2019).

Due to the potential of the food matrix and intestinal content to bind chemicals, the safety evaluation of veterinary drug residues requires the determination of the oral (unbound) dose fraction available to microorganisms (Piñeiro and Cerniglia, 2020), which can be carried out by using different methodologies (VICH, 2019). However, this requirement is not contemplated in the safety evaluation of food additives. Only a limited number of studies monitored the presence of the additive in the sample, but not for the purpose of calculating oral (unbound) dose fraction available to microorganisms.

Serrano *et al.* (2021) found saccharin in the stools of most mice and several humans participating in their trial. The purpose was to evaluate if the saccharin dose used was sufficient to reach the colon, where it could interact with the microbiota.

In a transgenerational study, Olivier-Van Stichelen, Rother and Hanover (2019) evaluated the presence of acesulfame K and sucralose in the mothers' blood, faeces and breast milk. In dams, sucralose was mainly detected in stools, while acesulfame K was detected in blood and stools. Both sweeteners were detected in breast milk but at lower concentrations than the other samples. The sweetener levels were undetectable or very low in pre-weaning pups from treated mothers. However, sucralose was detected only in stools. Acesulfame-K was detected in the urine of newborns and 14-day-old pups. Unless there was an accidental exposure of pups to the sweeteners (e.g. coprophagia, maternal behaviour or drinking water containing the sweeteners), the results suggested vertical transmission of sweeteners.

Chassaing *et al.* (2021) monitored the presence of CMC in human faecal and urine samples before, during and after treatment. The additive was found in high quantities in stool samples but not in urine, indicating low bioavailability.

Several studies determined titanium (Ti) in different samples. Li *et al.* (2019) evaluated the potential absorption of TiO_2 and determined titanium in various tissues after treating mice with varying TiO_2 sizes. Titanium was only found in the blood of animals treated with the smallest particle size (25 nm non-food grade TiO_2). Yan *et al.* (2022) did not detect titanium in colonic tissue after treating mice with food-grade or nano TiO_2. Cao *et al.* (2020) determined titanium content in stools from treated mice and untreated humans. The purpose of this exercise was not to evaluate TiO_2 bioavailability but to give an indication of whether the experimental dose was representative of TiO_2 occurrence in humans. The scientists found as much as nine times more titanium in mice than in humans.

Enzymes from both the host and the microbiome can metabolize food additives, affecting their bioactivity and availability. Some additives may only become bioactive or accessible to certain microbes after enzymatic modification. This will be addressed in the "Effects of the microbiota on food additives" section.

CAUSE-EFFECT: ASSOCIATIONS AND CAUSALITY

While some studies evaluated the impact of food additives on the gut microbiome, others also investigated effects in the host. One question that many of these studies tried to answer is whether changes in the gut microbiome caused by the additive exposure influenced the effects observed in the host. Although the question seems simple, the confirmation of this relationship is currently very challenging for several reasons (Cani, Moens de Hase and Van Hul, 2021):

> the lack of definitions for healthy gut microbiome and dysbiosis;

> the complexity of the gut microbiome composition and intracommunity dynamics, as well as the limited information on the taxa acting as functional drivers under the different environmental and physiological contexts;

> while the influence of the microbiome in various physiological functions has been reported and acknowledged (e.g. immune system, digestion), there are still many knowledge gaps in the microbiome-host relationship that need to be further investigated. These include, for example, distinguishing and measuring the extent to which both host and microbial factors contribute to health outcomes;

> individual variability and the influence of confounding factors (e.g. diet, lifestyle, environmental factors) make it difficult to extrapolate findings across populations and establish universal causal relationships; and

> the limited number of longitudinal studies over extended periods, which allows for tracking changes in the gut microbiome and health outcomes.

There are varying levels of evidence and methods that scientists have used to link disturbances in the microbiome with adverse effects observed in the host, and ultimately with non-communicable diseases (NCDs), following exposure to food additives. In the absence of host endpoints or markers, some research claimed associations between microbiome changes and disorders based on the mere comparisons between observed altered bacterial taxa and those previously associated with specific disorders (e.g. obesity). Of those evaluating effects in the host, the connection between the microbiome and host effects was often speculative, as no statistical models were applied to investigate possible associations or correlations. Less frequently, scientists applied correlation analysis to microbiome and host metrics or implemented methodologies to evaluate causality. Therefore, many of the associations claimed by many research groups were of speculative nature. Tierney *et al.* (2022) evaluated scientific works reporting associations between the gut microbiome and health outcomes and found inconsistencies in one-third of 581 associations between taxa and disease and in more than 90 percent of the studies linking gut microbes with type 1 and type 2 diabetes. It is important to highlight that correlations and associations imply co-occurrence but not causation. In addition, correlations do not detect cause-effect relationships (Xia, 2020). However, a properly determined association and further investigation of underlying mechanisms involved in the microbiome-host relationship are steps towards determining causation. Correlation and association analysis in microbiome studies has been addressed in depth by Xia (2020).

There is a tendency for researchers evaluating the gut microbiome and health to report unidirectionally, focusing primarily on the impact of the gut microbiome on the host. However, it overlooks the bidirectional nature of interactions between the gut microbiome and the host and the possibility that changes in the gut microbiome can result from the host's response to the additive or any other dietary or environmental factors. It is also possible that microbial disturbances are bystanders to host effects (or disease) (Walter *et al.*, 2020). While crosssectional studies do not contribute to directionality, longitudinal studies can be useful in investigating causality and its direction.

Causality implies a cause-effect relationship, which requires that one variable directly leads to changes in another one. Causal relationships are complex and multidimensional (Walter *et al.*, 2020). It is also important to keep in mind that the causes of disease are often multifactorial and identifying which one(s) is key or sufficient to initiate the development of disease is essential to implementing preventive measures (Lucas and McMichael, 2005). Demonstrating causality is challenging and rarely proven by a single experiment. The evidence has to be provided by reliable methodologies and well-designed studies (Weed, 2022). Criteria for establishing causation in disease were first defined by Bradford Hill over five decades ago and have provided a background framework against which exposures can be evaluated as cause factors (Hill, 2015; Lucas and McMichael, 2005).

These criteria have been adopted and updated in the WHO/IPCS mode of action framework (Meek *et al.*, 2014). There are different methods for inferring causality, all of them having their own limitations. These include, for example, computational approaches (e.g. mendelian randomization) (Lv, Quan and Zhang, 2021), randomized controlled trials (Zabor, Kaizer and Hobbs, 2020), faecal microbial transplantation, or human microbiota-associated murine models (Walter *et al.*, 2020).

One of the most common approaches to demonstrate causality is using germ-free (GF) mice. These animals are bred and raised free of microorganisms under stringent environmental conditions. In exposure or clinical studies, these animals receive "altered" microbiota from treated or diseased individuals by a procedure called faecal microbiota transplant (FMB). Reproducing the condition phenotype in the GF mice would be a step toward demonstrating causality. Because GF mice are resource-intensive and expensive, an alternative is the use of normal laboratory animals treated with an antibiotic cocktail to deplete the microbiota. Both approaches have advantages and disadvantages, which have to be considered when interpreting results (Kennedy, King and Baldridge, 2018).

Transplantation of intestinal microbiota from a donor – or pooled material from several donors – into a GF animal is a challenging procedure requiring the understanding and control of many factors (Gheorghe *et al.*, 2021; Hanssen, de Vos and Nieuwdorp, 2021; Secombe *et al.*, 2021):

> type of recipient. Germ-free (GF) or mice with depleted microbiota;

> genetic background of the recipient GF-mice. It influences the composition of the transplanted microbiota (Wos-Oxley *et al.*, 2012). Transplants within the same species (mice-mice), using the same strain (recipient-donor) will result in less selective pressure on the donor microbiota (Gheorghe *et al.*, 2021). A list of common mouse strains typically used in FMT has been compiled by (Gheorghe *et al.*, 2021);

> age and gender of the recipient animal;

> housing conditions (e.g. coprophagy and microbial contamination from external sources);

> differences in diets between the donor and recipient, which are known to rapidly influence the composition of the gut microbiota, e.g. high-fat diets used to induce obesity (Murphy *et al.*, 2010);

> microbiota handling (collection, processing, storage); and

> transplantation features (dose, route, duration and frequency).

Like many other areas of microbiome research, there is a need for standardized protocols to handle and carry out studies in GF mice, which is evidenced by the diversity in approaches used in the few studies where these animals have been used to investigate causation (Table 7).

TABLE 7. **STUDIES CONDUCTING MICROBIOTA TRANSPLANT EXPERIMENTS VIA ORAL GAVAGE**

REFERENCE	COMPOUND	PURPOSE	DONORS AND RECIPIENTS
(Hanawa *et al.*, 2021)	Acesulfame K	Reproduction of intestinal damage	Donor: C57BL/6 (caecal content) Recipient: ABX* treated C57BL/6
(Suez *et al.*, 2014)	Saccharin	Reproduction of metabolic effects, impaired glycaemic tolerance	Donor: C57BL/6 (stools) Recipient: GF Swiss Webster
(Suez *et al.*, 2022)	Saccharin, sucralose, aspartame, stevia	Reproduction of impaired glycaemic response	Donor: Healthy human (stools) Recipient: GF Swiss Webster
(Nettleton *et al.*, 2020)	Aspartame, rebaudioside A	Reproduction of metabolic changes, impaired glycaemic response	Donor: Sprague-Dawley rat (caecal content) Recipient: GF mice (strain not specified)
(Chassaing *et al.*, 2015)	CMC, P80	Reproduction of low-grade inflammation and metabolic syndrome	Donor: Swiss Webster (caecal content) Recipient: GF Swiss Webster
(Chassaing *et al.*, 2017)	CMC, P80	Reproduction of inflammatory potential	No donor. ASF* consortium Recipient: GF C57BL/6
(Viennois *et al.*, 2017)	CMC, P80	Reproduction of gene expression of proliferation and apoptosis factors	Donor: Swiss Webster (caecal content) Recipient: GF Swiss Webster
(Rousta *et al.*, 2021)	CMC, P80	Reproduction of colitis (IL-10 deficient GF mice)	Donor: Human with IBD* (stools) Recipient: GF IL-10 deficient mice (129SvEv background)
(Li *et al.*, 2020b)	P80	Susceptibility to radiation-induced GI tract toxicity	Donor: C57BL/6 (stools) Recipient: ABX treated C57BL/6
(Jin *et al.*, 2021)	P80	Reproduction of Intestinal inflammation and barrier dysfunction	Donor: C57BL/6 (stools) Recipient: ABX* treated C57BL/6
(Yin *et al.*, 2021)	κ-carrageenan (KCO*)	Explore pro-inflammatory effects of KCO and KCO-degrading bacteria	Donor: Healthy human (stools) > Bioreactor Recipient: GF Kunming mice
(Wu *et al.*, 2021)	λ-carrageenan	Reproduction of intestinal inflammatory effects	Donor: C57BL/6 (stools) Recipient: GF C57BL/6
(Wu *et al.*, 2022)	κ-carrageenan	Reproduction of intestinal inflammatory effects	Donor: C57BL/6 (stools) Recipient: GF C57BL/6
(Li *et al.*, 2019)	Titanium dioxide	Reproduction of mucus layer alterations	Donor: C57BL/6 (stools) Recipient: ABX* treated C57BL/6
(Cao *et al.*, 2020)	Titanium dioxide	Reproduction of inflammatory response in colon	Donor: C57BL/6 (stools) Recipient: ABX* treated C57BL/6
(Yan *et al.*, 2022)	Titanium dioxide	Reproduction of intestinal damage	Donor: ICR mice† (stools) Recipient: Conventional ICR mice

* ABX: Antibiotic; ASF: Altered Schaedler flora; KCO k-carrageenan oligosaccharides (~4.5 kDa); IBD Inflammatory bowel disease.

† Transplant using rectal enemas.

Sources: See References

Experiments to prove causality between diet-induced microbiome alterations and health outcomes have to be well designed, conducted and controlled. The procedures should be well described in published peer-reviewed papers to allow the expert reviewers and readers to assess the science quality or identify limitations. Unfortunately, several transplant experiments evaluated in this review were not well detailed, lacking the information necessary to properly assess the outcomes (e.g. Chassaing *et al.*, 2015; Suez *et al.*, 2014; Viennois *et al.*, 2017). Unfortunately, this is not an uncommon practice (Secombe *et al.*, 2021). The following are some of the factors identified, which can influence the study outcome:

- number of donors and recipients: Are all mice used as donors? Are donor samples pooled or transplanted individually?;
- limited information related to measures taken to ensure the absence of the test additive in the microbiota sample, which could influence the outcomes in the transplanted animal. For example, Wu *et al.* (2021) implemented a 7-day clearance period to reduce the likelihood of λ-carrageenan residues from stools being transferred to recipients;
- whether transplant is conducted in repeated doses and for how long. It has been reported that repeated gavage can increase the similarity of the transplanted microbiota to that of the donor (Choo and Rogers, 2021a);
- whether the procedures are sufficiently lengthy to allow the transplant to successfully colonize the recipient, achieve stability, and reproduce the effects observed in the donor? In GF mice, a transplanted microbiota can take about 28 days to stabilize (Choo and Rogers, 2021a). For example, Hanawa *et al.* (2021) carried out the transplantation in repeated doses for five days and tested the intestinal integrity 24 hours after the last dose. The researchers did not observe any changes, which could be due to insufficient time for potential changes to develop;
- often, engraftment stability is not monitored (or not reported) to assess the procedure's success and the taxa lost in the process. Microbiota stability, changes in community structure, or depleted taxa are factors that should be considered carefully when interpreting results and drawing conclusions;
- some research groups used different mouse strains as donors and recipients (Suez *et al.*, 2022; Suez *et al.*, 2014) or different rodent species (Nettleton *et al.*, 2020). Although different mouse strains may lead to similar microbiota composition, the use of the same strain is optimal to reduce the selective pressure exerted by the recipient on the donor microbiota (Gheorghe *et al.*, 2021); and
- that diets differed between donors and recipients (low and high-fat diets) (Cao *et al.*, 2020; Suez *et al.*, 2014), which has been shown to alter the microbiota structure and gene expression rapidly, within just one day (Turnbaugh *et al.*, 2009b).

Although transplant experiments are valuable in investigating the causal relationship between the gut microbiome and health outcomes, there is a need for more rigourous and reproducible experimental designs and methodologies to guarantee accurate conclusions and avoid erroneous attributions (Walter *et al.*, 2020). For more in-depth

information, several publications address considerations, challenges and limitations of faecal microbiota transplants in experimental animals (Choo and Rogers, 2021a; Gheorghe *et al.*, 2021; Hanssen, de Vos and Nieuwdorp, 2021), experimental protocols (Choo and Rogers, 2021b) and guidelines for reporting animal faecal transplantation studies (Secombe *et al.*, 2021).

Ultimately, even when there is evidence of the causal relationship in animal models, it remains to be seen if results obtained from FMT in GF animals are translatable to humans. More recent approaches have been proposed to investigate causality, although they are not free from challenges and limitations (Corander, Hanage and Pensar, 2022; Wade and Hall, 2020).

RESULT INTERPRETATION AND REPORTING

After reviewing the reporting and interpretation of results, certain aspects require attention as they influence the accuracy of statements and messages that are provided to the reader.

Generally, statistically significant results – often based on *p*-values alone – between treatment and control groups in both the microbiota and host experiments seem treated as effects, adverse or beneficial. However, statistically significant results do not necessarily translate into biologically relevant outcomes, for which it is necessary to consider the magnitude of the effect and the context in which it occurs (Solla *et al.*, 2018). There is controversy about the suitability and use of *p*-values in research, which will not be described here. Still, it is important to acknowledge that this debate exists and can significantly influence the interpretation of study results and conclusions (Montero, Hedeland and Balgoma, 2023; Smith, 2020; Solla *et al.*, 2018).

In general, null results (from microbial and physiological metrics) are often overlooked. The researchers typically decide on which markers to include in a study for a reason. Therefore, whether they are statistically significant or not, they should be included in the result interpretation. Null outcomes are not synonymous with

the absence of information. They are informative and valid results that form part of the overall evidence produced by the study. Assigning a disproportionate weight to specific outcomes without justification risks introducing bias into the interpretation and conclusions.

Although the concept of dysbiosis is often mentioned as a study outcome, its meaning is subject to the individual interpretation of the research group. Such interpretation frequently relies on obsolete concepts (e.g. Firmicutes/Bacteroidetes ratio). These situations can lead to overstatements and confusion about the actual impact of food additives on the gut microbiome and the consequent influence on health effects. For example, reporting dysbiosis after observing any type of change in the microbial community in the absence of the host, as observed in an *in vitro* study evaluating several non-nutritional sweeteners (Vamanu *et al.*, 2019). As already discussed by others, this is not an uncommon observation due to the lack of a consensus definition for dysbiosis (Brüssow, 2020). Scientists are responsible for promoting a healthy science (Yue, Segre and Chang, 2019) and should be more critical and careful when discussing dysbiosis to improve the ability to predict health outcomes and provide more accurate insights into the microbiome's contribution to health and disease (Hooks and O'Malley, 2017).

Additional considerations for interpreting and reporting findings:

> Researchers should remain objective and avoid seeking only data or interpretations supporting their hypotheses.

> Interpretations should be closely tied to the research context and data collected. Avoid drawing conclusions that go beyond what the data can support. The language used should describe findings accurately. Scientists should avoid overinterpreting results and using amplifying qualifiers like "profound" or "extreme" to describe changes in the microbiome unless the data unequivocally supports such claims. Unfortunately, the use of emotionally-charged terminology (positive and negative) in scientific publications is increasing (Edlinger, Buchrieser and Wood, 2023; Vinkers, Tijdink and Otte, 2015), which is something to be avoided as a recommendation for best practices in reporting.

> Discussions should acknowledge study limitations and uncertainties. Unfortunately, they are not often reported.

> Generalizing results beyond the study conditions and research data should be avoided. This includes, for example, generalizing findings from a single additive to an entire class of additives, such as extrapolating results from saccharin to all sweeteners or from carboxymethyl cellulose to all emulsifiers. Food additives are chemically different from each other, even within a class, and are not expected to behave equally in a biological system, like the one formed by the gut microbiome and humans. Exaggerations and generalizations are often included in the title and abstract of scientific publications, therefore having a higher impact. Such practices are speculative, can mislead the readers by setting unrealistic expectations, and influence their opinion, especially that of non-experts or the general public.

At the same time, it is essential to encourage the critical thinking of scientists when reading and evaluating findings reported by peers. Otherwise, there is a risk of compromising the integrity and validity of their own research. To avoid these situations, the researchers should:

> conduct a comprehensive literature review to capture a wide range of perspectives and findings, acknowledging areas of agreement and contention within the field. Researchers should avoid selecting and citing only those studies that support their hypothesis while ignoring conflicting evidence;

> ensure the studies cited are directly relevant to the research question and meet high scientific standards. Evaluate the methodologies, sample sizes, and contexts of the studies (e.g. route of exposure, doses) to ensure they are appropriate for supporting scientific arguments. Citing a study without fully understanding its context, methodology, or limitations can misrepresent its findings; and

> check the original citation, when possible. Relying on secondary sources without checking the original research can perpetuate misinformation or misinterpretation.

While some publishing houses or journals have established guidelines for data reporting that can be beneficial during the planning phase of a study, this practice is not uniformly adopted across all publications. Some guidelines for reporting data have been published and can be helpful to support researchers. These include, for example, the ARRIVE guideline for reporting animal research (Percie du Sert *et al.*, 2020) and the STORMS checklist for reporting human microbiome research (Mirzayi *et al.*, 2021). In addition, the OECD has developed a framework (OECD, 2023) and templates for reporting OMIC data (OECD, 2024a).

By adhering to these practices, researchers can maintain the integrity of their work, contribute valuable insights to their field, and ensure that their findings are interpreted and applied appropriately by, for example, risk assessors, clinicians, regulators and the general public.

EFFECTS OF THE MICROBIOTA ON FOOD ADDITIVES

The gut microbiome constitutes a broad range of enzymes, allowing it to participate in digestion processes and the biotransformation of dietary chemicals that take place in the gut. Some of these enzymes are of microbial origin only, therefore expanding the host's metabolic capacity (Koppel, Maini Rekdal and Balskus, 2017). Although many microbial enzymes are known, more research is needed to identify and annotate those participating in metabolite biotransformation with the support of omics tools (Jia *et al.*, 2022).

The enzymatic repertoire confers the microbial community the potential to modify the activity, bioavailability and toxicity of chemicals. In addition, after some chemicals are absorbed and transformed in liver, they can be secreted back to the intestinal lumen, where they can be deconjugated and re-activated by the microbiome. Some compounds can be re-absorbed, entering the enterohepatic

circulation[38] (FAO and WHO, 2009a; Collins and Patterson, 2020). Entering this cycle can prolong the half-life of the compound and consequently the exposure of the host to the chemical.

Although several reports have indicated the participation of the colonic microbiota, specifically *Bacteroides* species (Gardana *et al.*, 2003), in the cleavage of the glycoside linkage of steviol glucosides (Magnuson *et al.*, 2016; Wingard *et al.*, 1980), limited reference was made to this microbial activity in the studies included in this review. The steviol product, following microbial hydrolysis in the colon of steviol glycosides, is absorbed and conjugated in the liver to steviol glucuronide. However, the excretion differs between humans and rats. In humans the glucuronide moiety is excreted in the urine, while in rats it is secreted back into the intestinal lumen, deconjugated and eliminated in the faeces (Magnuson *et al.*, 2016).

Xiang *et al.* (2021) observed that *Lactobacillus reuteri, Bacteroides fragilis* and *Escherichia coli* (from mice) were involved in the xylitol metabolism by a cross-feeding[39] mechanism. Xylitol dehydrogenase was determined to be the core enzyme involved in the process.

He *et al.* (2021) reported the ability of *Bacteroides ovatus* and *Enterococcus faecalis* (from mouse stools) to metabolize the azo dye Red 40 (Allura red, E129) by azo reduction into two metabolites, i.e. cresidine-4-sulfonate sodium salt (CSA-Na) and 1-amino-2-naphthol-6-sulfonate sodium salt (ANSA-Na). Only ANSA-Na induced colitis in susceptible animals (dysregulated IL-23 expression). Similar results were reported for Yellow 6 (E110) but not with non-azo dyes (Red 3 and Blue 1). Azoreductase activity by human gut microbiota members has previously been described (Zahran *et al.*, 2019).

Yin *et al.* (2021) observed the capacity of *Bacteroides xylanisolvens* (isolated from humans) to degrade κ-carrageenan oligosaccharides (~4.5 kDa), which became more efficient in the presence of *Escherichia coli*, probably due to cross-feeding between the bacteria. Only this carrageenan fraction induced intestinal inflammation in germ-free mice. However, HMW carrageenan remained unaltered κ-carrageenan (> 100 kDa). Sun *et al.* (2019) also observed the fermentation of hydrolysed κ-carrageenan *in vitro*. Of note, food-grade carrrageenans are mainly HMW. Following reports indicating the activity of *Desulfovibrio* from marine samples to reduce sulfur groups of carrageenans, Yin *et al.* (2021) evaluated if this activity could be carried out also by the gut commensal bacteria. However, they did not observe the removal of sulfate groups from the different κ-carrageenan products tested (HMW and hydrolysed sample).

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[38] Enterohepatic circulation. Intestinal reabsorption of material that has been excreted through the bile followed by transfer back to the liver, making it available for biliary excretion again.

[39] Cross-feeding. Interaction between microorganisms in which molecules resulting from the metabolism of one microorganism (referred to as the provider or producer) are further metabolized by another (referred to as the receiver, or beneficiary) (Mataigne *et al.*, 2021, p. 3).

EFFECTS OF FOOD ADDITIVES ON THE GUT MICROBIOTA AND THE HOST

Before delving into this topic, it is necessary to clarify some points to align the expectations and understanding of this section. As discussed previously, the differences in research questions, study design and methodological approaches differ significantly amongst studies, making it challenging to compare results and identify commonalities, consistent patterns and trends. In addition to these challenges – which have been extensively discussed in previous sections – it is necessary to acknowledge that each study comes with its own set of limitations, which, although not explicitly addressed in some studies, play a relevant role in interpreting results.

For all these reasons, and due to differences in scientific rigour and quality across studies, it is essential to exert a cautious approach when discussing the impact of food additives on the gut microbiome and the consequent influence of the microbial population on health outcomes. Understanding and ensuring a well-informed and critical analysis of the available evidence reinforces the importance of avoiding inaccurate statements, premature conclusions and the dissemination of potentially misleading information to the scientific community, regulators and the general public.

This section will explore general aspects of studies investigating the impact of food additives on the gut microbiome and the subsequent potential influence of the intestinal bacterial community in host health outcomes. Specific outcomes are reported in the "Study summaries" section and summarized in the tables available in Annex III. However, it is important to note that most of these summaries report statistically significant results mainly, with limited mentions of null results (excluding those studies reporting no effects). Another clarification about this section is that the discussion is limited to the directionality "gut microbiome influence on the host outcomes" and will not cover the reverse or bystander effects because the studies evaluated did not consider or address this plausible explanation.

Research evaluating the impact of food additives on the gut microbiome and the potential subsequent health effects on the host was conducted in two different contexts, aiming to address different research questions:

- health, by including healthy animals or healthy human subjects in the study (or evaluating the microbiota from these *in vitro*). This context is relevant for studying the impact of food additives on the healthy population and the risk for adverse health effects or disease. However, the concept of health (in the context of chemical exposure) is complex, as healthy individuals are not equally healthy. Interventional studies recruited individuals "apparently" healthy without any seeming physiological alterations and not taking certain medications, for example, antimicrobials or anti-inflammatory drugs; and
- disease, experimenting in animals with a predisposition to a particular disorder, genetic alteration or where the disorder is induced, e.g. obesity, colitis, cancer. These individuals are representative of diseased or vulnerable populations, making it possible to evaluate the influence of the chemical on the onset or

progression of the disorder, whether it aggravates or ameliorates the condition. However, of relevance for food additive microbiome studies, an ADI is applicable to the general healthy population, not to individuals who have developed NCDs.

The following are some general observations focusing exclusively on the gut microbiome response to food additives. All studies investigated, to a different extent, the bacterial community structure, often with discussions evolving around taxa associated with disease, focusing on reduced diversity and increases of "detrimental" taxa or decreases in the abundance of "beneficial" species. Many studies observed some degree of disturbance in the microbial community structure, and those investigating various doses often reported dose-dependent responses. Microbial changes tended to be diverse and scattered, making it difficult, especially in cross-sectional studies, to identify consistent patterns following the exposure of the gut microbiome to food additives, whether *in vivo* or *in vitro*. Such variability is not surprising, given the differences in models, experimental conditions and analytical approaches.

When the functional microbiota was evaluated, effects typically considered detrimental included, for example, decreases in SCFAs (particularly butyrate) or increases in microbial pro-inflammatory structural components, e.g. flagellin and LPS. For a given food additive, it is difficult to define the impact of the substance on SCFA production, given that they are not systematically assessed, and it is not clear which levels should be indicative of an adverse effect. Similarly to observations in the microbial taxonomy, trends cannot be identified due to differences in study designs and diverging results.

Regarding LPS and flagellin, compounds belonging to the microbe-associated molecular patterns (MAMPs) (Chu and Mazmanian, 2013), it is important to note that, due to the evolutionary adaptation of gut microbes to the host, their chemical structure has been modified, especially the moiety responsible for the virulence (Mohr *et al.*, 2022; Zhao and Maynard, 2022). While they remain strong stimulants of the immune system, the modifications also allow the bacteria to be distinguished from pathogens and be tolerated by the host. Different LPS and flagellin variants, often evaluated in faecal samples, differ in their capacity to bind intestinal receptors (TLR4 and TLR5) and lead to varying effects on cytokine production and immune responses (Clasen *et al.*, 2023; Mohr *et al.*, 2022). It has been reported that LPS from certain bacteria, e.g. *Bacteroides vulgatus* mpk, could even modulate the immune response and help re-establish the intestinal immune balance and prevent colitis (Steimle *et al.*, 2016; Waidmann *et al.*, 2003). Therefore, the biological implications of changes in these compounds depend not only on the levels but also on the LPS and flagellin variants. Although more research is needed to better understand the diversity and activity of LPS and flagellin (Di Lorenzo *et al.*, 2019), it is important to consider this variability when interpreting changes in their levels in dietary exposure studies.

Research conducted by Chassaing's team to evaluate CMC and P80, consistently monitored bioactive LPS and flagellin levels (in cell cultures expressing receptors TLR4 and TLR5) or related gene-expression reported significant increases under

experimental conditions *in vitro* (Chassaing *et al.*, 2017) and in animal studies (Chassaing *et al.*, 2015; Viennois *et al.*, 2020; Viennois and Chassaing, 2021; Viennois *et al.*, 2017), which were interpreted as signs of pro-inflammatory microbiota. However, no altered levels were found in the human trial by CMC (P80 not evaluated), except in two subjects (Chassaing *et al.*, 2021). Monitoring MAMPs profile alone, e.g. *in vitro* studies, should not be used to predict host outcomes because specific host receptors and loss of gut barrier function are essential for exacerbating metabolic disorders (Ha, Lam and Holmes, 2014).

Dietary components can impact how microbes communicate among themselves. Reports have indicated that aspartame, sucralose, saccharin and steviol glycosides can affect microbial communication systems (Quorum Sensing) by inhibiting signalling molecules (autoinducers) of some gram-negative bacteria (Markus *et al.*, 2021; Markus *et al.*, 2020). However, the impact of such alterations on the microbial function and potentially in the host remains unexplored, although some have suggested their potential application as biomarkers of inflammatory disorders (e.g. IBD), colorectal cancer and neurological diseases (Dicks, 2022). The Quorum Sensing system has not been a common target in the studies included in this review. For example, by monitoring the metabolome, Bian *et al.* (2017b) detected changes in metabolites related to the Quorum Sensing system after sucralose treatment in mice.

Another underexplored microbial aspect in the manuscripts included in this review has been the potential of food additives to influence antimicrobial resistance. Enrichment of AMR genes was reported after treatment with sucralose and saccharin (Bian *et al.*, 2017b; Bian *et al.*, 2017c), while the silver nanoparticles did not affect the expression levels of silver resistance genes (Hadrup *et al.*, 2012). *In vitro* studies have shown that sucralose, saccharin, acesulfame K and aspartame (used as 90 percent growth inhibition of *Acinetobacter baylyi* or *Escherichia coli* K12) could promote the transformation of extracellular DNA in gram-positive and gram-negative and induced plasmid persistence in transformants (Yu *et al.*, 2021a) and conjugation of antimicrobial resistance genes (Yu *et al.*, 2021b). Because the physiological environment and the gut microbiota community may influence the transfer of genetic material, the authors of these studies indicated the need to verify and validate these results *in vivo*.

In general, discussions of studies reporting negative outcomes tend to highlight what is considered by researchers as unfavourable results and limited consideration is made to frequent null results or those microbial outcomes that researchers often refer to as "beneficial" (e.g. increases in diversity or beneficial bacteria, butyrate or decreases in Proteobacteria). Differences in the meaning and interpretation of microbial disturbances by the different researchers are partly due to the lack of reference consensus definitions for healthy microbiomes and dysbiosis. In a recent meeting organized by FAO on the consideration of microbiome data for risk assessment, experts acknowledged that dysbiosis should not be viewed only as an undesirable and unstable state of the gut microbiome but should also encompass the health status of the host (FAO, 2024).

It is challenging to understand and determine the meaning of changes in the microbial community, whether they are normal fluctuations, the result of adaptation or are biologically relevant, and whether such disturbances are reversible. More research is needed to characterize the physiological relevance of microbial changes after chemical exposure, especially those that are long term.

The overall body of research has focused on investigating the potential involvement of the gut microbiome in chronic disorders, especially concentrating on intestinal health (e.g. immune responses, barrier functions), inflammation at the intestinal level (e.g. IBD[40] [Sunderhauf *et al.*, 2020]) or systemically, metabolic disorders, cancer and neurobehavioural conditions. This scientific interest responds to growing concerns about the potential link between lifestyle and dietary changes, including the increase in consumption of Westernized – ultra-processed foods in particular – and increases in the prevalence of chronic disorders. These types of food are characterized as being low in dietary fibres and high in sugars and fat, which are known to influence microbial community structure and function. Additionally, these foods contain food additives, prompting scientists and physicians to explore whether these additives could play a role in triggering or advancing chronic diseases, with the gut microbiome potentially serving as a mediating factor.

In general, the following are different types of outcomes reported by the studies evaluated:

> No disturbances were found, or changes were only seen in either the microbiota or the host. Some treatments did not induce significant changes, contradicting other studies evaluating the same additive—for example, saccharin and sucralose.

[40] Inflammatory bowel disease (IBD) comprises a group of chronic, immune-mediated inflammatory disorders of the human gastrointestinal tract, i.e. Crohn's disease (CD) and ulcerative colitis (UC). Although there has been considerable research on IBD in recent years, the specific cause(s) remains unclear.

- Statistically significant gut microbiome disturbances were often suggested as contributors to host effects. Unfortunately, these links were often speculative, and only a few researchers designed studies to investigate causality or underlaying mechanisms involved in the interaction between the additive, the gut microbiota, and the host.
- Effects affected sensitive or predisposed individuals only.
- In disease models, exposure to the additive aggravated or ameliorated the condition induced in experimental animals.

NON-NUTRITIONAL SWEETENERS

In general, most human interventional studies reported limited or no effects compared to findings in animal models. Experimental doses were more realistic than those used in animal studies, which, due to ethical reasons and to comply with regulatory recommendations, never exceeded the JECFA ADI of the sweetener. However, trials are typically conducted in small groups of individuals – although in higher numbers than many animal studies – and are short in duration, a limitation often noted because long-term exposures more accurately represent the food additive intake. Epidemiological studies aimed to uncover potential links between non-nutritional sweetener consumption and chronic disorders. However, establishing these connections is challenging due to the influence of confounding factors in this type of study.

Research evaluating aspartame, acesulfame K, sucralose, saccharin and steviol glycosides (often rebaudioside A) primarily focused on endpoints related to the inflammatory response and metabolic effects (lipid and glucose) in the context of obesity or type 2 diabetes.

Glucose homeostasis has been a primary endpoint in several human interventional trials and animal studies, often assessed by OGTT or by monitoring other glucose-related parameters, e.g. fasting glucose or fasting insulin. Despite OGTT being regarded as an indicator of risk for developing diabetes and an early marker of impaired glucose homeostasis, there is high intra-individual variability in oral glucose tolerance tests (OGTT) studies, with a coefficient of variation of up to 16.7 percent (Sacks, 2011). Many variables influence this test, requiring standardization to facilitate consistency and comparison of results from different studies. For example, in mice, such variables include the length of the fasting period before the OGTT, dose and route of administration (Pedro, Tsakmaki and Bewick, 2020). These factors varied among the different studies monitoring the effects of food additives on OGTT and the gut microbiome. Although the OGTT test can potentially identify a pathological phenotype, the evaluation of glucose homeostasis requires a more detailed assessment to investigate possible implications (Bowe *et al.*, 2014). For example, to evaluate glucose homeostasis after non-nutritive sweetener supplementation, Serrano *et al.* (2021) and Suez *et al.* (2022) conducted insulin tolerance tests and measured GLP-1, glucagon and C-reactive protein levels in addition to the OGTT.

As multiple factors can influence glucose tolerance, it is essential to ensure that any change is a deviation from the normal glycaemic response by implementing proper controls. Glendinning *et al.* (2020) implemented up to eight different controls in a mouse study investigating non-nutritional sweeteners (saccharin, sucralose, acesulfame K) (Table 8). The studies included in this review implemented some but not all the controls defined in Table 8, with the number of controls differing from study to study.

TABLE 8. **CONTROLS TO HELP DETECT TRUE CHANGES IN STUDIES EVALUATING GLUCOSE TOLERANCE**

CONTROL	PURPOSE
Within-subject design.	Control for any pre-existing differences in glucose tolerance across mice.
Experiment overpowering (n > min number of mice recommended by power analysis).	Reduced risk of type 2 error.
For GTT, use a glucose dose that elicits an intermediate glycaemic response.	Reduce floor or ceiling effects during GTT.
Use an isoacceptable concentration of test compound (e.g. non-nutritional sweetener).	Control sweetness intensity.
Use sweetener in vehicle (water or glucose solution).	Control any effects of vehicle.
Conduct exposure in two independent experiments.	Confirm that results are reproducible.
Positive control (e.g. glucose).	Confirm that the measurement system can detect diet-induced changes in metabolic response.
Negative control (e.g. chow and water diet)	Confirm that changes in metabolic response are mediated by the sweetener.

Source: Adapted from **Glendinning *et al.*** 2020. Low-calorie sweeteners cause only limited metabolic effects in mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* 318(1): R70-R80. https://doi.org/10.1152-ajpregu.00245.2019

As research is often conducted in mice, it is necessary to consider that the translatability of findings from OGTT in mice to humans is challenging due to diverging metabolic responses to the OGTT (Bruce *et al.*, 2021).

Human trials evaluating saccharin and sucralose have led to contradictory results. Serrano *et al.* (2021) did not observe changes in microbial diversities and composition nor changes in body weight or glucose homeostasis in individuals participating in the randomized, double-blind, placebo-controlled study (n=13/group). On the contrary, at similar or slightly lower doses but using a commercial formulation, Suez *et al.* (2014) reported differences in the microbiota and glucose homeostasis in 4 out of 7 individuals (non-usual consumers of non-nutritive sweeteners). These individuals were selected from a previous epidemiological study that found correlations between the consumption of non-caloric sweeteners and some clinical parameters related to metabolic syndrome. To further evaluate the potential causal link between the gut microbiome and glucose tolerance, faecal material from selected two responders and two non-responders was transplanted to GF mice, reproducing the results observed in humans.

In a recent randomized interventional trial to evaluate commercial formulations containing saccharin or sucralose, aspartame or stevia, Suez *et al.* (2022) observed microbial changes and altered glucose responses in OGTT in the sucralose and saccharin group. Such responses were reproduced in GF-mice receiving faecal material from top saccharin responders in the OGTT test. Although volunteers who took aspartame and stevia did not show altered (OGTT) responses, researchers conducted FMT using samples from these nonresponders, specifically those with the highest OGTT values. This procedure resulted in significant differences in OGTT outcomes compared to the control group. In the assessment of causality, the purpose of FMT is to "reproduce" in transplanted animals the alterations observed in treated individuals. Therefore, observing statistically significant responses in the OGTT only in transplanted GF animals (not in the donors) requires careful interpretation, which could be due to chance or bias and not a causal effect. In this study, the diet was not fully standardized as it also included real-life meals. Therefore, dietary differences could also influence the results. In addition, OGTTs were performed by the participating individuals in their houses (not by trained personnel in a clinical setting) after fasting, which could range from 7 to 12 hours, potentially influencing the variability in glycaemic responses. Although differences in the glycaemic responses between responders and non-responders were significant, the differences were not marked, leading to questions about whether they were clinically relevant.

Other human studies reported no or limited effects of sweeteners. In a human randomized, double-blind trial study, the 7-day **sucralose** intervention at doses corresponding to 75 percent of the ADI did not alter glycaemic and insulinaemic responses or the microbiome (Thomson *et al.*, 2019). However, the authors reported different microbial profiles between individuals with higher or lower insulinaemic responses, which were independent of the sucralose treatment. This study contributes to the findings of other research teams, suggesting that responses to certain food additives are individual and vary from person to person. This observation calls for more detailed research on an individual basis.

In another randomized, double-blind interventional study, the sequential treatments with aspartame and sucralose or sucralose and aspartame did not alter the gut microbiota (Ahmad, Friel and Mackay, 2020a) and had no influence on glucose metabolism and insulin sensitivity (Ahmad, Friel and MacKay, 2020b).

One cross-sectional study compared the microbiome of aspartame and acesulfame K consumers and non-consumers, with differences limited mainly to bacterial diversity (Frankenfeld *et al.*, 2015).

In one human randomized interventional study (Beards, Tuohy and Gibson, 2010), chocolate supplemented with mannitol alone or combined with bulking agents (polydextrose [PDX] or resistant starch) did not change bowel activity but promoted the growth of Lactobacilli. Only the blend of maltitol-PDX increased Lactobacilli and SCFA propionate and butyrate.

Two observational human studies were conducted in connection with the consumption of artificially sweetened beverages (ASBs). Ramne *et al.* (2021) did

not find associations between ASB consumption and the gut microbiota. The researchers indicate that their findings very modestly support the triad of artificial sweeteners, the gut microbiota and the risk of cardiometabolic disorders. However, Laforest-Lapointe *et al.* (2021) reported the positive association between gestational consumption of ASBs, and the BMI, some urine metabolites and shifts in microbiota structure (especially the depletion of some Bacteroides species) in infants.

Most studies evaluating sweeteners were conducted in animal models. Research evaluating **aspartame** was conducted in obesity-induced animals (at doses below the ADI: 40 mg/kg bw/day), with metabolic effects more likely due to the high-fat diet than to the alterations of the faecal microbiome reported by the authors (Nettleton *et al.*, 2020; Palmnas *et al.*, 2014). This possibility is further supported by the fact that aspartame is quickly broken down, and its components are absorbed in the early segments of the small intestine (Magnuson *et al.*, 2016). Therefore, limited interaction of the intestinal microbiota and the sweetener is expected at distal intestinal segments.

The only animal study evaluating **acesulfame K** at a dose equivalent to its ADI (15 mg/kg bw/day) did not influence the host or the microbiome (Uebanso *et al.*, 2017b). Bian *et al.* (2017a) and Hanawa *et al.* (2021) evaluated acesulfame at doses between 2 and 10 times higher than the sweetener ADI, respectively. Bian's group suggested that microbial alterations could be involved in the development of metabolic alterations, diabetes and associated chronic disorders. On the contrary, Hanawa discarded the involvement of the gut microbiota in the intestinal alterations and speculated that the microbial disturbances could result from intestinal injury. Limited interaction between acesulfame K and the gut microbiota is expected, especially in the large intestine, as it is almost completely absorbed in the small intestine (Magnuson *et al.*, 2016).

Most **saccharin** is partly absorbed in the small intestine, with smaller quantities reaching the last section of the gastrointestinal tract (Magnuson *et al.*, 2016). Saccharin studies were methodologically very heterogeneous, and the results were contradictory. While doses at the saccharin ADI (5 mg/kg bw/day) have been reported to alter the microbiome and influence glucose dysregulation (Suez *et al.*, 2014), no effects on the microbiome or no influence in the host alterations have been reported at different dose levels (above or below the ADI) in obese animals (Becker *et al.*, 2020; Serrano *et al.*, 2021). Following treatment with high saccharin doses, Sunderhauf *et al.* (2020) reported improvement of colitis by modulating the disturbed gut microbiome, while Bian *et al.* (2017c) suggested the influence of the disturbed microbiota in the observed liver inflammatory effects in the host.

Sucralose, which is poorly absorbed, has been the sweetener most studied, but the research purposes were very different, and the study designs very heterogeneous. The different study conditions included doses below or several times above the ADI (15 mg/kg bw/day), different forms (commercial preparation or pure), different exposure periods, diets (standard or HFD), and so on (for details, see Annex III.4.). For this reason, finding commonalities or trends in the microbial disturbances reported by the different studies is challenging. Except for the human trial by Suez *et al.* (2022), discussed above, none of the studies were designed to evaluate causal links between microbial changes and host alterations.

Researchers evaluating sucralose had a particular interest in the function and integrity of the liver and intestine or glucose homeostasis. Rodriguez-Palacios *et al.* (2018b) reported dysbiosis and intestinal inflammation only at the highest dose of sucralose (Splenda) tested in a model of ileal Crohn's disease. After maternal exposure to sucralose during gestation and lactation, Dai *et al.* (2020) reported dysbiosis and alterations consistent with low-grade intestinal inflammation in mice at weaning and exacerbated HFDinduced hepatic steatosis later in life. Within ADI levels, sucralose was reported to induce hepatic fibrosis in HFD-fed mice (Xi *et al.*, 2020) and altered hepatic metabolism, with the potential contribution of dysbiosis in non-alcoholic fatty liver (Shi *et al.*, 2021). Based on altered hepatic markers, Bian *et al.* (2017b) suggested that sucralose could increase the risk of hepatic inflammation.

Sucralose did not impact weight gain and energy balance in HFD-induced obesity (Xi *et al.*, 2020) or lead to weight loss in animals fed standard rodent chow (Abou-Donia *et al.*, 2008; Wang *et al.*, 2018) or an HFD (Sanchez-Tapia *et al.*, 2020)

The effects of sucralose on glucose homeostasis varied depending on the study conditions. At high doses and in conjunction with HFD, sucralose led to glucose tolerance and insulin resistance, which was concomitant to enriched LPS genes and LPS-producing bacteria (Sanchez-Tapia *et al.*, 2020). There is evidence that microbial LPS could participate in the pathogenesis of insulin resistance (Liang *et al.*, 2013). Of note, sucrose, also evaluated in the work of Sanchez-Tapia *et al.* (2020), had a higher impact than sucralose. On the contrary, lower doses of sucralose given for a more extended period improved glucose tolerance and insulin sensitivity in HFD-fed mice (Xi *et al.*, 2020).

At doses higher than the ADI, Li *et al.* (2020a) suggested that sucralose-disturbed microbiota could be involved in the tumourigenesis of colorectal cancer and potentially increase the risk of colitis-associated colorectal cancer. Guo *et al.* (2021) reported that sucralose exacerbated the colitis in mice by mechanisms potentially involving microbial dysbiosis and the alteration of the intestinal barrier. In both studies, microbial alterations included an increased abundance of Firmicutes and decreased *Bifidobacterium*. While Proteobacteria increased in the colitis model, it was reduced in colorectal cancer.

Other studies involving sucralose (Li *et al.*, 2020a), CMC and P80 (discussed below) (Viennois and Chassaing, 2021; Viennois *et al.*, 2017) have evaluated the potential involvement of the gut microbiome on the tumourigenesis and colorectal cancer following the additive treatment. The International Cancer Microbiome Consortium published a consensus statement on the role of the human microbiome in carcinogenesis and research directions (Scott *et al.*, 2019). Based on existing evidence from animal and human studies, the experts *considered that there is currently no direct evidence that the human commensal microbiome is a key determinant in the aetiopathogenesis of cancer. However, expert opinion was that the microbiome is one apex of a tripartite, multidirectional interactome alongside environmental factors and an epigenetically/genetically vulnerable host that combine to cause cancer*.

In addition, the experts addressed the definition for dysbiosis (in the context of cancer) indicating that it *should be considered a persistent departure of the host microbiome from the health-associated homeostatic state (consisting of mutualists and commensals), towards a cancer promoting and/or sustaining phenotype (parasitism or amensalism). This dysbiosis is specific to the individual and thus can only be defined by prospective longitudinal analysis*. This group of experts noted that while the direct contribution of the gut microbiome to cancer ethiopathogenesis remains unproven, the majority concurred with the hypothesis.

Studies evaluating **steviol glycosides**, which are cleaved by the colonic microbiota (Magnuson *et al.*, 2016), focused mainly on investigating their impact on glucose regulation and inflammatory responses, often within the context of obesity.

Many of the reported beneficial effects of steviol glycosides were observed at doses higher than the JECFA ADI (4 mg/kg bw/day). For example, they have been reported to ameliorate the impact of high-caloric diets on glucose homeostasis and hepatic alterations (Xi *et al.*, 2020), to improve the inflammatory response, chronic kidney disease and gut dysbiosis associated with the disorder (Mehmood *et al.*, 2020) or result in diet-dependent anti-inflammatory effects (Sanchez-Tapia *et al.*, 2020). Increases in the abundance *Akkermansia muciniphila* were a commonality identified in the works by Xi *et al.* (2020) and Sanchez-Tapia *et al.* (2020). Considering studies evaluating low doses, Nettleton *et al.* (2020) reported that rebaudioside A, in the context of a high-caloric diet and obesity, could promote adiposity early in life, and disrupt glucose control in mothers and the gut microbiota of lactating offspring and mothers. However, Becker *et al.* (2020) observed that an HFD had a more critical role than the sweetener in influencing microbial and physiological outcomes. In lean animals, Rebaudioside A did not affect body weight, glucose tolerance or insulin resistance (Nettleton *et al.*, 2019).

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High concentrations of **sugar alcohols** (e.g. xylitol, sorbitol, and so on) are known to induce osmotic diarrhoea in susceptible individuals (Grembecka, 2015; Mäkinen, 2016; Zuo *et al.*, 2021) and disturb the gut microbiota composition in a dose-dependent manner (Xiang *et al.*, 2021; Zuo *et al.*, 2021). Hattori *et al.* (2021) reported the potential of the gut microbiota to prevent diarrhoea by degrading sorbitol. Xylitol is also utilized by microbial members (*Lactobacillus reuteri, Bacteroides fragilis,* and *Escherichia coli*), possibly by following crossfeeding mechanisms (Xiang *et al.*, 2021). Although low doses of xylitol (0.2 mg/kg bw or below) can disturb the gut microbiota in a dose- and diet-dependent manner, they don't seem sufficient to alter the expression of inflammatory markers or lipid metabolism and did not change HFD-induced alterations (Uebanso *et al.*, 2017a). Xylitol-induced microbial shifts might influence the metabolism of certain dietary components, e.g. isoflavone daidzein, which could have beneficial effects (Tamura, Hoshi and Hori, 2013).

Sweeteners have also been evaluated in **combination**, primarily in commercial formulations containing several compounds (multiple sweeteners, bulking agents) mixed in different proportions, which in many cases are not disclosed. This makes the assessments challenging and product-specific. A potential challenge posed by combined sweeteners is the difficulty in discerning whether potential effects are due to one or multiple components and whether these are additive, synergistic or even antagonistic.

Commercial products Splenda® (containing sucralose) or Svetia® (containing sucralose and steviol glycosides) led to inflammatory responses in the small intestine, metabolic deregulation and metabolic alterations (Martínez-Carrillo *et al.*, 2019). Maternal (early life) exposure to a mixture of sucralose and acesulfame-K at the ADI or twice the ADI led to metabolic dysregulation and a bacterial profile similar to that observed in individuals with metabolic disorders and obesity (Olivier-Van Stichelen, Rother and Hanover, 2019). Falcon *et al.* (2020) did not observe alterations after chronic treatment of rats with yoghurt supplemented with a low dose of a commercial NNS containing saccharin and sodium cyclamate.

NON-SWEETENER FOOD ADDITIVES

In vivo studies in animals models evaluating **carboxymethyl cellulose** (CMC) and **polysorbate 80** (P80) were initiated by Chassaing *et al.* (2015). After testing several concentrations, the highest dose (1 percent), showing more evident effects *in vitro*, was selected for subsequent investigations by this and other research groups. This dose is higher than available exposure estimates for CMC and P80 (Cox *et al.*, 2020). Chassaing *et al.* (2015) suggested that alterations of the microbiota (diversity, composition, microbial encroachment and pro-inflammatory potential) caused by P80 and CMC were necessary and sufficient to promote the observed low-grade inflammation and metabolic syndrome, especially affecting susceptible individuals. Other authors reported gender- and emulsifier-dependent effects on the microbiota, with a greater inflammatory potential of CMC over P80 (Rousta *et al.*, 2021) or distinct neurobehavioural effects (Holder *et al.*, 2019). Additional research by Chassaing's team also concluded that P80 and CMC-altered microbiota was necessary to exacerbate the observed intestinal inflammatory response, i.e. colitis, and the development of intestinal adenomas in susceptible individuals (Viennois and Chassaing, 2021; Viennois *et al.*, 2017). P80 was also found to exacerbate indomethacin-induced ileitis, characterized by an increase in the abundance of the Proteobacteria *Proteus mirabilis* (Furuhashi *et al.*, 2020) and the gastrointestinal toxicity caused by radiation (Li *et al.*, 2020b).

The only human study investigating a non-sweetener additive consisted of a short interventional trial evaluating CMC (Chassaing *et al.*, 2021). This study found no- or limited effects in inflammatory markers, glucose homeostasis and the microbiota, with the exception of two treated individuals showing microbial encroachment. What is encroachment, and what are the potential implications? It is known that the mucus layer plays a fundamental role in protecting the mucosa from chemical and microbiological hazards as well as physical stress. The lack of it, deficiencies in the mucus structure or disruption of this layer through different mechanisms can facilitate the approximation or access of bacteria to the epithelium (referred as encroachment by Chassaing's research group). This can result in bacterial translocation into the lamina propria and the consequent elicitation of an immune response (Steffen, Berg and Deitch, 1988). It has been suggested that intestinal inflammation, e.g. colitis, is mediated by the microbiota, and the degree of severity depends on its composition (Johansson and Hansson, 2016). Chassaing's team evaluated encroachment as an endpoint in all their studies assessing CMC and P80 in humans and animals. They defined encroachment as the reduction of the distance between the epithelium and the nearest bacteria to this intestinal surface (Chassaing *et al.*, 2021; Chassaing *et al.*, 2015; Viennois and Chassaing, 2021; Viennois *et al.*, 2017). However, a better characterization of encroachment would provide greater insight into the potential risk for intestinal detrimental effects, as it is unclear if there is a general approximation of the microbiota distribution to the epithelium layer and the type of the closest bacteria to the epithelial surface, whether pathobionts or commensal bacteria members of the mucosal-associated microbiota.

The main interest of most CMC and P80 studies was to evaluate whether the microbial community could play a role in metabolic syndrome and chronic low-grade inflammation after exposure to the additive. For complex conditions (e.g. metabolic syndrome) or those lacking universally agreed-upon definitions (e.g. low-grade inflammation), researchers should clarify or provide their own definitions. It will improve the transparency of both the research methodology and the interpretation of findings.

Metabolic syndrome is a collection of conditions or risk factors for metabolic disorders (e.g. diabetes, coronary heart disease). Different organizations have defined metabolic syndrome, including the World health Organization (WHO) (Huang, 2009; WHO, 1999). Although such definitions consider similar components (e.g. obesity, hyperglycemia, dyslipidemia, hypertension), they differ on specific details (e.g. endpoints and thresholds). Some have argued that these older definitions should be revised and consider recent scientific information, including other risk factors such as altered gut microbiome as a potential contributor to chronic low-grade inflammation, obesity, hyperglycemia and dyslipidemia (Dabke, Hendrick and Devkota, 2019).

Contrary to metabolic syndrome, low-grade inflammation lacks a formal definition. It is considered a subclinical unresolved chronic inflammatory state and considered a risk factor for metabolic disorders (e.g. obesity, diabetes) and other chronic diseases (Marialaura *et al.*, 2016; Minihane *et al.*, 2015). As inflammatory responses are complex, the best approach to characterize the inflammatory status is to rely on multiple biomarkers of different nature (e.g. molecular, cellular). However, there are no specific and sensitive biomarkers for low-grade inflammation (Minihane *et al.*, 2015). For example, some studies, including those evaluating the inflammatory potential of the diet, have used a low-grade inflammation (INFLA) score, a composite parameter measuring C-reactive protein, leukocyte and platelet counts and the granulocyte to lymphocyte ratio in blood samples (Marialaura *et al.*, 2016; Mignogna *et al.*, 2022). However, proposed biomarkers of low-grade inflammation do not have the same sensitivity and predictive value and can be sample-dependent (e.g. serum vs. faeces) (Minihane *et al.*, 2015). In addition, it is necessary to establish the functional range or threshold values to be used as a reference for the diagnosis of low-grade chronic inflammation (Soares *et al.*, 2022). In the evaluation of P80 and CMC, Chassaing's research team rated lowgrade inflammation based on reduced colon length, increased weight of organs, and increased levels of lipocalin-2 LPS and flagellin. It is challenging to understand how the scientists interpret the inflammatory status without a defined scoring, especially when not all these markers are altered.

In vivo studies evaluating **glycerol monolaurate or glycerol monocaprylate** were conducted by the same research group. These studies have important methodological and reporting limitations. Researchers reported limited effects or the ability of the emulsifiers to modulate the gut microbiome and ameliorate or prevent HFD-induced metabolic changes in a dose–response manner (Mo *et al.*, 2019; Zhang, Feng and Zhao, 2021; Zhao *et al.*, 2019; Zhao *et al.*, 2020; Zhao *et al.*, 2022). However, at the lowest doses tested, the same research group reported microbial dysbiosis and the development of metabolic syndrome and low-grade inflammation (Jiang *et al.*, 2018).

The main purpose of studies investigating **carrageenan** was to explore the potential of different forms of this algae-derived compound to induce intestinal inflammation and microbial disturbance. It is important to highlight and acknowledge that carrageenan products used in several research works (e.g. hydrolysed, LMW carrageenans) differed from those used in food (food grade). Therefore, such studies should not be included in the risk assessment of carrageenans as food additives. The following comments are only based on research using food-grade carrageenans. The pro-inflammatory potential of carrageenan seemed dependent on the dose, type (λ-, κ- or ι-carrageenan) and vehicle (water vs. food matrix) (Mi *et al.*, 2020; Shang *et al.*, 2017; Wu *et al.*, 2022). High doses of κ-carrageenan were reported to worsen the severity of *Citrobacter rodentium*-induced colitis (Wu *et al.*, 2022).

Maltodextrin exacerbated colitis in a dose–response manner, while no effects were observed in healthy animals (Laudisi *et al.*, 2019). Short-term treatment with **lecithin** from two different plant sources (soybean and rapeseed) led to distinct metabolic changes and microbial profiles, which, according to the researchers, could be indicative of a specific beneficial impact on metabolic and intestinal health (Robert *et al.*, 2021).

Products resulting from the microbial reduction of azo **colorants** allura red and sunset yellow led to colitis in predisposed animals overexpressing IL-23 (He *et al.*, 2021).

Similar to what has been seen in carrageenan studies and as already discussed earlier, non-food grade **titanium dioxide** has been used in several research works (average diameter ≤ 25 nm). Again, such studies do not represent realistic oral exposures and therefore should not be included in food safety assessments. Of the 17 studies included in this review, only one *in vitro* study (Dudefoi *et al.*, 2017) and four animal studies (Cao *et al.*, 2020; Pinget *et al.*, 2019; Yan *et al.*, 2022; Zhu *et al.*,

2022) explicitly indicated the use of food-grade titanium dioxide. Cao *et al.* (2020) and Yan *et al.* (2022) compared food-grade and non-food-grade titanium dioxide, with effects more marked after exposure to the smaller sizes (non-food-grade nanoparticles). In one of these studies, colonic inflammation and dysbiosis were observed at doses approximately 100 times greater than the estimated daily intake for humans, and seemed influenced by the diet (Cao *et al.*, 2020). Yan *et al.* (2022) indicated dose-dependent effects and suggested that the altered microbiota might be involved in the mechanisms of titanium dioxide toxicity (reported as disruption of the intestinal barrier). The doses used in these two last studies did not represent realistic exposure scenarios. Following titanium dioxide treatment, Pinget *et al.* (2019) observed a dose-dependent disruption of gut homeostasis (intestinal inflammation), while Zhu *et al.* (2022) reported the aggravation of atherosclerosis in a model of obesity. In both cases, the authors suggested the influence of gut microbiota changes on the reported effects.

Nanosilver has not been evaluated by JECFA and has very limited applications as a food additive. It was unclear whether nanosilver used in the different studies reviewed was of food-grade quality. Most researchers reported some degree of microbial disturbance and no or limited physiological alterations (Bredeck *et al.*, 2021; Hadrup *et al.*, 2012; Wilding *et al.*, 2016). Only Williams *et al.* (2015) reported dose-, size- and gender-dependent effects affecting the gut microbiota and the gene expression of intestinal markers. These researchers suggested additional research to evaluate the physiological implications of such changes.

In summary, it is not possible to draw clear conclusions about the impact of each food additive on the gut microbiome and its potential influence on health outcomes for several reasons discussed earlier. In general, the overall strength of evidence and conclusions are challenged by (1) the variability in scientific rigour among studies; (2) the low statistical power, which increases the risk of false positive and false negative results; (3) the diversity of experimental conditions, methodologies and finding reporting; and (4) contradictive outcomes (e.g. sucralose and saccharin).

It is necessary to acknowledge that all studies had limitations and that no single research can provide definite answers. While well-designed research studies add information to the overall body of evidence, poor-quality research only generates confusing statements and misinformation. The scientific community and publishing houses should prioritize quality.

Microbial shifts are often suggested as contributors to the alterations observed in the host and made extensive to chronic disorder (e.g. metabolic syndrome, inflammatory bowel disease), with stronger or weaker supporting evidence. Still, many of the associations are speculative, and the meaning of microbial changes remains unclear, whether they result from the direct exposure of the food additive, the host-food additive interaction or both. The evolutionarily informed framework for understanding the microbiota suggests that the characteristics of the Western gut microbiome, often perceived as maladaptive due to its association with various modern diseases, may represent an adaptive response to the unique environmental conditions of industrialized societies. This perspective postulates that the evolutionary selection process may have favoured certain microbial functions beneficial in the context of Western lifestyles, leading to the loss of other functions that were less necessary in this new environment (Reese and Kearney, 2019). Thus, the changes in the gut microbiota composition could be seen as a natural evolutionary adaptation rather than a straightforward negative shift. Although this is another plausible explanation for the microbial shifts often observed following food additive exposure, it is clear that more (rigourous) research is necessary to better understand and characterize the nature of such microbial changes, whether stable or sporadic, and their biological relevance for the host (desirable or undesirable). Also, more research is necessary to better understand the driving factors for the onset and progression of chronic diseases.

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CHAPTER 6
GUT MICROBIOME DATA AND FOOD ADDITIVE RISK ASSESSMENT

Given that the gut microbiome can alter the toxicity and bioavailability of chemicals and affect overall health, it becomes necessary to consider incorporating microbiome data into the risk assessment of regulated chemicals, including food additives. Integrating such data could provide deeper insights into the safety of food additives by offering mechanistic explanations for toxicity or acting as predictive markers for potential adverse effects. However, this integration should be approached with caution due to the complexities and current limitations of microbiome research and the existing knowledge gaps. Decisions must be carefully weighed in regulatory science because of their impact on agrifood systems and public health. For this reason, such decisions must be based on robust and reliable scientific evidence.

On December 2023, following the publication of the critical evaluations of studies investigating the impact of pesticide residues (FAO, 2023b), veterinary drug residues (FAO, 2023c) and microplastics (FAO, 2023a) on the gut microbiome and health, the FAO convened a multidisciplinary group of experts, including risk assessors and microbiome ecologists (FAO, 2024). This activity aimed to discuss the limitations and challenges of applying microbiome science in chemical risk assessments. The discussions facilitated the identification of gaps and key actions needed to incorporate microbiome data into the risk assessment process, marking a significant step toward enhancing food safety and public health strategies. The following points combine some of the main conclusions of the technical meeting, and key aspects identified in this review.

There is a need for consensus **microbiome-related definitions** of relevance to risk assessment. These include healthy microbiome, dysbiosis and microbiome resilience. Healthy microbiome is challenging to identify due to interindividual variability and the lack of clarity on the health concept. The challenges for characterizing the features of a healthy microbiome makes it difficult to define dysbiosis. However, both should be considered stable and non-transient states of the microbial community, needing to encompass both the gut microbiome and the host.

Another term relevant to the assessment of food additives is microbiome resilience, which refers to the ability of the gut microbiome to resist and recover from stresses, such as exposure to dietary components.

Microbiome science needs to be robust and reproducible. Reproducibility can be accomplished by harmonizing study designs, employing suitable models and using standardized and validated analytical methodologies, including omics technologies. The harmonization of existing standards and methodologies can be carried out by collaborative activities involving different stakeholders and standard-setting organizations such as the International Organization for Standardization (ISO) (ISO, 2024), the European Committee for Standardization (CEN), or the National Institute of Standards and Technology (NIST) (NIST, 2024).

Studies conducted by a research team should be reproducible by independent research groups. However, as observed in the many scientific manuscripts included in this review, publications often lack methodological details needed to mimic the original research. Similar conclusion has been reached by Eaton *et al.* (2018), participants in the Reproducibility Project on Cancer Biology (OSF, 2024). These researchers highlighted the need for clear descriptions of experimental methodologies to ensure accurate reproduction of experimental studies, after they failed to replicate the study conducted previously by Arthur *et al.* (2012) entitled "*intestinal inflammation targets cancer-inducing activity of the microbiota*". The lack of methodological details occurs regardless of the impact factor of the journal, e.g. studies on saccharin (Suez *et al.*, 2014) or CMC (Chassaing *et al.*, 2015) published in the *Nature* journal. Extended methodological details are often included in supplemental information, which is a document or a set of files independent from the main article and typically available online for download. Publishing houses have to ensure that this information is complete and available. Part of the methodologies used in the human interventional trial evaluating CMC (Chassaing *et al.*, 2021) was only described in supplemental information but was not included among the files available online. After two attempts to inform the journal about this issue and request the relevant files, they have remained unresponsive. Detailed methodologies are critical to risk assessors in evaluating the suitability and quality of methodologies applied.

The availability of **guidelines, guidance and best practice documents**, especially those based on consensus and developed through scientific rigour, are key support for scientists. Some existing guidelines can be updated or adapted to include specifics of microbiome investigations, such as the guidelines for toxicological studies developed by the Organisation for Economic Co-operation and Development (OECD) (OECD 2024c; 2024d; 2024e). Annex III. contains several relevant guidance and best practice documents, some based on consensus and others developed by independent research groups.

Research should consider **realistic exposure scenarios** to ensure the relevance of findings. Experimental substances (additives) should be of food-grade quality. The doses should reflect consumption rates and habits, and exposure durations should reflect long-term or lifetime consumption of food additives.

There is a need for more research aiming to clarify the involvement of the gut microbiome in the biotransformation of food additives, which can influence the bioavailability, toxicity and pharmacokinetics of these substances. In addition, studies evaluating the interaction between food additives, the gut microbiome and the host should consider existing toxicological evaluations of the compound.

Additional research efforts should aim to understand and identify microbiome signatures (e.g. **keystone taxa and function) of biological relevance**.

It is necessary to identify and validate robust, predictive and sensitive **microbiome-related endpoints and biomarkers** (e.g. keystone taxa and metabolite-related metabolites). Due to the symbiotic nature of the gut microbiome and host relationship, biomarkers could be defined as sets encompassing microbiome and host metrics. Ideally, such metrics should have reference values that allow the distinction between normal and abnormal or identify levels or degrees of concern. The integration of OMICs, metagenomics and metabolomics in particular, can help in the identification of health and disease-relevant microbiome-related biomarkers (Puig-Castellví *et al.*, 2023).

Research should include the evaluation of **baselines** and monitoring of the gut microbiota during **clearance** periods (after treatment) to understand microbiome resilience in response to chemical exposure. A chemical disrupting the microbiome's balance, causing long-lasting changes or impairing its ability to recover, could indicate potential health risks for the host. A resilient microbiome that can quickly return to its baseline state after chemical exposure might suggest transient effects and a lower health risk. Clearance periods can also help identify delayed effects of the food additive.

Research inferring **causality** (including directionality) and investigating underlying **mechanisms** are needed to clarify and demonstrate the involvement of the gut microbiome on adverse health outcomes (or vice versa) following exposure to the additive. Faecal microbiota transplant (FMT) and human interventional trials provide a higher strength of evidence due to their direct assessment of causality. However, more efforts are needed to standardize procedures, define endpoints, and enhance analytical power, which will provide a more accurate estimation of magnitude of the effect (Hanssen, de Vos and Nieuwdorp, 2021).

More efforts are needed to understand the **translatability** of microbiome outcomes obtained from *in vitro*, *in vivo*, or *ex vivo* studies to the human exposure context. Applying data from *in vivo* and *in vitro* studies to the human context requires careful consideration. *In vitro* experiments lack the host responses that can modulate the dynamics of the gut microbiome. When working with laboratory animals, significant anatomical and physiological differences in the gastrointestinal tracts compared to humans, variations in the gut microbiome, and differences in the mechanisms of metabolic diseases pose significant challenges to translating findings across species (Douglas, 2018; Hugenholtz and de Vos, 2018; Vo, Lynch and Roberts, 2019). Translatability can be improved by (1) using human microbiota in relevant *in vitro* or *ex vivo* containing anatomically and physiologically relevant components, like M-SHIME or gut-on-a-chip (under development), respectively; (2) selecting

animals with a gut physiology and microbiome closer to humans (e.g. pigs); and (3) validating findings obtained *in vitro*, *ex vivo*, from animal models or by conducting clinical trials with human subjects.

Ultimately, developing an assessment **framework** that weights and ranks the evidence from microbiome data would guide assessors and enhance transparency in the assessment process. Frameworks for risk assessment considering omics data have been proposed, including the one based on the adverse output pathway (AOP) (Piña *et al.*, 2018). With origins in toxicology and ecotoxicology, the AOP concept can be expanded and applied to other fields. It describes how a specific molecular event, like a food additive acting as a molecular trigger, affects several layers of the organization, with outcomes at the ecosystem or population level (Ankley *et al.*, 2010). The AOP framework has been useful in establishing the correlation between the initial molecular interaction and a truly adverse outcome, which is relevant to risk assessment (Piña *et al.*, 2018). Some initiatives have evaluated how high-throughput molecular-level datasets can support (chemical) risk assessments using the AOP framework (Brockmeier *et al.*, 2017). The OECD has a programme addressing AOP (OECD, 2024b) and has published a *Guidance Document on Developing and Assessing Adverse Outcome Pathways* (OECD, 2017), and it has also been considered in the WHO/IPCA mode of action framework (Meek *et al.*, 2014).

The critical evaluation of studies presented in this review, along with previous FAO reports covering pesticide residues (FAO, 2023b), veterinary drug residues (FAO, 2023c), and microplastics (FAO, 2023a), emphasizes the very essential need to enhance the quality and rigour of research. This responsibility extends beyond the scientists designing studies, conducting research, and communicating their findings. The quality of research is also the responsibility of publishing houses and peer reviewers. Given the interdisciplinary nature of microbiome science, collaborative efforts incorporating all relevant expertise should be implemented at every phase, from the inception of research to its publication.

CHAPTER 7
CONCLUSIONS

The review focused on evaluating the current state of research investigating the impact of select food additives on the gut microbiome and their potential health implications. Specifically, this project critically assessed scientific evidence, identified the quality and reliability of existing studies, and highlighted aspects of research needing improvement before incorporating microbiome data into food additive safety risk assessments. General findings included variability in research quality, scientific rigour, research questions, study designs, and analytical approaches, which led to cases of contradictory findings (e.g. sucralose and saccharin), challenges to compare research outcomes and making definite conclusions. Result interpretation does not always include all study results, typically excluding null results from the overall body of evidence generated by the research, which risks introducing bias and inaccuracies. Also, reporting and stated conclusions are often speculative, which can include exaggerating the interpretation of outcomes or generalizing findings by extending the outcomes of a specific study to the additive class or food additives in general.

Based on the findings of this review, there is a critical need to improve the rigour of research design to understand how food additives interact with the gut microbiome and their subsequent effects on human health. Additional efforts should focus on standardizing and harmonizing methodologies, employing realistic exposure research scenarios, and investigating causality and underlying mechanisms. The complexity of identified needs highlights the importance of using multidisciplinary approaches throughout all research levels (from study design to peer-review process), harmonization and standardization. The implementation of such approaches will ensure that all aspects will be covered by the appropriate expertise. Addressing these aspects is key to improving the quality, robustness and reliability of data needed for risk assessment. To bridge the gap between current knowledge and regulatory requirements, it is imperative to ensure that findings from animal and *in vitro* models are translatable to the human contexts and also to develop a framework for the assessment of microbiome data, thereby providing a solid scientific foundation for risk assessors to evaluate the implications of food additives on health.

Under specific experimental conditions, some food additives have been shown to modify the gut microbiome. However, it remains unclear whether these changes result in a dysbiotic or dysfunctional microbiome, whether they impact microbiome resilience, and to what extent they contribute to adverse effects in the host. Although only a limited number of studies involving animal models and humans have explored

the causal role of the gut microbiome in health effects caused by certain food additives (e.g. saccharin, CMC), further research is necessary to produce more robust evidence.

NEXT STEPS TO CLOSE THE KNOWLEDGE AND RESEARCH GAPS

To better understand the gut microbiome's influence on host outcomes (e.g. adverse health effects) following food additive consumption, it is necessary to refine several aspects of research. The following points are proposed to bridge current knowledge gaps, foster innovation, and tailor studies to meet the specific demands of chemical risk assessment, ensuring that the science is robust and practically applicable. By implementing these measures, researchers can contribute to a more comprehensive understanding of the microbiome's role in health and disease, ultimately supporting evidence-based decision-making and advancing public health. The measures are grouped into different categories related to:

1. study design
2. interpretation of results and communication of findings
3. research to investigate knowledge gaps
4. validation, standardization, harmonization and guidelines
5. other

STUDY DESIGN

> Clearly define *a priori* the research question, the hypothesis and study purpose.

> Define the number of animals used in the different experimental groups based on statistical power calculated *a priori*, during the study planning. Ideally, the number should be sufficiently and ethically high to increase the likelihood that the observed effects result from exposure and not chance.

> Select the most appropriate statistical approaches *a priori*, ideally collaborating with biostatisticians.

> Identify confounding factors and implement control measures to minimize their potential influence in the study results.

> Carefully select the diet and provide the relevant information in the scientific publication.

> Investigate realistic food additive doses, selected based on reference dietary values (e.g. high percentile additive consumption estimates in humans, ADI) and considering dietary habits.

> Investigate multiple doses to allow the preparation of dose–response curves, in ranges allowing for no observed effects at the low end. Doses could be determined in preliminary or pilot studies, using ADI as reference values, if available, or estimated consumption or exposures of the population to the additive.

> Deliver the additive (*in vivo* studies or human interventional trials) in vehicles that resemble typical additive-containing matrices.

- Control food additive consumption (drinking water and food intake) in animal studies. To maintain a constant daily food additive intake, the dosage should be updated based on changes in food or water consumption and body weight.
- Implement proper control groups.
- Conduct longitudinal studies to assess the dynamics of the gut microbiome, which help identify transient changes, patterns or deviations of concern (always in conjunction with host alterations). Longitudinal studies are useful to pinpoint sequence of events: changes in the microbiome precede changes in the host, indicating the potential influence of the gut microbiome in the host; changes in the host appear before changes in the gut microbiome, suggesting the potential effect of the host on the microbiome.
- Evaluate baselines for the gut microbiota and host parameters.
- Include wash-off/clearance periods to monitor the reversibility of observed effects or the emergence of delayed effects. These periods should be reasonably long to permit the different values to recover, while avoiding incurring ethical issues or cost burden.
- Implement proper sampling, sample handling and storage, ideally following existing guidelines.
- Select the sampling site based on the research question and consider the food additive's bioaccesibility, bioavailability and toxicokinetics (ADME).
- Researchers should refer to standarized and harmonized research protocols such as the OECD guidelines (i.e. animal selection, doses, husbandry, control groups).

INTERPRETATION OF RESULTS AND REPORTING OF FINDINGS

- Consider that statistically significant results alone do not necessarily translate into adverse effects.
- Consider the overall amount of evidence produced by studies by considering not only statistically significant outcomes but also including null results.
- Explore and clearly state the challenges and limitations of the study.
- Consider how treatments represent typical or estimated intake scenarios and consumption patterns scenarios.
- Consider available toxicokinetics and toxicological information about the compound being evaluated.
- Consider the possibility of alternative plausible explanations.
- Use terminology accurately in publications avoiding generalizations, overstatements and clearly indicate when discussing facts or speculating content (e.g. typically conveyed in the form of opinions, views, thoughts, and so on). Ensure the title and abstracts reflect the facts derived from findings and conclusions while avoiding speculation.

- Report detailed and complete procedures and research methodologies used. Provide them in supplementary information of the journal if space is limited in the main manuscript.
- Exercise caution when citing the work of peers. It is important to consult the original research to critically assess the study's conditions, methodology and the reporting of results.

RESEARCH TO INVESTIGATE KNOWLEDGE GAPS

- Research should go beyond taxonomical markers and expand into the functional microbiome. Although any microbiome effect on test compound toxicokinetics can be identified during ADME testing, which is a required component of food additive assessment, their potential participation in adverse health effects or physiopathological processes requires further investigation.
- Research is needed to identify and validate physiologically relevant microbiome-related biomarkers and endpoints. Research is also needed to identify thresholds that help distinguish normal ranges from adverse effects.
- Investigate the role of non-bacterial gut microbiome members in the interactions with the food additive and the host.
- Investigate the potential influence of generalist[41] and specialist[42] bacteria, and their co-participation along with the host, in the development of adverse effects.
- Investigate the suitability (fit-for-purpose) of artificial microbiome consortia to evaluate the safety of food additives.
- Investigate the influence of genders, age and vulnerable populations in food-additive exposure studies.
- Research to better understand the influence of caloric diets on metabolic effects when evaluating the impact of food additives on the gut microbiome and their potential influence on health outcomes. These needs are based on diverging reports indicating either the caloric diets or the altered microbiome as main contributors to metabolic alterations.

VALIDATION, STANDARDIZATION, HARMONIZATION AND GUIDELINES

- Validate and standardize models and develop guidelines for using the most suitable model to address specific microbiome research questions. For example, *in vitro* models could be suitable to study the potential capacity of the gut microbiome to biotransform food additives (e.g. by monitoring single microorganisms or multiple in cross-feeding processes), while those looking for physiological interactions should be conducted *in vivo*, or *ex-vivo*, when monitoring specific local effects involved in the disruption of the gut barrier (e.g. intestinal permeability).

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41 Generalist microorganisms are able to adapt to diverse habitats (Sriswasdi, Yang and Iwasaki, 2017, p.2).

42 Specialist microorganisms are adapted to specific habitats (Sriswasdi, Yang and Iwasaki, 2017, p.2).

- Validate and standardize analytical methodologies, including omics. Develop or update best practice guidelines.
- Identify and standardize diets to improve result consistency and study reproducibility. These could include standard diets and diets relevant to specific dietary habits or specific health conditions, e.g. high-caloric or Western diets linked to metabolic disorders or atherosclerosis.
- Develop guidance to investigate microbiome baseline.
- Develop guidance for monitoring factors affecting food additive intake in animal studies (i.e. body weight, food and water consumption), including methods for calculating or updating dosages to ensure a constant daily intake of the food additive.
- Standardize and develop best practice guidance for FMT experiments.
- Standardized clinical tests, e.g. glucose tolerance tests, histopathological examination, grading and interpretation.
- Develop guidance and training to support assessors in the interpretation of microbiome-related omics data.

OTHER

- Avoid using terminology and concepts that are outdated or not accurate, e.g. microflora, Firmicutes:Bacteroidetes ratio. The ratio Firmicutes:Bacteroidetes ratio as a microbial marker for obesity is unsuited for this purpose, and it has led to many contradictory results (Cani, Moens de Hase and Van Hul, 2021).
- Promote a higher quality of microbiome research and peer-review processes.
- Train scientists for better planning, designing and conducting dietary exposure research involving animal models and the microbiome as well as reporting scientific results.


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ANNEX I. GLOSSARY OF RISK ASSESSMENT TERMS

Unless specified, the following concepts are defined in the guidance document *Principle and Methods for the Risk Assessment of Chemical in Food* (Environmental health criteria 240) (FAO and WHO, 2009a) or in the *Codex Alimentarius Commission Procedural Manual* (28th Edition) (FAO and WHO, 2023a).

Acceptable daily intake (ADI). The estimate of the amount of a chemical in food or drinking-water, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk to the consumer. It is derived on the basis of all the known facts at the time of the evaluation. The ADI is expressed in milligrams of the chemical per kilogram of body weight (a standard adult person weighs 60 kg). It is applied to food additives, residues of pesticides and residues of veterinary drugs in food.

Acceptable Daily Intake "Not Specified" (NS) is a term applicable to a food substance of very low toxicity for which, on the basis of the available data (chemical, biochemical, toxicological, and other), the total dietary intake of the substance, arising from its use at the levels necessary to achieve the desired effect and from its acceptable background levels in food, does not, in the opinion of JECFA, represent a hazard to health (FAO and WHO, 1995).

Adverse effect. Change in the morphology, physiology, growth, development, reproduction or lifespan of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences.

Bioavailability. For food additives, contaminants and pesticide residues, a term referring to the proportion of a substance that reaches the systemic circulation unchanged after a particular route of administration. For veterinary drug residues in food, it is used to reflect the fraction that can be released from the food matrix and is available for absorption.

Biomarkers. Indicators of changes or events in human biological systems. Biomarkers of exposure refer to cellular, biochemical or molecular measures that are obtained from biological media such as human tissues, cells or fluids and are indicative of exposure to a substance. Biomarkers of effect refer to biological changes that represent an alteration in endogenous body constituents (e.g. depression of cholinesterase levels as an indicator of exposure to pesticides).

Chronic exposure. A continuous or intermittent long-term contact between an agent and a target.

Codex Alimentarius Commission (CAC). CAC was formed in 1962 to implement the Joint FAO/WHO Food Standards Programme. It is an intergovernmental body made up of more than 170 member nations, the delegates of which represent their own countries. CAC's work of harmonizing food standards is carried out through various committees, such as the Codex Committee on Food Additives (CCFA), the Codex Committee on Contaminants in Food (CCCF), the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) and the Codex Committee on Pesticide Residues (CCPR). The Joint FAO/WHO Expert Committee on Food Additives serves as the advisory body to CAC on all scientific matters concerning food additives, food contaminants, naturally occurring toxicants and residues of veterinary drugs in food. The Joint FAO/WHO Meeting on Pesticide Residues serves as the advisory body to CAC on all scientific matters concerning pesticide residues.

Dietary exposure assessment. The qualitative and/or quantitative evaluation of the likely intake of chemicals (including nutrients) via food, beverages, drinking-water and food supplements. Synonymous with: Intake assessment.

Dose. Total amount of an agent administered to, taken up by or absorbed by an organism, system or (sub)population.

Dose–response. Relationship between the amount of an agent administered to, taken up by or absorbed by an organism, system or (sub)population and the change developed in that organism, system or (sub)population in reaction to the agent.

Dose–response relationship. Relationship between the amount of an agent administered to, taken up by or absorbed by an organism, system or (sub)population and the change developed in that organism, system or (sub)population in reaction to the agent. Related terms: Concentration–effect relationship, Dose–effect relationship.

Elimination. The expelling of a substance or other material from the body (or a defined part thereof), usually by a process of extrusion or exclusion, but sometimes through metabolic transformation.

End-point. Qualitative or quantitative expression of a specific factor with which a risk may be associated as determined through an appropriate risk assessment.

Enterohepatic circulation. Intestinal reabsorption of material that has been excreted through the bile followed by transfer back to the liver, making it available for biliary excretion again.

Exposure. Concentration or amount of a particular agent that reaches a target organism, system or (sub)population in a specific frequency for a defined duration.

Exposure assessment. Evaluation of the exposure of an organism, system or (sub) population to an agent (and its derivatives). Exposure assessment is one of the steps in the process of risk assessment.

Exposure scenario. A set of conditions or assumptions about sources, exposure pathways, amounts or concentrations of agents involved and exposed organisms, systems or (sub)populations (i.e. numbers, characteristics, habits) used to aid in the evaluation and quantification of exposures in a given situation.

Food additive. In the Codex Alimentarius Commission context, any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food, whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment, packing, packaging, transport or holding of such food results, or may be reasonably expected to result, (directly or indirectly) in it or its by-products becoming a component of or otherwise affecting the characteristics of such foods. The term does not include contaminants or substances added to food for maintaining or improving nutritional qualities.

Hazard. Inherent property of an agent or situation having the potential to cause adverse effects when an organism, system or (sub)population is exposed to that agent.

Hazard assessment. A process designed to determine the possible adverse effects of an agent or situation to which an organism, system or (sub)population could be exposed. The process includes hazard identification and hazard characterization. The process focuses on the hazard, in contrast to risk assessment, where exposure assessment is a distinct additional step.

Hazard characterization. The qualitative and, wherever possible, quantitative description of the inherent properties of an agent or situation having the potential to cause adverse effects. This should, where possible, include a dose–response assessment and its attendant uncertainties. Hazard characterization is the second stage in the process of hazard assessment and the second step in risk assessment.

Hazard identification. The identification of the type and nature of adverse effects that an agent has an inherent capacity to cause in an organism, system or (sub) population. Hazard identification is the first stage in hazard assessment and the first step in the process of risk assessment.

Health-based guidance value. A numerical value derived by dividing a point of departure (a no-observed-adverse-effect level, benchmark dose or benchmark dose lower confidence limit) by a composite uncertainty factor to determine a level that can be ingested over a defined time period (e.g. lifetime or 24 h) without appreciable health risk. Related terms: Acceptable daily intake, Provisional maximum tolerable daily intake, Provisional tolerable monthly intake, Provisional tolerable weekly intake, Tolerable daily intake.

Intake. For the purposes of food and feed risk assessment, the amount of a substance (including nutrients) ingested by a person or an animal as part of its diet (via food, beverages, drinking water and food supplements). This term does not refer to whole foods. The "intake" of whole foods is termed "food consumption".

Joint FAO/WHO Expert Committee on Food Additives (JECFA). An expert committee that has been meeting since 1956. JECFA has been engaged in collecting and evaluating scientific data on food additives and making recommendations on safe levels of use. This has been accomplished 1) by elaborating specifications for the identity and purity of individual food additives that have been toxicologically tested and are in commerce and 2) by evaluating toxicological data on these food additives and estimating acceptable intakes by humans.

In 1972, the scope of the evaluations was extended to include contaminants in food, whereas in 1987, the scope was extended even further to include residues of veterinary drugs in food. When evaluating the latter compounds, maximum residue limits are recommended based upon acceptable intakes estimated by the Committee and data relating to Good Practice in the Use of Veterinary Drugs.

JECFA is a technical committee of specialists acting in their individual capacities. Each JECFA is a separately constituted committee. When the term "JECFA" or "the Committee" is used without reference to a specific meeting, it is meant to imply the common policy or combined output of the separate meetings over the years.

Lowest-observed-adverse-effect level (LOAEL). Lowest concentration or amount of a substance, found by experiment or observation, that causes an adverse alteration of morphology, functional capacity, growth, development or lifespan of the target organism distinguishable from normal (control) organisms of the same species and strain under the same defined conditions of exposure.

Lowest-observed-effect level (LOEL). Lowest concentration or amount of a substance, found by experiment or observation, that causes any alteration of morphology, functional capacity, growth, development or lifespan of the target organism distinguishable from normal (control) organisms of the same species and strain under the same defined conditions of exposure.

Margin of safety. The margin between the health-based guidance value (reference dose) and the actual or estimated exposure dose or concentration.

Maximum Use Level of an additive is the highest concentration of the additive determined to be functionally effective in a food or food category and agreed to be safe by the Codex Alimentarius Commission. It is generally expressed as mg additive/kg of food (FAO and WHO, 1995).

Mechanism of action. The specific biochemical interaction through which a substance produces an effect on a living organism or in a biochemical system. Related term: Mode of action.

Model. A set of constraints restricting the possible joint values of several quantities; a hypothesis or system of beliefs regarding how a system works or responds to changes in its inputs. The purpose of a model is to represent as accurately and precisely as necessary with respect to particular decision objectives a particular system of interest.

Mode of action. A biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. A mode of action describes key cytological and biochemical events—that is, those that are both measurable and necessary to the observed effect—in a logical framework. Related term: Mechanism of action.

No-observed-adverse-effect level (NOAEL). Greatest concentration or amount of a substance, found by experiment or observation, that causes no adverse alteration of morphology, functional capacity, growth, development or lifespan of the target organism distinguishable from those observed in normal (control) organisms of the same species and strain under the same defined conditions of exposure.

No-observed-effect level (NOEL). Greatest concentration or amount of a substance, found by experiment or observation, that causes no alteration of morphology, functional capacity, growth, development or lifespan of the target organism distinguishable from those observed in normal (control) organisms of the same species and strain under the same defined conditions of exposure.

Pharmacodynamics. The study of the physiological effects of drugs on the body or on microorganisms or parasites within or on the body, the mechanisms of drug action and the relationship between drug concentration and effect. Related term: Toxicodynamics.

Pharmacokinetics. Description of the fate of drugs in the body, including a mathematical account of their absorption, distribution, metabolism and excretion. Related term: Toxicokinetics.

Risk. The probability of an adverse effect in an organism, system or (sub)population caused under specified circumstances by exposure to an agent.

Risk analysis. A process for controlling situations where an organism, system or (sub) population could be exposed to a hazard. The risk analysis pro-process consists of three components: risk assessment, risk management and risk communication.

Risk assessment. A process intended to calculate or estimate the risk to a given target organism, system or (sub)population, including the identification of attendant uncertainties, following exposure to a particular agent, taking into account the inherent characteristics of the agent of concern as well as the characteristics of the specific target system. The risk assessment process includes four steps: hazard identification, hazard characterization (Related term: Dose–response assessment), exposure assessment and risk characterization. It is the first component in a risk analysis process. Related term: Safety assessment.

Risk characterization. The qualitative and, wherever possible, quantitative determination, including attendant uncertainties, of the probability of occurrence of known and potential adverse effects of an agent in a given organism, system or (sub)population, under defined exposure conditions. Risk characterization is the fourth step in the risk assessment process.

Risk communication. Interactive exchange of information about (health or environmental) risks among risk assessors, managers, news media, interested groups and the general public.

Risk management. Decision-making process involving considerations of political, social, economic and technical factors with relevant risk assessment information relating to a hazard so as to develop, analyse and compare regulatory and non-regulatory options and to select and implement appropriate regulatory response to that hazard.

Safety factor. A composite (reductive) factor applied by the risk assessment experts to the no-observed-adverse-effect level (NOAEL) or other reference point, such as the benchmark dose or benchmark dose lower confidence limit, to derive a reference dose that is considered safe or without appreciable risk, such as an acceptable daily intake or tolerable daily intake (the NOAEL or other reference point is divided by the safety factor to calculate the reference dose). The value of the safety factor depends on the nature of the toxic effect, the size and type of population to be protected, and the quality of the toxicological information available. Related terms: Assessment factor, Uncertainty factor.

Toxicodynamics. The process of interaction of chemical substances with target sites and the subsequent reactions leading to adverse effects. Related term: Pharmacodynamics.

Toxicokinetics. The process of the uptake of potentially toxic substances by the body, the biotransformation they undergo, the distribution of the substances and their metabolites in the tissues, and the elimination of the substances and their metabolites from the body. Both the amounts and the concentrations of the substances and their metabolites are studied. The term has essentially the same meaning as pharmacokinetics, but the latter term should be restricted to the study of pharmaceutical substances. Related term: Pharmacokinetics.

Uncertainty factor. Reductive factor by which an observed or estimated no-observed-adverse-effect level or other reference point, such as the benchmark dose or benchmark dose lower confidence limit, is divided to arrive at a reference dose or standard that is considered safe or without appreciable risk. Related terms: Assessment factor, Safety factor.

Variability. Heterogeneity of values over time, space or different members of a population. Variability implies real differences among members of that population. For example, in exposure assessment, different individuals have different intakes and susceptibilities. In relation to human exposure assessment, differences over time for a given individual are referred to as intraindividual variability; differences over members of a population at a given time are referred to as interindividual variability.

Weight of evidence. A process in which all of the evidence considered relevant to a decision is evaluated and weighted.

ANNEX II. **LITERATURE SEARCH STRATEGY**

With the purpose of supporting the FAO Food Systems and Food Safety Division (ESF) in expanding the knowledge about compounds affecting the well-being and health of humans there is ongoing collaboration between FAO and the University of Bari Aldo Moro Dept Soil Plant and Food Sciences. The activity described here aims to list all the scientific publications together with the relative metadata on food additives-gut microbiome interactions and the potential implications for human health.

The first steps aimed to select recently updated and comprehensive databases, define the best strategy plan for querying them and identify and prioritize the class of food additives often investigated in connection with the gut microbiome and health. The food additive prioritization also took existing concerns into consideration about the potential influence of certain food additive classes in the development of chronic diseases, including metabolic and inflammatory disorders.

The source selection relied on three different databases encompassing every area of evaluation. In detail, we selected (i) PUBMED DB for indexed articles and abstracts of medical, health care, and preclinical journals; (ii) WEB OF SCIENCE DB as interdisciplinary database; and (iii) SCOPUS DB for other sources such as grey literature. An initial search strategy was adopted to establish the occurrence of each defined functional class of additives within literature. Following the list of additives shared by FAO (https://www.fao.org/gsfaonline/reference/techfuncs.html), the database query was carried out with a restricted publication time lapse ranging from 2010 to the date of database query (September 2021–June 2022). In this phase, original articles, reviews (narrative and systematic) and other editorial material (letters, notes, book chapters, conference abstracts) investigating diversity and function of the human gut microbiome or surrogate *in vivo* (clinical trial and animal model) and *in vitro* models were collected.

Based on Codex Class Names and the International Numbering System (INS) for Food Additives (CAC/GL 36-1989) the above-mentioned databases were queried by including the recognized functional additive classes and their interactions with human gut microbiota. The first queries involving some functional food additive classes generated a large number of investigations whereas some others resulted in only few (or any) publications. Therefore, a second search step was carried out to query the databases using the subclasses of additives as key terms (e.g. “acidifier” or “pH adjusting agent” instead of “acidity regulators”).

Below is an example of the first query string:

"additive classes/sub-classes AND (human OR *in vivo* OR *in vitro*) AND (microbiota OR microbiome)"

Following the preliminary investigation, the top priority food additive classes to be investigated were:

- Sweeteners
- Emulsifiers, stabilizers, thickeners, gelling agents, and foaming agents
- Preservatives
- Colorants

After the definition of key terms within Boolean queries, the search strategy based upon controlled vocabularies was adapted for each one of the databases. In PubMed, the search method also included the MESH terminology.

Sweeteners (January 2010 – December 2021)

The first used string to query PUBMED was:

"MESH: ("Sweetening Agents/administration and dosage"[Mesh] OR "Sweetening Agents/adverse effects"[Mesh] OR "Sweetening Agents/analysis"[Mesh] OR "Sweetening Agents/antagonists and inhibitors"[Mesh] OR "Sweetening Agents/blood"[Mesh] OR "Sweetening Agents/immunology"[Mesh] OR "Sweetening Agents/isolation and purification"[Mesh] OR "Sweetening Agents/metabolism"[Mesh] OR "Sweetening Agents/pharmacokinetics"[Mesh] OR "Sweetening Agents/pharmacology"[Mesh] OR "Sweetening Agents/physiology"[Mesh] OR "Sweetening Agents/poisoning"[Mesh] OR "Sweetening Agents/statistics and numerical data"[Mesh] OR "Sweetening Agents/therapeutic use"[Mesh] OR "Sweetening Agents/toxicity"[Mesh] OR "Sweetening Agents/urine"[Mesh]) AND (microbiota OR microbiom*)".

A more generic query line was also used at the class level:

"(sweete*) AND (microbiota OR microbiom*)"

To cover all fields of sweeteners, compounds were individually searched (https://www.fao.org/gsfaonline/additives/results.html?techFunction=26&searchBy=tf).

"(Acesulfame potassium OR Advantame OR Alitame OR Aspartame OR Aspartame acesulfame salt OR Calcium cyclamate OR Calcium saccharin OR Cyclamic acid OR Erythritol OR Isomalt OR Lactitol OR Maltitol OR Maltitol syrup OR Mannitol OR Neotame OR Polyglycitol syrup OR Potassium saccharin OR Rebaudioside OR Saccharin OR Sodium cyclamate OR Sodium saccharin OR Sorbitol OR Sorbitol syrup OR Stevia OR Sucralose OR Thaumatin OR Xylitol) AND (microbiota OR microbiom*)"

"(Acesulfame potassium OR Advantame OR Alitame OR Aspartame OR Aspartame acesulfame salt OR Calcium cyclamate OR Calcium saccharin OR Cyclamic acid OR Erythritol OR Isomalt OR Lactitol OR Maltitol OR Maltitol syrup OR Mannitol OR Neotame OR Polyglycitol syrup OR Potassium saccharin OR Rebaudioside OR Saccharin OR Sodium cyclamate OR Sodium saccharin OR Sorbitol OR Sorbitol syrup OR Stevia OR Sucralose OR Thaumatin OR Xylitol) AND (sweete*) AND (microbiota OR microbiom*)"

WEB OF SCIENCE (WoS) as interdisciplinary database covering all scientific areas was enquired as below. General class search string:

"(sweete*) AND (microbiota OR microbiom*) (Topic) AND microbiota OR microbiom* (All Fields)"

Individual sweetener terms (title, abstract, author keywords, and keywords plus) were searched as follow:

"(Acesulfame*potassium OR advantage OR alihame OR Aspartame OR Aspartame*acesulfame*salt OR Calcium*cyclamate OR Calcium*saccharin OR Cyclamic*acid OR Erythritol OR Isomalt OR Lactitol OR Maltitol OR Maltitol*syrup OR Mannitol OR Neotame OR Polyglycitol*syrup OR Potassium*saccharin OR Rebaudioside OR Saccharin OR Sodium*cyclamate OR Sodium*saccharin OR Sorbitol OR Sorbitol*syrup OR Stevia OR Sucralose OR Thaumatin OR Xylitol) (Topic) AND microbiota OR microbiom*".

An additional search was applied, by including the general "sweete*" term in all the searchable fields (All Fields):

"(Acesulfame*potassium OR advantage OR alihame OR Aspartame OR Aspartame*acesulfame*salt OR Calcium*cyclamate OR Calcium*saccharin OR Cyclamic*acid OR Erythritol OR Isomalt OR Lactitol OR Maltitol OR Maltitol*syrup OR Mannitol OR Neotame OR Polyglycitol*syrup OR Potassium*saccharin OR Rebaudioside OR Saccharin OR Sodium*cyclamate OR Sodium*saccharin OR Sorbitol OR Sorbitol*syrup OR Stevia OR Sucralose OR Thaumatin OR Xylitol) (Topic) and microbiota OR microbiom* (All Fields) and sweete* (All Fields)".

In SCOPUS, we used the following lines.

General class search string:

"TITLE-ABS-KEY ((sweete*) AND (microbiota OR microbiom*)) AND PUBYEAR > 2009"

Individual sweeteners:

"TITLE-ABS-KEY ((acesulfame*potassium OR advantame OR alitame OR aspartame OR aspartame*acesulfame*salt OR calcium*cyclamate OR calcium*saccharin OR cyclamic*acid OR erythritol OR isomalt OR lactitol OR maltitol OR maltitol*syrup OR mannitol OR neotame OR polyglycitol*syrup OR potassium*saccharin OR rebaudioside OR saccharin OR sodium*cyclamate OR sodium*saccharin OR sorbitol OR sorbitol*syrup OR stevia OR sucralose OR thaumatin OR xylitol) AND (microbiota OR microbiom*)) AND PUBYEAR > 2009"

Individual classes + sweete*:

"TITLE-ABS-KEY ((acesulfame*potassium OR advantame OR alitame OR aspartame OR aspartame*acesulfame*salt OR calcium*cyclamate OR calcium*saccharin OR cyclamic*acid OR erythritol OR isomalt OR lactitol OR maltitol OR maltitol*syrup OR mannitol OR neotame OR polyglycitol*syrup OR potassium*saccharin OR rebaudioside OR saccharin OR sodium*cyclamate OR sodium*saccharin OR sorbitol OR sorbitol*syrup OR stevia OR sucralose OR thaumatin OR xylitol) AND (sweete*) AND (microbiota OR microbiom*)) AND PUBYEAR > 2009"

All obtained results were merged into a unique file that allowed the removal of duplicates within and among the different databases.

The exclusion criteria were defined to filter the query results:

> Only studies written in English
> Non-mammalian or non-experimental animal studies
> No studies using sugars as marker of intestinal permeability
> No studies on sugars not investigated as food additive
> No studies on community microbiota other than the gut (e.g. oral microbiota)

Based on the preliminary database query, the next group of food additives researched included several compounds belonging to two or more of the following classes: emulsifiers, stabilizers, thickeners, gelling agents, and foaming agents.

The generic research was conducted employing the same criteria used for "sweeteners" class. After the definition of key terms within Boolean queries, the search strategy was adapted for each database. The time period queried was January 2010 – date of database search (January – March 2022).

Emulsifier

PUBMED. "(emulsifier*) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE. "(emulsifier*) AND (microbiota OR microbiom*)" set Topic from 2010.

SCOPUS. "(emulsifier*) AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

Stabilizer

PUBMED "(stabilizer*) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE. "(stabilizer*) AND (microbiota OR microbiom*)" set Topic from 2010.

SCOPUS. "(stabilizer*) AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

Thickener

PUBMED. "(thickener*) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE. "(thickener*) AND (microbiota OR microbiom*)" set Topic from 2010.

SCOPUS. "(thickener*) AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

Gelling agent

PUBMED. "(gelling agent*) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE. "("gelling agent" OR "gelling agents") AND (microbiota OR microbiom*)" set Topic from 2010.

SCOPUS. "(gelling AND agent*) AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

Foaming agent

PUBMED. "(foaming*agent*) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE. "("foaming agent" OR "foaming agents") AND (microbiota OR microbiom*)" set All Fields from 2010. 0 results found. "(foaming*agent*) AND (microbiota OR microbiom*)"

SCOPUS. "(foaming AND agent*) AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

ADDITIONAL SEARCH STRATEGIES WERE CONDUCTED TARGETING EMULSIFIERS.

MESH TERMINOLOGY WAS ALSO USED: IN PUBMED

MESH database. "("Emulsifying Agents/administration and dosage"[Mesh] OR "Emulsifying Agents/adverse effects"[Mesh] OR "Emulsifying Agents/agonists"[Mesh] OR "Emulsifying Agents/analysis"[Mesh] OR "Emulsifying Agents/immunology"[Mesh] OR "Emulsifying Agents/metabolism"[Mesh] OR "Emulsifying Agents/organization and administration"[Mesh] OR "Emulsifying Agents/pharmacokinetics"[Mesh] OR "Emulsifying Agents/pharmacology"[Mesh] OR "Emulsifying Agents/physiology"[Mesh] OR "Emulsifying Agents/standards"[Mesh] OR "Emulsifying Agents/therapeutic use"[Mesh] OR "Emulsifying Agents/toxicity"[Mesh]) AND (microbiota OR microbiom*)" set from 2010.

The literature search was expanded to include single compounds classified under the emulsifier functional class according to Codex Alimentarius (https://www.fao.org/gsfaonline/reference/techfuncs.html).

Unique terms: "("acetoglyceride*" OR "acetylated monoglycerides" OR "acetylated distarch" OR "acid-treated starch" OR "agar-agar" OR "gelose" OR "alginic acid" OR "ammonium alginate" OR "ammonium polyphosphate" OR "ammonium phosphatide" OR "beeswax" OR "bone phosphate" OR "calcium polyphosphate" OR "calcium stearoyl" OR "candelilla wax" OR "carob bean gum" OR "algaroba" OR "carob gum" OR "locust bean gum" OR "carrageenan" OR "cassia gum" OR "castor oil" OR "ricinus oil" OR "CITREM" OR "DATEM" OR "calcium pyrophosphate" OR "dicalcium pyrophosphate" OR "dioctyl sodium sulfosuccinate" OR "docusate sodium" OR "DSS" OR "dipotassium hydrogen phosphate" OR "dibasic potassium phosphate" OR "dipotassium hydrogen orthophosphate" OR "dipotassium phosphate" OR "disodium diphosphate" OR "disodium dihydrogen pyrophosphate" OR "disodium Pyrophosphate" OR "disodium hydrogen phosphate" OR "dibasic sodium phosphate" OR "disodium acid phosphate" OR "disodium phosphate" OR "secondary sodium phosphate" OR "distarch phosphate" OR "ethyl hydroxyethyl cellulose" OR "ester gum" OR "guar" OR "gum arabic" OR "acacia gum" OR "arabic gum" OR "acacia senegal" OR "acacia seyal" OR "gum ghatti" OR "hydroxypropyl cellulose" OR "modified cellulose" OR "hydroxypropyl distarch phosphate" OR "hydroxypropyl methyl cellulose" OR "hydroxypropyl starch" OR "kadaya" OR "karaya" OR "katilo" OR "kullo" OR "sterculia" OR "konjac" OR "konnyaku" OR "lactitol" OR "lactit" OR "lecithin" OR "magnesium stearate" OR "hydrogenated glucose syrup" OR "maltitol syrup" OR "methyl cellulose" OR "cellulose methyl ether" OR "methyl ethyl cellulose" OR "MEC" OR "microcrystalline cellulose" OR "cellulose gel" OR "glyceryl monooleate" OR "glyceryl monostearate" OR "GMS" OR "monoolein" OR "monopalmitin" OR "monostearin" OR "oxidized starch" OR "pectin*" OR "potassium tripolyphosphate" OR "pentasodium tripolyphosphate" OR "sodium tripolyphosphate" OR "triphosphate" OR "dimethylpolysiloxane" OR "polyethylene glycol" OR "macrogol" OR "PEG" OR "glycerin fatty acid esters" OR "polyglycerol fatty acid esters" OR "polyglycerol esters" OR "polyoxyethylene" OR "polysorbate" OR "polyvinylpyrrolidone" OR "povidone" OR "PVP" OR "potassium alginate" OR "monobasic potassium phosphate" OR "potassium acid phosphate" OR "potassium lactate" OR "powdered cellulose" OR "processed eucheuma seaweed" OR "PES" OR "semi-refined carrageenan" OR "propylene glycol" OR "methyl glycol" OR "propanediol" OR "propylene glycol alginate" OR "Quillaia" OR "quillaja" OR "quillay" OR "sodium alginate" OR "sodium aluminium phosphate" OR "SALP" OR "kasal" OR "sodium carboxymethyl cellulose" OR "cellulose gum" OR "CMC" OR "monosodium citrate" OR "sodium citrate monobasic" OR "Monobasic Sodium Phosphate" OR "Monosodium Dihydrogen Orthophosphate" OR "Monosodium Monophosphate" OR "Sodium Acid Phosphate" OR "Sodium Biphosphate" OR

"Sodium lactate" OR "Sodium polyphosphate" OR "Graham's salt" OR "Sodium hexametaphosphate" OR "Sodium tetrapolyphosphate" OR "Sodium stearoyl lactylate" OR "Sodium Stearoyl-2-Lactylate" OR "Sorbitan monolaurate" OR "Sorbitan laurate" OR "Sorbitan monooleate" OR "Sorbitan monopalmitate" OR "Sorbitan monostearate" OR "Sorbitan tristearate" OR "Starch acetate" OR "Starch sodium octenyl succinate" OR "Sucroglycerides" OR "Sucrose Oligoesters" OR "Sucrose acetate isobutyrate" OR "SAIB" OR "Sucrose fatty acid esters" OR "Tannic acid" OR "Gallotannic acid" OR "Tannins" OR "Potassium pyrophosphate" OR "Tetrapotassium pyrophosphate" OR "Tetrasodium diphosphate" OR "Sodium pyrophosphate" OR "Tetrasodium pyrophosphate" OR "TOSOM" OR "Tragacanth gum" OR "Triacetin" OR "Calcium phosphate" OR "Precipitated calcium phosphate" OR "Triethyl citrate" OR "Tripotassium phosphate" OR "Trisodium citrate" OR "Trisodium phosphate" OR "Sodium phosphate" OR "Tribasic sodium phosphate" OR "Xanthan gum" OR "Xylitol") AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE

Unique terms: "("acetoglyceride*" OR "acetylated diglycerides" OR "acetylated monoglycerides" OR "acetylated distarch" OR "acetylated oxidized" OR "acid-treated starch" OR "agar-agar" OR "ceylon isinglass" OR "chinese isinglass" OR "japanese isinglass" OR "bengal isinglass" OR "gelose" OR "japan agar" OR "layor carang" OR "alginic acid" OR "alkaline treated starch" OR "ammonium alginate" OR "ammonium polyphosphate" OR "ammonium phosphatide" OR "beeswax" OR "bleached starch" OR "bone phosphate" OR "calcium dihydrogen diphosphate" OR "acid calcium pyrophosphate" OR "monocalcium dihydrogen pyrophosphate" OR "calcium polyphosphate" OR "calcium stearoyl" OR "candelilla wax" OR "carob bean gum" OR "algaroba" OR "carob gum" OR "locust bean gum" OR "carrageenan" OR "danish agar" OR "eucheuman" OR "furcellaran agar" OR "hypnean" OR "iridophycan" OR "irish moss gelose" OR "cassia gum" OR "castor oil" OR "ricinus oil" OR "CITREM" OR "citroglyceride" OR "DATEM" OR "dicalcium diphosphate" OR "calcium pyrophosphate" OR "dicalcium pyrophosphate" OR "dioctyl sodium sulfosuccinate" OR "docusate sodium" OR "DSS" OR "dipotassium hydrogen phosphate" OR "dibasic potassium phosphate" OR "dipotassium acid phosphate" OR "dipotassium hydrogen monophosphate" OR "dipotassium hydrogen orthophosphate" OR "dipotassium monophosphate" OR "dipotassium phosphate" OR "secondary potassium phosphate" OR "disodium diphosphate" OR "acid sodium pyrophosphate" OR "disodium dihydrogen diphosphate" OR "disodium dihydrogen pyrophosphate" OR "disodium Pyrophosphate" OR "disodium hydrogen phosphate" OR "dibasic sodium phosphate" OR "disodium acid phosphate" OR "disodium hydrogen monophosphate" OR "disodium phosphate" OR "secondary sodium phosphate" OR "distarch phosphate" OR "ethyl hydroxyethyl cellulose" OR "ester gum" OR "guar" OR "gum cyamopsis" OR "gum arabic" OR "acacia gum" OR "arabic gum" OR "acacia senegal" OR "acacia seyal" OR "gum ghatti" OR "hydroxypropyl cellulose" OR "cellulose hydroxypropyl ether" OR "modified cellulose" OR "hydroxypropyl distarch phosphate" OR "hydroxypropyl methyl cellulose" OR "hydroxypropyl starch" OR "kadaya" OR "karaya" OR "katilo" OR "kullo" OR "kutterra" OR "sterculia" OR "konjac" OR "konnyaku" OR "konnyaleu" OR "lactoglyceride*" OR "lactitol" OR "lactit" OR "lactobiosit" OR "lactositol" OR "lecithin" OR "magnesium stearate" OR "*maltitol" OR "hydrogenated glucose syrup" OR "hydrogenated maltose" OR "maltitol syrup" OR "methyl cellulose" OR "cellulose methyl ether" OR "methyl ethyl cellulose" OR "MEC" OR "microcrystalline cellulose" OR "cellulose gel" OR "glyceryl monooleate" OR "glyceryl monoplamitate" OR "glyceryl monostearate" OR "GMS" OR "monoolein" OR "monopalmitin" OR "monostearin" OR "monostarch phosphate" OR "oxidized starch" OR "pectin*" OR "pentapotassium tripolyphosphate" OR "potassium tripolyphosphate" OR "pentasodium tripolyphosphate" OR "sodium tripolyphosphate" OR "triphosphate" OR "phosphated distarch phosphate" OR "polydimethylsiloxane" OR "dimethylpolysiloxane" OR "dimethylsilicone fluid" OR "dimethylsilicone oil" OR "polydimethylsiloxane"

OR "polyethylene glycol" OR "macrogol" OR "PEG" OR "glycerin fatty acid esters" OR "polyglycerol fatty acid esters" OR "polyglycerol esters" OR "polyoxyethylene" OR "polysorbate" OR "polyvinylpyrrolidone" OR "povidone" OR "PVP" OR "potassium alginate" OR "potassium dihydrogen phosphate" OR "monobasic potassium phosphate" OR "monopotassium dihydrogen monophosphate" OR "monopotassium dihydrogen orthophosphate" OR "monopotassium monophosphate" OR "potassium acid phosphate" OR "potassium biphosphate" OR "potassium dihydrogen phosphate" OR "potassium lactate" OR "potassium polyphosphate" OR "potassium metaphosphate" OR "powdered cellulose" OR "processed eucheuma seaweed" OR "PES" OR "PNG-carrageenan" OR "semi-refined carrageenan" OR "propylene glycol" OR "methyl glycol" OR "propanediol" OR "propylene glycol alginate" OR "hydroxypropyl alginate" OR "propane 1,2-diol alginate" OR "Quillaia" OR "bois de panama" OR "panama bark extract" OR "quillai" OR "quillaja" OR "quillay" OR "soapbark extract" OR "sodium alginate" OR "sodium aluminium phosphate" OR "SALP" OR "kasal" OR "sodium calcium polyphosphate" OR "sodium carboxymethyl cellulose" OR "cellulose gum" OR "CMC" OR "sodium cellulose glycolate" OR "sodium dihydrogen citrate" OR "monosodium citrate" OR "sodium citrate monobasic" OR "sodium dihydrogen phosphate" OR "Monobasic Sodium Phosphate" OR "Monosodium Dihydrogen Monophosphate" OR "Monosodium Dihydrogen Orthophosphate" OR "Monosodium Monophosphate" OR "Sodium Acid Phosphate" OR "Sodium Biphosphate" OR "Sodium Dihydrogen Phosphate" OR "Sodium lactate" OR "Sodium polyphosphate" OR "Graham's salt" OR "Sodium hexametaphosphate" OR "Sodium tetrapolyphosphate" OR "Sodium stearoyl lactylate" OR "Sodium Stearoyl-2-Lactylate" OR "Sodium stearoyl lactate" OR "Sorbitan monolaurate" OR "Sorbitan laurate" OR "Sorbitan monooleate" OR "Sorbitan monopalmitate" OR "Sorbitan monostearate" OR "Sorbitan tristearate" OR "Starch acetate" OR "Starch sodium octenyl succinate" OR "Stearyl citrate" OR "Sucroglycerides" OR "Sucrose Oligoesters" OR "Sucrose acetate isobutyrate" OR "SAIB" OR "Sucrose fatty acid esters" OR "Tannic acid" OR "Gallotannic acid" OR "Tannins" OR "Tetrapotassium diphosphate" OR "Potassium pyrophosphate" OR "Tetrapotassium pyrophosphate" OR "Tetrasodium diphosphate" OR "Sodium pyrophosphate" OR "Tetrasodium pyrophosphate" OR "TOSOM" OR "Tragacanth gum" OR "Triacetin" OR "Tricalcium phosphate" OR "Calcium phosphate" OR "Precipitated calcium phosphate" OR "Tricalcium phosphate" OR "Triethyl citrate" OR "Ethyl citrate" OR "Tripotassium phosphate" OR "Trisodium citrate" OR "Ethyl citrate" OR "Trisodium diphosphate" OR "Acid trisodium pyrophosphate" OR "Trisodium monohydrogen diphosphate" OR "Trisodium phosphate" OR "Sodium phosphate" OR "Tribasic sodium phosphate" OR "Xanthan gum" OR "Xylitol") AND (microbiota OR microbiom*)" set Topic from 2010.

SCOPUS

Concerning unique terms, Scopus DB accepts only 50 terms per query, so queries were divided into five parts and the results were recomposed. set Title/Abstract/Keywords from 2010.

Unique terms – Part 1: "("acetoglyceride*" OR "acetylated*diglycerides" OR "acetylated*monoglycerides" OR "acetylated*distarch" OR "acetylated*oxidized" OR "acid-treated*starch" OR "agar-agar" OR "ceylon*isinglass" OR "chinese*isinglass" OR "japanese*isinglass" OR "bengal*isinglass" OR "gelose" OR "japan*agar" OR "layor*carang" OR "alginic*acid" OR "alkaline*treated*starch" OR "ammonium*alginate" OR "ammonium*polyphosphate" OR "ammonium*phosphatide" OR "beeswax" OR "bleached*starch" OR "bone*phosphate" OR "calcium*dihydrogen*diphosphate" OR "acid*calcium*pyrophosphate" OR "monocalcium*dihydrogen*pyrophosphate" OR "calcium*polyphosphate" OR "calcium*stearoyl" OR "candelilla*wax" OR "carob*bean*gum" OR "algaroba" OR "carob*gum" OR "locust*bean*gum" OR "carrageenan" OR "danish*agar" OR "eucheuman" OR "furcellaran*agar" OR "hypnean" OR "iridophycan"

OR "irish*moss*gelose" OR "cassia*gum" OR "castor*oil" OR "ricinus*oil" OR "CITREM" OR "citroglyceride" OR "DATEM" OR "dicalcium*diphosphate" OR "calcium*pyrophosphate" OR "dicalcium*pyrophosphate") AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

Unique terms – part 2: "("dioctyl*sodium*sulfosuccinate" OR "docusate*sodium" OR "DSS" OR "dip otassium*hydrogen*phosphate" OR "dibasic*potassium*phosphate" OR "dipotassium*acid*phosphate" OR "dipotassium*hydrogen*monophosphate" OR "dipotassium*hydrogen*orthophosphate" OR "dipotassium*monophosphate" OR "dipotassium*phosphate" OR "secondary*potassium*phosphate" OR "disodium*diphosphate" OR "acid*sodium*pyrophosphate" OR "disodium*dihydrogen*diphosphate" OR "di sodium*dihydrogen*pyrophosphate" OR "disodium*Pyrophosphate" OR "disodium*hydrogen*phosphate" OR "dibasic*sodium*phosphate" OR "disodium*acid*phosphate" OR "disodium*hydrogen*monoph osphate" OR "disodium*phosphate" OR "secondary*sodium*phosphate" OR "distarch*phosphate" OR "ethyl*hydroxyethyl*cellulose" OR "ester*gum" OR "guar" OR "gum*cyamopsis" OR "gum*arabic" OR "acacia*gum" OR "arabic*gum" OR "acacia*senegal" OR "acacia*seyal" OR "gum*ghatti" OR "hydroxypropyl*cellulose" OR "cellulose*hydroxypropyl*ether" OR "modified*cellulose" OR "hydroxypropy l*distarch*phosphate" OR "hydroxypropyl*methyl*cellulose" OR "hydroxypropyl*starch" OR "kadaya" OR "karaya" OR "katilo" OR "kullo" OR "kutterra" OR "sterculia" OR "konjac" OR "konnyaku" OR "konnyaleu") AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

Unique terms – part 3: "("lactoglyceride*" OR "lactitol" OR "lactit" OR "lactobiosit" OR "lactositol" OR "lecithin" OR "magnesium*stearate" OR "*maltitol" OR "hydrogenated*glucose*syrup" OR "hydrogenated*maltose" OR "maltitol*syrup" OR "methyl*cellulose" OR "cellulose*methyl*ether" OR "methyl*ethyl*cellulose" OR "MEC" OR "microcrystalline*cellulose" OR "cellulose*gel" OR "glyceryl*monooleate" OR "glyceryl*monoplamitate" OR "glyceryl*monostearate" OR "GMS" OR "monoolein" OR "monopalmitin" OR "monostearin" OR "monostarch*phosphate" OR "oxidized*starch" OR "pectin*" OR "pentapotassium*tripolypho sphate" OR "potassium*tripolyphosphate" OR "pentasodium*tripolyphosphate" OR "sodium*tripolyphosphate" OR "triphosphate" OR "phosphated*distarch*phosphate" OR "polydimethylsiloxane" OR "dimethylpolysiloxane" OR "dimethylsilicone*fluid" OR "dimethylsilicone*oil" OR "polydimethylsiloxane" OR "polyethylene*glycol" OR "macrogol" OR "PEG" OR "glycerin*fatty*acid*esters" OR "polyglycerol*fatty*acid*esters" OR "polyglycerol*esters" OR "polyoxyethylene" OR "polysorbate" OR "polyvinylpyrrolidone" OR "povidone") AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

Unique terms – part 4: "("Sodium*Acid*Phosphate" OR "Sodium*Biphosphate" OR "Sodium*Dihydrogen*Phosphate" OR "Sodium*lactate" OR "Sodium*polyphosphate" OR "Graham's*salt" OR "Sodium*hexametaphosphate" OR "Sodium*tetrapolyphosphate" OR "Sodium*stearoyl*lactylate" OR "Sodium*Stearoyl-2-Lactylate" OR "Sodium*stearoyl*lactate" OR "Sorbitan*monolaurate" OR "Sorbitan*laurate" OR "Sorbitan*monooleate" OR "Sorbitan*monopalmitate" OR "Sorbitan*monostearate" OR "Sorbitan*tristearate" OR "Starch*acetate" OR "Starch*sodium*octenyl*succinate" OR "Stearyl*citrate" OR "Sucroglycerides" OR "Sucrose*Oligoesters" OR "Sucrose*acetate*isobutyrate" OR "SAIB" OR "Sucrose*fatty*acid*esters" OR "Tannic*acid" OR "Gallotannic*acid" OR "Tannins" OR "Tetrapotassium*diphosphate" OR "Potassium*pyrophosphate" OR "Tetrapotassium*pyrophosphate" OR "Tetrasodium*diphosphate" OR "Sodium*pyrophosphate" OR "Tetrasodium*pyrophosphate" OR "TOSOM" OR "Tragacanth*gum" OR "Triacetin" OR "Tricalcium*phosphate" OR "Calcium*phosphate" OR "Precipitated*calcium*phosphate" OR "Tricalcium*phosphate" OR "Triethyl*citrate" OR "Ethyl*citrate" OR "Tripotassium*phosphate" OR "Trisodium*citrate" OR "Ethyl*citrate" OR "Trisodium*diphosphate" OR "Acid*trisodium*pyrophosphate") AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

Unique terms - part 5: "("Trisodium*monohydrogen*diphosphate" OR "Trisodium*phosphate" OR "Sodium*phosphate" OR "Tribasic*sodium*phosphate" OR "Xanthan*gum" OR "Xylitol") AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

All obtained results were merged into a unique file allowing the removal of all duplicates within and among the different databases.

Exclusion criteria were defined to filter the query results and included:

> articles without microbiota or microbiome in title/abstract;

> languages different from English;

> document types different from Articles and Review (grey literature);

> oral microbiota (oral, plaque, dental, caries);

> animal studies (exception made for mammals animal model, e.g. pigs, rodents);

> studies with DSS used as inducers of inflammation;

> studies on the effects of additives on specific bacteria from commercial sources; and

> studies on the effects of additives on food microbiota.

The search was further expanded using specific food additive compounds under the stabilizer and thickener classes. The time period queried was January 2010 – date of database search (March–June 2022). Compounds considered in the emulsifier queries were excluded.

STABILIZER

PUBMED

Unique terms: "("Aluminium ammonium sulfate" OR "Ammonium dihydrogen phosphate" OR "Ammonium polyphosphate" OR "Bromelain" OR "Calcium acetate" OR "Calcium alginate" OR "Calcium carbonate" OR "Calcium chloride" OR "Calcium dihydrogen phosphate" OR "Calcium hydrogen phosphate" OR "Calcium polyphosphate" OR "Calcium stearoyl lactylate" OR "Calcium sulfate" OR "Cross-linked sodium carboxymethyl cellulose" OR "Curdlan" OR "Cyclodextrin" OR "Diammonium hydrogen phosphate" OR "Dipotassium hydrogen phosphate" OR "Disodium diphosphate" OR "Disodium ethylenediaminetetraacetate" OR "Disodium hydrogen phosphate" OR "Gellan gum" OR "Invertases" OR "Magnesium chloride" OR "Magnesium hydrogen phosphate" OR "Pentapotassium triphosphate" OR "Pentasodium triphosphate" OR "Polyoxyethylene (20) sorbitan monolaurate" OR "Polyoxyethylene (20) sorbitan monooleate" OR "Polyvinylpyrrolidone" OR "Potassium carbonate" OR "Potassium chloride" OR "Potassium dihydrogen citrate" OR "Potassium dihydrogen phosphate" OR "Potassium hydrogen carbonate" OR "Sodium L(+)-tartrate" OR "Sodium aluminium phosphate" OR "Sodium carbonate" OR "Sodium dihydrogen phosphate" OR "Sodium gluconate" OR "Sodium hydrogen carbonate" OR "Sodium polyphosphate" OR "Sodium stearoyl lactylate" OR "Sorbitan monolaurate" OR "Sorbitan monooleate" OR "Sorbitan monostearate" OR "Sorbitan tristearate" OR "Tamarind seed polysaccharide" OR "Tara gum" OR "Tetrasodium diphosphate" OR "Tricalcium citrate" OR "Tricalcium phosphate" OR "Trimagnesium phosphate" OR "Tripotassium citrate" OR "Tripotassium phosphate" OR "Trisodium phosphate") AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE

Unique terms: "("Aluminium ammonium sulfate" OR "Ammonium dihydrogen phosphate" OR "Ammonium polyphosphate" OR "Bromelain" OR "Calcium acetate" OR "Calcium alginate" OR "Calcium carbonate" OR "Calcium chloride" OR "Calcium dihydrogen diphosphate" OR "Calcium dihydrogen phosphate" OR "Calcium hydrogen phosphate" OR "Calcium polyphosphate" OR "Calcium stearoyl lactylate" OR "Calcium sulfate" OR "Cross-linked sodium carboxymethyl cellulose" OR "Cross-linked-cellulose gum" OR "Curdlan" OR "Cyclodextrin" OR "Diammonium hydrogen phosphate" OR "Dicalcium diphosphate" OR "Dipotassium hydrogen phosphate" OR "Disodium diphosphate" OR "Disodium ethylenediaminetetraacetate" OR "Disodium hydrogen phosphate" OR "Gellan gum" OR "Invertases" OR "Magnesium chloride" OR "Magnesium dihydrogen diphosphate" OR "Magnesium dihydrogen phosphate" OR "Magnesium hydrogen phosphate" OR "Pentapotassium triphosphate" OR "Pentasodium triphosphate" OR "Polydextroses" OR "Polyoxyethylene (20) sorbitan monolaurate" OR "Polyoxyethylene (20) sorbitan monooleate" OR "Polyoxyethylene (20) sorbitan monostearate" OR "Polyoxyethylene (20) sorbitan tristearate" OR "Polyvinylpyrrolidone" OR "Potassium carbonate" OR "Potassium chloride" OR "Potassium dihydrogen citrate" OR "Potassium dihydrogen phosphate" OR "Potassium hydrogen carbonate" OR "Potassium polyphosphate" OR "Potassium sodium L" OR "Sodium L(+)-tartrate" OR "Sodium aluminium phosphate" OR "Sodium calcium polyphosphate" OR "Sodium carbonate" OR "Sodium dihydrogen phosphate" OR "Sodium gluconate" OR "Sodium hydrogen carbonate" OR "Sodium polyphosphate" OR "Sodium stearoyl lactylate" OR "Sorbitan monolaurate" OR "Sorbitan monooleate" OR "Sorbitan monostearate" OR "Sorbitan tristearate" OR "Tamarind seed polysaccharide" OR "Tara gum" OR "Tetrapotassium diphosphate" OR "Tetrasodium diphosphate" OR "Tricalcium citrate" OR "Tricalcium phosphate" OR "Trimagnesium phosphate" OR "Tripotassium citrate" OR "Tripotassium phosphate" OR "Trisodium diphosphate" OR "Trisodium phosphate") AND (microbiota OR microbiom*)" set Topic from 2010.

SCOPUS

Scopus DB accepts only 50 terms per query, so queries were divided into two parts and then the results were recomposed. set Title/Abstract/Keywords from 2010.

Unique terms - Part 1: "("Aluminium*ammonium*sulfate" OR "Ammonium*dihydrogen*phosphate" OR "Ammonium*polyphosphate" OR "Bromelain" OR "Calcium*acetate" OR "Calcium*alginate" OR "Calcium*carbonate" OR "Calcium*chloride" OR "Calcium*dihydrogen*diphosphate" OR "Calcium*dihydrogen*phosphate" OR "Calcium*hydrogen*phosphate" OR "Calcium*polyphosphate" OR "Calcium*stearoyl*lactylate" OR "Calcium*sulfate" OR "Cross-linked*sodium*carboxymethyl*cellulose" OR "Cross-linked-cellulose*gum" OR "Curdlan" OR "Cyclodextrin" OR "Diammonium*hydrogen*phosphate" OR "Dicalcium*diphosphate" OR "Dipotassium*hydrogen*phosphate" OR "Disodium*diphosphate" OR "Disodium*ethylenediaminetetraacetate" OR "Disodium*hydrogen*phosphate" OR "Gellan*gum" OR "Invertases" OR "Magnesium*chloride" OR "Magnesium*dihydrogen*diphosphate" OR "Magnesium*dihydrogen*phosphate" OR "Magnesium*hydrogen*phosphate" OR "Pentapotassium*triphosphate" OR "Pentasodium*triphosphate" OR "Polydextroses" OR "Polyoxyethylene*(20)*sorbitan*monolaurate" OR "Polyoxyethylene*(20)*sorbitan*monooleate" OR "Polyoxyethylene*(20)*sorbitan*monostearate" OR "Polyoxyethylene*(20)*sorbitan*tristearate" OR "Polyvinylpyrrolidone" OR "Potassium*carbonate" OR "Potassium*chloride") AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

Unique terms - Part 2: "("Potassium*dihydrogen*citrate" OR "Potassium*dihydrogen*phosphate" OR "Potassium*hydrogen*carbonate" OR "Potassium*polyphosphate" OR "Potassium*sodium*L" OR "Sodium*L(+)-tartrate" OR "Sodium*aluminium*phosphate" OR "Sodium*calcium*polyphosphate" OR "Sodium*carbonate" OR "Sodium*dihydrogen*phosphate" OR "Sodium*gluconate" OR "Sodium*hydrogen*carbonate" OR "Sodium*polyphosphate" OR "Sodium*stearoyl*lactylate" OR "Sorbitan*monolaurate" OR "Sorbitan*monooleate" OR "Sorbitan*monostearate" OR "Sorbitan*tristearate" OR "Tamarind*seed*polysaccharide" OR "Tara*gum" OR "Tetrapotassium*diphosphate" OR "Tetrasodium*diphosphate" OR "Tricalcium*citrate" OR "Tricalcium*phosphate" OR "Trimagnesium*phosphate" OR "Tripotassium*citrate" OR "Tripotassium*phosphate" OR "Trisodium*diphosphate" OR "Trisodium*phosphate") AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

THICKENER

PUBMED

Unique terms: "("Calcium lactate" OR "Ethyl cellulose" OR "Glycerol" OR "Polyvinyl alcohol" OR "Pullulan" OR "Talc" OR "Glycerin" OR "PVOH" OR "Vinyl alcohol polymer" OR "Talcum") AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE

Unique terms: "("Calcium lactate" OR "Ethyl cellulose" OR "Glycerol" OR "Polyvinyl alcohol" OR "Pullulan" OR "Talc" OR "Glycerin" OR "PVOH" OR "Vinyl alcohol polymer" OR "Talcum") AND (microbiota OR microbiom*)" set Topic from 2010.

SCOPUS

Unique terms: "("Calcium*lactate" OR "Ethyl*cellulose" OR "Glycerol" OR "Polyvinyl*alcohol" OR "Pullulan" OR "Talc" OR "Glycerin" OR "PVOH" OR "Vinyl*alcohol*polymer" OR "Talcum") AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

Based on the preliminary search activities, the next group of priority food additive classes included colours, preservatives, sequestrants and humectants. The time period queried was January 2010 – date of database search (March–June 2022). The search strategy was the same as described previously for the other classes.

COLOURS

PUBMED

General. "(Colour) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

Unique terms: "("Paprika extract" OR "Allura red AC" OR "Amaranth" OR "Annatto extract" OR "Azorubine" OR "Beet red" OR "Brilliant black" OR "Brilliant blue FCF" OR "Brown HT" OR "Canthaxanthin" OR "Caramel" OR "Carmines" OR "β-Carotene" OR "Chlorophylls" OR "Curcumin" OR "Erythrosine" OR "Fast green FCF"

OR "Grape skin extract" OR "Indigotine" OR "Iron oxide" OR "Lutein" OR "Lycopene" OR "Ponceau 4R" OR "Quinoline yellow" OR "Riboflavin 5'-phosphate sodium" OR "Riboflavin" OR "Sunset yellow FCF" OR "Tartrazine" OR "Titanium dioxide" OR "Zeaxanthin" OR "FD&C Red No.40" OR "Carmoisine" OR "Beetroot Red" OR "Black BN" OR "Black PN" OR "Brilliant Black BN" OR "FD&C Blue No.1" OR "Chocolate brown HT" OR "Ammonia caramel" OR "Sulfite ammonia caramel" OR "Carmine" OR "Cochineal carmine" OR "C.I. Food Orange 5" OR "Natural beta-carotene" OR "Sodium copper chlorophyllin" OR "Copper chlorophyll" OR "C.I. Natural Yellow 3" OR "Diferuloymethane" OR "Turmeric yellow" OR "FD&C Red No. 3" OR "FD&C Green No. 3" OR "ENO" OR "FD&C Blue No. 2" OR "Indigo Carmine" OR "Cochineal Red A" OR "New Coccine" OR "Riboflavin 5'-phosphate ester monosodium salt" OR "FD&C Yellow No. 6" OR "FD&C Yellow No. 5") AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE

General. "(Colour) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

Unique terms: "("Paprika extract" OR "Allura red AC" OR "Amaranth" OR "Annatto extract" OR "Azorubine" OR "Beet red" OR "Brilliant black" OR "Brilliant blue FCF" OR "Brown HT" OR "Canthaxanthin" OR "Caramel" OR "Carmines" OR "Carotenal, beta-apo-8'-" OR "β-Carotene" OR "Carotenoic acid, ethyl ester, beta-apo-8'-" OR "Chlorophylls" OR "Curcumin" OR "Erythrosine" OR "Fast green FCF" OR "Grape skin extract" OR "Indigotine" OR "Iron oxide" OR "Lutein" OR "Lycopene" OR "Ponceau 4R" OR "Quinoline yellow" OR "Riboflavin 5'-phosphate sodium" OR "Riboflavin" OR "Sunset yellow FCF" OR "Tartrazine" OR "Titanium dioxide" OR "Zeaxanthin" OR "CI (1975) No.16035" OR "CI Food Red 17" OR "FD&C Red No.40" OR "CI (1975) No. 16185" OR "CI Food Red 9" OR "Naphtol Rot S." OR "Carmoisine" OR "CI (1975) No. 14720" OR "CI Food Red 3" OR "Beetroot Red" OR "Black BN" OR "Black PN" OR "Brilliant Black BN" OR "CI (1975) No. 28440" OR "CI Food Black 1" OR "CI (1975) No. 42900" OR "CI Food Blue 2" OR "FD&C Blue No.1" OR "Chocolate brown HT" OR "CI (1975) No. 20285" OR "CI Food Brown 3" OR "CI (1975) No 40850" OR "CI Food Orange 8" OR "Caustic caramel" OR "Plain caramel" OR "Caustic sulfite caramel" OR "Ammonia caramel" OR "Sulfite ammonia caramel" OR "Carmine" OR "CI (1975) No. 75470" OR "CI Natural Red 4" OR "Cochineal carmine" OR "C.I. Food Orange 6" OR "C.I. Food Orange 5" OR "Carotenes-natural" OR "CI Food Orange 5" OR "Mixed carotenes" OR "Natural beta-carotene" OR "C.I. Food Orange 7 (Ethyl Ester)" OR "C.I. (1975) No. 75810" OR "Potassium copper chlorophyllin" OR "Sodium copper chlorophyllin" OR "C.I. (1975) No. 75810" OR "CI Natural Green 3" OR "Magnesium chlorophyll" OR "Magnesium phaeophytin" OR "C.I. (1975) No. 75810" OR "CI Natural Green 3" OR "Copper chlorophyll" OR "Copper phaeophytin" OR "C.I. Natural Yellow 3" OR "Diferuloymethane" OR "Kurkum" OR "Turmeric yellow" OR "C.I. (1975) No. 45430" OR "C.I. Food Red 14" OR "FD&C Red No. 3" OR "C.I. Food Green 3" OR "CI (1975) No. 42053" OR "FD&C Green No. 3" OR "ENO" OR "Enociania" OR "C.I. Food Blue 1" OR "CI (1975) No. 73015" OR "FD&C Blue No. 2" OR "Indigo Carmine" OR "C.I. Pigment Black 11" OR "CI (1975) No. 77499" OR "C.I. Pigment Black 11" OR "CI (1975) No. 77499" OR "C.I. Pigment Red 101" OR "C.I. Pigment Red 102" OR "CI (1975) No. 77491" OR "C.I. Pigment Yellow 42" OR "C.I. Pigment Yellow 43" OR "CI (1975) No. 77492" OR "CI (1975) No. 16255" OR "CI Food Red 7" OR "Cochineal Red A" OR "New Coccine" OR "CI (1975) No. 47005" OR "CI Food Yellow 13" OR "Vitamin B2 Ester Monosodium Salt" OR "Riboflavin 5'-phosphate ester monosodium salt" OR "Vitamin B2 phosphate ester monosodium salt" OR "CI (1975) No. 15985" OR "CI Food Yellow 3" OR "Crelborange S" OR "FD&C Yellow No. 6" OR "CI (1975) No. 19140" OR "CI Food Yellow 4" OR "FD&C Yellow No. 5") AND (microbiota OR microbiom*)" set Topic from 2010.

SCOPUS

General. "(Colour) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

Unique terms: "("Paprika*extract" OR "Allura*red*AC" OR "Amaranth" OR "Annatto*extract" OR "Azorubine" OR "Beet*red" OR "Brilliant*black" OR "Brilliant*blue*FCF" OR "Brown*HT" OR "Canthaxanthin" OR "Caramel" OR "Carmines" OR "Carotenal,*beta-apo-8'-" OR "β-Carotene" OR "Carotenoic*acid,*ethyl*ester,*beta-apo-8'-" OR "Chlorophylls" OR "Curcumin" OR "Erythrosine" OR "Fast*green*FCF" OR "Grape*skin*extract" OR "Indigotine" OR "Iron*oxide" OR "Lutein" OR "Lycopene" OR "Ponceau*4R" OR "Quinoline*yellow" OR "Riboflavin*5'-phosphate*sodium" OR "Riboflavin" OR "Sunset*yellow*FCF" OR "Tartrazine" OR "Titanium*dioxide" OR "Zeaxanthin" OR "CI*(1975)*No.16035" OR "CI*Food*Red*17" OR "FD&C*Red*No.40" OR "CI*(1975)*No.*16185" OR "CI*Food*Red*9" OR "Naphtol*Rot*S." OR "Carmoisine" OR "CI*(1975)*No.*14720" OR "CI*Food*Red*3" OR "Beetroot*Red" OR "Black*BN" OR "Black*PN" OR "Brilliant*Black*BN" OR "CI*(1975)*No.*28440" OR "CI*Food*Black*1" OR "CI*(1975)*No.*42900" OR "CI*Food*Blue*2" OR "FD&C*Blue*No.1" OR "Chocolate*brown*HT" OR "CI*(1975)*No.*20285" OR "CI*Food*Brown*3" OR "CI*(1975)*No*40850" OR "CI*Food*Orange*8" OR "Caustic*caramel" OR "Plain*caramel" OR "Caustic*sulfite*caramel" OR "Ammonia*caramel" OR "Sulfite*ammonia*caramel" OR "Carmine" OR "CI*(1975)*No.*75470" OR "CI*Natural*Red*4" OR "Cochineal*carmine" OR "C.I.*Food*Orange*6" OR "C.I.*Food*Orange*5" OR "Carotenes-natural" OR "CI*Food*Orange*5" OR "Mixed*carotenes" OR "Natural*beta-carotene" OR "C.I.*Food*Orange*7*(Ethyl*Ester)" OR "C.I.*(1975)*No.*75810" OR "Potassium*copper*chlorophyllin" OR "Sodium*copper*chlorophyllin" OR "C.I.*(1975)*No.*75810" OR "CI*Natural*Green*3" OR "Magnesium*chlorophyll" OR "Magnesium*phaeophytin" OR "C.I.*(1975)*No.*75810" OR "CI*Natural*Green*3" OR "Copper*chlorophyll" OR "Copper*phaeophytin" OR "C.I.*Natural*Yellow*3" OR "Diferuloymethane" OR "Kurkum" OR "Turmeric*yellow" OR "C.I.*(1975)*No.*45430" OR "C.I.*Food*Red*14" OR "FD&C*Red*No.*3" OR "C.I.*Food*Green*3" OR "CI*(1975)*No.*42053" OR "FD&C*Green*No.*3" OR "ENO" OR "Enociania" OR "C.I.*Food*Blue*1" OR "CI*(1975)*No.*73015" OR "FD&C*Blue*No.*2" OR "Indigo*Carmine" OR "C.I.*Pigment*Black*11" OR "CI*(1975)*No.*77499" OR "C.I.*Pigment*Black*11" OR "CI*(1975)*No.*77499" OR "C.I.*Pigment*Red*101" OR "C.I.*Pigment*Red*102" OR "CI*(1975)*No.*77491" OR "C.I.*Pigment*Yellow*42" OR "C.I.*Pigment*Yellow*43" OR "CI*(1975)*No.*77492" OR "CI*(1975)*No.*16255" OR "CI*Food*Red*7" OR "Cochineal*Red*A" OR "New*Coccine" OR "CI*(1975)*No.*47005" OR "CI*Food*Yellow*13" OR "Vitamin*B2*Ester*Monosodium*Salt" OR "Riboflavin*5'-phosphate*ester*monosodium*salt" OR "Vitamin*B2*phosphate*ester*monosodium*salt" OR "CI*(1975)*No.*15985" OR "CI*Food*Yellow*3" OR "Crelborange*S" OR "FD&C*Yellow*No.*6" OR "CI*(1975)*No.*19140" OR "CI*Food*Yellow*4" OR "FD&C*Yellow*No.*5") AND (microbiota OR microbiom*)' set Title/Abstract/Keywords from 2010.

PRESERVATIVE

PUBMED

General. "(Preservative) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

MESH database. "("Preservatives, Pharmaceutical/adverse effects"[Mesh] OR "Preservatives, Pharmaceutical/metabolism"[Mesh] OR "Preservatives, Pharmaceutical/poisoning"[Mesh] OR "Preservatives, Pharmaceutical/toxicity"[Mesh]) AND (microbiota OR microbiom*)".

Unique terms: "("Benzoyl peroxide" OR "Calcium benzoate" OR "Calcium propionate" OR "Calcium sorbate" OR "Carbon dioxide" OR "Dimethyl dicarbonate" OR "Diphenyl" OR "Hexamethylene tetramine" OR "Lysozyme" OR "Methyl para-hydroxybenzoate" OR "Natamycin" OR "Nisin" OR "ortho-Phenylphenol" OR "Potassium acetate" OR "Potassium benzoate" OR "Potassium metabisulfite" OR "Potassium nitrate" OR "Potassium nitrite" OR "Potassium propionate" OR "Potassium sorbate" OR "Potassium sulfite" OR "Sodium acetate" OR "Sodium benzoate" OR "Sodium hydrogen sulfite" OR "Sodium metabisulfite" OR "Sodium nitrate" OR "Sodium nitrite" OR "Sodium ortho-phenylphenol" OR "Sodium propionate" OR "Sodium sulfite" OR "Sorbic acid" OR "Sulfur dioxide" OR "Benzoyl superoxide" OR "Carbonic Acid Anhydride" OR "Dry Ice" OR "Dimethyl Pyrocarbonate" OR "DMDC" OR "Ethylparaben" OR "Hexamine" OR "Methenamine" OR "Methylparaben" OR "Methyl p-Oxybenzoate" OR "Natamycin" OR "Nitre" OR "Saltpetre" OR "Sodium Bisulfite") AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE

General. "(Preservative) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

Unique terms: "("Benzoyl peroxide" OR "Calcium benzoate" OR "Calcium propionate" OR "Calcium sorbate" OR "Carbon dioxide" OR "Dimethyl dicarbonate" OR "Diphenyl" OR "Ethyl para-hydroxybenzoate" OR "Hexamethylene tetramine" OR "Lauric arginate ethyl ester" OR "Lysozyme" OR "Methyl para-hydroxybenzoate" OR "Natamycin" OR "Nisin" OR "ortho-Phenylphenol" OR "Potassium acetate" OR "Potassium benzoate" OR "Potassium metabisulfite" OR "Potassium nitrate" OR "Potassium nitrite" OR "Potassium propionate" OR "Potassium sorbate" OR "Potassium sulfite" OR "Sodium acetate" OR "Sodium benzoate" OR "Sodium hydrogen sulfite" OR "Sodium metabisulfite" OR "Sodium nitrate" OR "Sodium nitrite" OR "Sodium ortho-phenylphenol" OR "Sodium propionate" OR "Sodium sulfite" OR "Sorbic acid" OR "Sulfur dioxide" OR "Benzoyl superoxide" OR "Monocalcium benzoate" OR "Calcium propanoate" OR "Carbonic Acid Anhydride" OR "Dry Ice" OR "Dimethyl Pyrocarbonate" OR "DMDC" OR "Ethylparaben" OR "Ethyl p-Oxybenzoate" OR "Hexamine" OR "Methenamine" OR "Methylparaben" OR "Methyl p-Oxybenzoate" OR "Natamycin" OR "Orthoxenol" OR "Nitre" OR "Saltpetre" OR "Sodium Bisulfite" OR "Chile saltpetre" OR "cubic nitre" OR "Soda nitre" OR "Disodium sulfite") AND (microbiota OR microbiom*)" set Topic from 2010.

SCOPUS

General. "(Preservative) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

Scopus - Unique terms: "("Benzoyl*peroxide" OR "Calcium*benzoate" OR "Calcium*propionate" OR "Calcium*sorbate" OR "Carbon*dioxide" OR "Dimethyl*dicarbonate" OR "Diphenyl" OR "Ethyl*para-hydroxybenzoate" OR "Hexamethylene*tetramine" OR "Lauric*arginate*ethyl*ester" OR "Lysozyme" OR "Methyl*para-hydroxybenzoate" OR "Natamycin" OR "Nisin" OR "ortho-Phenylphenol" OR "Potassium*acetate" OR "Potassium*benzoate" OR "Potassium*metabisulfite" OR "Potassium*nitrate" OR "Potassium*nitrite" OR "Potassium*propionate" OR "Potassium*sorbate" OR "Potassium*sulfite" OR "Sodium*acetate" OR "Sodium*benzoate" OR "Sodium*hydrogen*sulfite" OR "Sodium*metabisulfite" OR "Sodium*nitrate" OR "Sodium*nitrite" OR "Sodium*ortho-phenylphenol" OR "Sodium*propionate" OR "Sodium*sulfite" OR "Sorbic*acid" OR "Sulfur*dioxide" OR "Benzoyl*superoxide" OR "Monocalcium*benzoate" OR "Calcium*propanoate" OR "Carbonic*Acid*Anhydride" OR "Dry*Ice" OR "Dimethyl*Pyrocarbonate" OR "DMDC" OR "Ethylparaben" OR "Ethyl*p-Oxybenzoate" OR "Hexamine" OR "Methenamine" OR "Methylparaben"

OR "Methyl*p-Oxybenzoate" OR "Natamycin" OR "Orthoxenol" OR "Nitre" OR "Saltpetre" OR "Sodium*Bisulfite" OR "Chile*saltpetre" OR "cubic*nitre" OR "Soda*nitre" OR "Disodium*sulfite") AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

SEQUESTRANT

PUBMED

General. "(Sequestrant) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

Unique terms: "("Ascorbic acid, L-" OR "Calcium disodium ethylenediaminetetraacetate" OR "Calcium gluconate" OR "Citric acid" OR "Glucono delta-lactone" OR "Isopropyl citrates" OR "Phosphoric acid" OR "Potassium gluconate" OR "Sodium acetate" OR "Sodium diacetate" OR "Sodium thiosulfate" OR "Tartaric acid" OR "Vitamin C" OR "Calcium disodium edetate" OR "Calcium disodium EDTA" OR "GDL" OR "D-Gluconic Acid Delta-Lactone" OR "Glucono-delta-lactone" OR "Gluconolactone" OR "Delta-Gluconolactone" OR "Rochelle salt" OR "Sodium hyposulfite") AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE

General. "(Sequestrant) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

Unique terms: "("Ascorbic acid, L-" OR "Calcium disodium ethylenediaminetetraacetate" OR "Calcium gluconate" OR "Citric acid" OR "Glucono delta-lactone" OR "Isopropyl citrates" OR "Malic acid, DL-" OR "Phosphoric acid" OR "Potassium gluconate" OR "Potassium sodium L(+)-tartrate" OR "Sodium acetate" OR "Sodium diacetate" OR "Sodium thiosulfate" OR "Tartaric acid" OR "Vitamin C" OR "Calcium disodium edetate" OR "Calcium disodium EDTA" OR "Calcium Di-D-Gluconate Monohydrate" OR "Calcium Di-Gluconate" OR "GDL" OR "D-Gluconic Acid Delta-Lactone" OR "Glucono-delta-lactone" OR "Gluconolactone" OR "Delta-Gluconolactone" OR "Isopropyl Citrate mixture" OR "2-Hydroxybutanedioic acid" OR "Pomalous Acid" OR "Potassium sodium dextro-tartrate" OR "Rochelle salt" OR "Seignette salt" OR "Sodium hyposulfite") AND (microbiota OR microbiom*)" set Topic from 2010.

SCOPUS

General. "(Sequestrant) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

Scopus - Unique terms: "("Ascorbic*acid,*L-" OR "Calcium*disodium*ethylenediaminetetraacetate" OR "Calcium*gluconate" OR "Citric*acid" OR "Glucono*delta-lactone" OR "Isopropyl*citrates" OR "Malic*acid,*DL-" OR "Phosphoric*acid" OR "Potassium*gluconate" OR "Potassium*sodium*L(+)-tartrate" OR "Sodium*acetate" OR "Sodium*diacetate" OR "Sodium*thiosulfate" OR "Tartaric*acid" OR "Vitamin*C" OR "Calcium*disodium*edetate" OR "Calcium*disodium*EDTA" OR "Calcium*Di-D-Gluconate*Monohydrate" OR "Calcium*Di-Gluconate" OR "GDL" OR "D-Gluconic*Acid*Delta-Lactone" OR "Glucono-delta-lactone" OR "Gluconolactone" OR "Delta-Gluconolactone" OR "Isopropyl*Citrate*mixture" OR "2-Hydroxybutanedioic*acid" OR "Pomalous*Acid" OR "Potassium*sodium*dextro-tartrate" OR "Rochelle*salt" OR "Seignette*salt" OR "Sodium*hyposulfite") AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

HUMECTANT

PUBMED

General. "(Humectant) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

Unique terms: "("Sodium malate") AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

WEB OF SCIENCE

General. "(Humectant) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

Unique terms: "("Sodium malate" OR "Sodium hydrogen malate" OR "Malic acid sodium salt") AND (microbiota OR microbiom*)" set Topic from 2010.

SCOPUS

General. "(Humectant) AND (microbiota OR microbiom*)" set Title/Abstract from 2010.

Unique terms: "("Sodium*malate" OR "Sodium*hydrogen*malate" OR "Malic*acid*sodium*salt") AND (microbiota OR microbiom*)" set Title/Abstract/Keywords from 2010.

Exclusion criteria were:

> articles without microbiota or microbiome in title/abstract;
> languages different from English;
> document types different from Articles and Review ((grey literature);
> oral microbiota (oral, plaque, dental, caries);
> animal studies (exception made for mammals animal model, e.g. pig, rodents);
> studies on the effects of additives on specific taxa evaluated *in vitro*; and
> studies on the effects of additives on food and soil microbiota.

ANNEX III. **SUMMARY TABLES**

ANNEX III.1. SUMMARY TABLES – ACESULFAME K

JECFA ADI: 0–15 mg/kg bw/day

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Male 4-week-old pups C57Bl/6J mice (8 mice per group)	15 mg/kg bw/d pure acesulfame-K (in drinking water) 8 weeks	Samples: faeces, caecal content qPCR, DGGE Caecal metabolome	No changes	No effects on host metabolism were observed	(Uebanso *et al.*, 2017b)
CD-1 mice, male and female (7-weeks old) (5 mice/group)	37.5 mg/kg bw/d gavage for 4 weeks	Samples: faeces 16S rRNA (V4) gene sequencing PICRUSt functional gene analysis Faecal metabolome	Gender-specific changes: Males: ↑ *Bacteroides, Anaerostipes, Sutterella.* Females: ↓ *Lactobacillus, Clostridium,* unassigned Ruminococcaceae and Oxalobacteraceae, ↑ *Mucispirillum* Gene enrichment analysis: ↑ males: carbohydrate metabolism pathways ↓ females: genes related to carbohydrate metabolism ↑ LPS and flagella synthesis (females), thiol-activated cytolysin (males)	Gender-dependent effects: ↑ body weight in males; no bw effect in females. Metabolome: Females: ↓ bacterial metabolism-related metabolites Males: ↑ energy metabolites, cholic acid; ↓ deoxycholic acid (DCA)	(Bian *et al.*, 2017a)
C57BL/6J mice (8-weeks old) (Control: 5 mice/ group, Ace-K: 4 mice/ group)	150 mg/kg bw/day (drinking water) for 8 weeks	Samples: caecal content 16S rRNA (V4) gene sequencing	↓ α-diversity, Proteobacteria, Bacteroidetes, *Bacteroides, Desulfovibrio*, Clostridiaceae, Lachnospiraceae, and Ruminococcaceae ↑ Verrocomicrobia, Actinobacteria Erysipelotrichacecae, *Akkermansia muciniphila, Bifidobacterium, Allobaculum*	Small intestine: damage, increased permeability. ↑ gene expression of proinflammatory cytokines **MFT** (treated animals): no intestinal inflammation	(Hanawa *et al.*, 2021)
Human cross-sectional study males, females 7/31 individuals: Ace-K consumers	Estimated from information reported in questionnaire: 1.7–33.2 mg/day 4-day monitoring	16S rRNA gene capillary sequencing PICRUSt functional gene analysis	Compared to non-consumers: > No differences in predicted gene function, composition and Firmicutes:Bacteroidetes ratio > Differences in bacterial diversity	Host not studied	(Frankenfeld *et al.*, 2015)

Sources: See References

ANNEX III.2. SUMMARY TABLES – ASPARTAME

JECFA ADI 0–40 mg/kg bw/day

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Sprague-Dawley rats male (n=10–12/group)	5 mg/kg bw/day (HFD) 7 mg/kg bw/day (normal chow) Both diets had their own control group without ASP 8 weeks	qRT-PCR (16S rRNA gene) (faeces)	Aspartame groups: ↑ *Clostridium leptum*, Enterobacteriaceae In HFD group: ↑ *Roseburia* Aspartame attenuated HFD-induced alterations: Firmicutes:Bacteroidetes ratio ↑ serum propionate	↑ fasting glucose levels, impaired insulin tolerance (several other alterations were diet dependent)	(Palmnas *et al.*, 2014)
Diet-induced obese Sprague-Dawley rats Dams, male/female offspring (n=10 group) GF mice (unknown strain), male	Obese dams: 5–7 mg/kg bw/day aspartame (High fat/High sucrose diet) during gestation and lactation Offspring post-weaning: control diet, no aspartame, until 18 weeks of age	qPCR specific bacteria linked to obesity (faeces) 16S rRNA (V3–V4) gene sequencing (caecal content) Caecal SCFAs	**Dams:** ↑ *Clostridium* cluster IV ↓ Enterococcaceae, *Enterococcus*, *Parasutterella* Compared to offpring, *Akkermansia muciniphila* and Enterobacteriaceae were higher in dams. **Offspring:** ↑ Porphyromonadaceae Lactobacilli: sex-specific variation **GF mice:** ↑ Porphyromonadaceae SCFA (dams) ↑ caecal propionate, butyrate, isobutyrate, isovalerate, valerate	**Dams:** Impaired Insulin sensitivity **Offspring:** Male: increased body fat (at weaning only). Impaired insulin sensitivity and altered glucose tolerance (W8) Female: increased body weight and body fat (at weaning only) **Offspring:** Mesolimbic reward gene expression	(Nettleton *et al.*, 2020)
Human cross-sectional study males, females 7/31 individuals: aspartame consumers	Estimated from information reported in questionnaire: 5.3–112 mg/day 4-day monitoring	16S rRNA gene capillary sequencing (faeces)	Compared to non-consumers: > No differences: functional, composition and Firmicutes:Bacteroidetes ratio > Differences in bacterial diversity	Not studied	(Frankenfeld *et al.*, 2015)
			See summary under sweetener combination table		(Ahmad, Friel and Mackay, 2020a)
Study reported in the saccharin table					(Suez *et al.*, 2022)

Sources: See References

ANNEX III.3. SUMMARY TABLES – SACCHARIN

JECFA ADI 0–5 mg/kg bw/day

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6 mice, adult 20 mice/group – normal chow 10 mice/group – HFD Germ-free Swiss Webster mice (for MFT)	10% Commercial saccharin (5% saccharin + 95% glucose) in drinking water – about 5 000 mg/kg bw/day (calculated based on liquid consumption ~20 ml/day, 20 g mice) Controls: 10% glucose or sucrose, water 11-week (normal chow, HFD) GF mice: 6 days after transplantation, normal chow	16S rRNA (V2) gene sequencing (faeces) Shotgun metagenomics	Normal chow: Reported dysbiosis ↑ *Bacteroides*, *Bacteroides vulgatus*, Clostridiales ↓ *Lactobacillus reuteri*, *Akkermansia muciniphila* ↑ Glycan-degradation pathways, propionate, acetate ↓ Glucose transport **Germ-free mice:** dysbiosis (no details) HFD: Microbiota not studied	Glucose intolerance (treated groups on normal chow, HFD and transplanted mice) (normal chow groups): Normal fasting plasma Insulin levels and insulin tolerance test	(Suez *et al.*, 2014)
C57BL/6 mice, male adult 20 mice/group – normal chow Germ-free Swiss Webster mice (for MFT)	Pure saccharin (0.1 mg/ml ~ ADI 5 mg/kg bw/day) in drinking water Control: water 5-week (HFD) GF mice: 6 days after transplantation, normal chow	16S rRNA (V2) gene sequencing (faeces) Shotgun metagenomics	HFD: dysbiosis (no details) ↑ bacterial chemotaxis, lipopolysaccharide synthesis pathways **Germ-free mice:** Microbiota not studied	Reported by authors: Glucose intolerance in treated and transplanted germ-free mice Normal fasting plasma Insulin levels	(Suez *et al.*, 2014)
In vitro Faecal culture Germ-free Swiss Webster mice (for MFT)	Saccharin (assumed pure) 9 days GF mice: 6 days after transplantation, normal chow	16S rRNA (V2) gene sequencing (faeces) Shotgun metagenomics	9 days **culture**: ↑ Bacteroidetes ↓ Firmicutes **GF mice:** ↑ *Bacteroides*; ↓ some Clostridiales ↑ Glycan-degradation pathways, ↓ Glucose transport	Reported by authors: Glucose intolerance (glucose test curve, at 2 h show no differences between treatment and controls)	(Suez *et al.*, 2014)
Human (n=7) (28–36 years old, healthy, non-usual NAS consumers) Germ-free Swiss Webster mice (for MFT)	**Humans:** Commercial saccharin 5 mg/kg bw/day (days 2–7) **GF mice:** normal chow/ liquid (faecal material D1 and D7 from two responders and two non-responders)	16S rRNA (V2) gene sequencing (faeces)	**Humans:** Microbiota of GTT responders different from non-responders **GF mice:** 1 individual: ↑ *Bacteroides fragilis*, *Weissella cibaria*, *Candidatus Arthromitus*	**Poor (?)** glucose response n=4 No responder n=3 **GF mice:** glucose intolerance	(Suez *et al.*, 2014)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Human (n=20) Healthy male and females Non NNS consumers Germ-free Swiss Webster mice (for MFT)	Commercial non-nutritive sweeteners (NNS) (glucose as filler), (US FDA ADIs based on 60 kg person): Stevia: 180 mg/day (74% ADI) Sucralose: 102 mg/day (34% ADI) Saccharin: 180 mg/day (20% ADI) Aspartame: 240 mg/day (8% ADI) 7-day pre-treatment (baseline) > 14-day treatment > 7-day clearance	Shotgun metagenomic sequencing (faeces)	Distinct microbiota (composition and function) – NNS-dependent	Saccharin and sucralose: ↑ glycaemic response (compared to group baseline). Glycaemic response differences were not evident during the follow-up period. Microbiome and metabolome correlate with glycaemic responses (treatment group and top GTT responders) FMT: ↑ glycaemic responses in transplanted GF mice with faecal microbiota from top GTT responders from all NNS and bottom saccharin responders, potentially dependent on individual's microbiome response to NNS.	(Suez *et al.*, 2022)
C57BL/6J mice (10/group: 5 male, 5 female) HFD (controls HFD and LFD)	Saccharin (5 mg/kg bw/day) 10 weeks (HFD) (stevia group – 5 mg/kg bw/day – HFD, also included in this study) Sweeteners in drinking water	16S rRNA (V4) gene sequencing (faeces)	Females: Differences in β-diversity ↑ *Akkermansia muciniphila*	HFD increased glucose levels and body weight (no effect from the sweetener)	(Becker *et al.*, 2020)
C57BL/6J mice (n=24/group)	5 mg/kg bw/day in drinking water 2 studies: 2–7 days after colitis induction 5 weeks prior colitis induction (followed by 30 d of induced colitis without saccharin)	16S rRNA (V1–V2) gene sequencing (faeces)	Change in β-diversity ↑ Bacteroidetes, Proteobacteria ↓ Firmicutes, *Bacillus cereus*	Protection against intestinal inflammation, improved colitis	(Sunderhauf *et al.*, 2020)
Human randomized DBPC interventional study (total 46 subjects, saccharin group n=13)	400 mg/day (2 capsules/day) ~ 4x ADI 2 weeks (+ 2 weeks clearance) (2 more groups: lactisole, lactisole+saccharin)	16S rRNA (V3–V4) gene sequencing (faeces) Faecal SCFA and metabolome	Null effects (no changes in α- β-diversities, composition)	Null effects (no changes in body weight or glucose homeostasis)	(Serrano *et al.*, 2021)
WT C57BL/6J and T1R2-defficient mice (n=23–28/group)	250 mg/kg bw/day in drinking water 10 weeks	16S rRNA (V3–V4) gene sequencing (faeces) Faecal SCFA and metabolome	Limited effects (moderate overtime intra-individual change in β-diversity)	Age-dependent increases glucose intolerance and ↑ SCFA (NOT due to saccharin consumption).	(Serrano *et al.*, 2021)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6J mice Pregnant and non-pregnant females (n= not specified)	0.066% saccharin (3 mg/kg bw/day) > 10% ethanol + 0.066% saccharin in water > 10% ethanol > 0.066% saccharin in drinking water 4 h/day for 2 weeks *Diet type not reported	qPCR 16S rRNA gene (faeces)	Ethanol changed the abundance of some bacterial groups depending on the presence of saccharin and the pregnancy status, affecting *Clostridium*, *Eubacterium* and *Helicobacter* groups.	Host not evaluated	(Labrecque *et al.*, 2015)
Landrace X Large White piglets Male and females, 28-days old (n=8/group)	0.015%, w/w SUCRAM (**FEED ADDITIVE**): saccharin and neohesperidin dihydrochalcone [NHDC]) in feed 2 weeks	16S rRNA (V1–V3) gene sequencing (caecal content) Caecal SCFAs	Prebiotic effects Microbial community shift (β-diversity) ↑ Lactobacillaceae (mainly *Lactobacillus* 4228), promoted by NHDC ↓ Ruminococcaceae, Veillonellaceae No change in SCFAs	Host not evaluated	(Daly *et al.*, 2016)
C57BL/6J mice, adult males (n=24/group)	0.3 mg/ml (~18-26 mg/kg bw/day) in drinking water 6 months (microbiota composition: also checkpoints at 0 and 3 months) Standard rodent pellets	16S rRNA (V4) gene sequencing (faeces) Faecal metabolome	(Diversity not studied) ↓ M3+M6: *Ruminococcus* ↓ M6 *Adlercreutzia*, *Dorea* ↑ M3: *Akkermansia*, *Oscillospira* ↑ M6: *Corynebacterium*, *Roseburia*, *Turicibacter* Gene enrichment: LPS, flagella, fimbriae, bacterial toxins, AMR	Possible hepatic inflammation ↑ expression hepatic pro-inflammatory markers (iNOS and TNF-α) ↓ some anti-inflammatory metabolites ↑ pro-inflammatory quinolinic acid Increased risk of inflammation	(Bian *et al.*, 2017c)

Sources: See References

ANNEX III.4. SUMMARY TABLES – SUCRALOSE

JECFA ADI 0–15 mg/kg bw/day

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Male Sprague-Dawley rats (n=10/group)	Splenda (1.1% sucralose, 1.1% glucose, 93.6% maltodextrin) 100, 300, 500 and 1 000 mg/kg bw/day (1.1, 3.3, 5.5, 11 mg sucralose/kg bw/day) gavage. 12-week treatment (n=10, of which 5 continue on a 12-week clearance) Control: water	Select cultured bacterial groups isolated from stools (total aerobes, total anaerobes, Lactobacilli, Enterobacteria, Clostridia, Bifidobacteria and *Bacteroides*)	After 12-week treatment: ↓ total aerobes, total anaerobes, Lactobacilli, Clostridia, Bifidobacteria and *Bacteroides* After 12-week clearance: ↓ total anaerobes	↓ Body weight, histological alterations of colon epithelium, increased expression of detoxification enzymes	(Abou-Donia *et al.*, 2008)* * The science quality of Abou-Donia's work evaluated by Brusick *et al.* (2009)
Male 4-weeks old pups C57Bl/6J mice (n=8 mice/group)	Low-dose: 1.4 mg/kg bw/d pure sucralose (in water) High-dose: 14.2 mg/kg bw/d (in water) Free access to standard food Control: distilled water 8 weeks	Samples: faeces, caecal content qPCR, DGGE Metabolome (caecal content)	↓ *Clostridium* XIVa (in faeces; dose-dependent) (qPCR. No changes by DGGE) ↓ caecal butyrate (dose-dependent)	Altered lipid metabolism: ↑ caecal: Ratio secondary:primary bile acids (dose dependent) ↑ hepatic cholesterol. No change in body weights	(Uebanso *et al.*, 2017b)
C57BL/6 mice (5-weeks old) (n=8 mice/group) *In vitro*: culture *E. coli*	Chow + sucralose sol. (2.5% w/v) ~ 3.3 mg/kg bw/day – (control without sucr.) HFD + sucralose sol. (2.5% w/v) ~ 1.5 mg/kg bw/day – (control without sucr.) *calculated from consumption 8 weeks treatment *In vitro*: 1.25, 2.5% (w/v)	Samples: faeces (0, 2, 7 weeks of treatment) 16S rRNA gene seq. (region not reported) a-diversity (Shannon index)	Phylum level: (+) Firmicutes (Sucr-HFD and transient in Sucr-chow) Genus level: (+) *Bifidobacterium* (Sucr-chow only) *In vitro*: dose-dependent colony reduction: IC50 *E. coli* HB101: 58.4 mM IC50 *E. coli* K-12: 63.3 mM	Controls: ↑ body weight in HFD (expected) Body weight: - ↓ in SUC-chow - no change in SUC+HFD Focus on microbiota: No other host parameters were evaluated.	(Wang *et al.*, 2018)
Wistar rats (lean) (6-weeks old) (n=6 mice/ group)	1.5% sucralose in drinking water (~600-1 200 mg/kg bw/day) fed control diet or HFD 4 weeks * This study also evaluated other sweeteners, including steviol glycosides (see corresponding table) and other caloric sweeteners.	16S rRNA (V3-V4) gene sequencing (faeces) Shotgun metagenomics SCFA	↓ α-diversity, HFD: Change in ß-diversity ↓ B/F ratio, *Akkermansia muciniphila* ↑ *Bacteroides fragilis* *Desulfovibrio* ↓ gene richness ↑ LPS genes ↑ faecal SCFA, primarily acetate	Metabolic effects ↓ body weight plasma LPS (metabolic endotoxemia) Insulin resistance and glucose intolerance	(Sanchez-Tapia *et al.*, 2020)
Mice (wild type) Strain and gender not reported (n=16 mice per group) (controls: HFD, and control chow) **This study focuses primarily on Reb A*	Sucralose: 97 mg/ml (~5 mg/kg bw/day) in drinking water fed HFD [calculated based on reported obese mouse weight 40 g and ~ 2 ml daily fluid intake] 15 weeks	16S rRNA (V4) gene sequencing (faeces)	High inter-individual variability Other results not discussed	Hepatic fibrosis. Improved glucose homeostasis and insulin sensitization compared to high carbohydrates/HFD or HFD groups control.	(Xi *et al.*, 2020)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6J mice Adult male (n=10/group)	Sucralose in drinking water: 1 mg/ml (5 mg/kg bw/day) [estimated 10 mg/kg bw/day] 6 weeks	16S rRNA (V4) gene sequencing (faeces)	Diversity not evaluated ↑ Christensenellaceae, Clostridiaceae, *Akkermansia, Roseburia, Turicibacter* ↓ Erysipelotrichaceae, *Dehalobacterium, Streptococcus, Ruminococcus* Gene enrichment: LPS, flagella, fimbriae, bacterial toxins, AMR	Altered faecal metabolome (66 metabolites identified: quorum sensing, amino acids, bile acids. ↓ expression hepatic iNOS, MMP-2 risk hepatic inflammation	(Bian *et al.*, 2017b)
SAMP1/YitFc (a spontaneous mouse model of CD-like ileitis), AKR/J mice (n=6/group)	Splenda (1% sucralose:99% maltodrextrin): 1, 3.5, 35 mg/ml in drinking water 6 weeks	16S rRNA (V4) gene sequencing (faeces) Shotgun metagenomics (focus on bacteria, virus screening) (faeces)	↑ α-, β-, δ-, ε-, γ-Proteobacteria ↑ *E. coli* (SAMP only)	↑ Myeloperoxidase activity (SAMP only) ↑ Intestinal inflammation (highest dose only)	(Rodriguez-Palacios *et al.*, 2018b)
C57BL/6 mice Mothers and offspring (n=not specified, different n for different tests)	Dams: > Sucralose: 0.1 mg/ml (5-15 mg/kg bw/day) in drinking water > 6 weeks (3W pregnancy+3W lactation), standard diet Offspring: > Maternal exposure to sucralose > Diet: 3W lactation > 5W standard diet > 4W HFD	16S rRNA (V3–V4) gene sequencing (faeces) Caecal SCFA	**3-week-old offspring:** ↑ α-diversity, Changed β-diversity ↑ Verrucomicrobia, Proteobacteria, *Blautia, Akkermansia, Escherichia/Shigella, Anaerostipes* ↓ Bacteroidetes, *Alistipes, Parabacteroides, Prevotellaceae, Clostridium* XIVa. ↓ butyrate **12-week-old offspring:** Changed β-diversity ↓ Proteobacteria	**3-week-old offspring:** Altered gut barrier Low-grade intestinal inflammation **12-week-old offspring:** Disturbed hepatic lipid –metabolism Sucralose exacerbates HFD-hepatic steatosis	(Dai *et al.*, 2020)
AOM/DSS CRC model – C57BL/6 mice (gender not specified) n=8 (not specified in methods (taken from one Figure)	1.5 mg/ml sucralose in drinking water [~estimated: 150 mg/kg bw/day] 6 weeks pre- + 36 days post-CRC induction Diet: standard rodent chow One sucralose group in healthy mice Control: healthy mice, no sucralose	qPCR (faeces)	**Sucralose-healthy mice:** ↓ Total bacteria ↑ Firmicutes **Sucralose-CRC mice:** ↓ Total bacteria, Proteobacteria ↑ Firmicutes, Actinobacteria **Both:** ↑ *Clostridium symbiosum, Peptostreptococcus anaerobius, P. stomatis* (this one not in sucralose-healthy mice) ↓ *Bifidobacterium*	Sucralose aggravated the induced colitis and increased number of colorectal tumours. (tissue damage, and altered tight junctions, inflammatory response, protease activity and tumour-associated signalling pathway molecules)	(Li *et al.*, 2020a)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6 mice (DSS induced colitis) Male (n=6/group)	1.5 mg/ml sucralose in drinking water [~estimated: 150–300 mg/kg bw/day] 6 weeks followed by 7 days with or without DSS (to induce colitis) Controls: no sucralose or DSS Diet: standard rodent chow	qPCR (faeces)	**Sucralose (vs. control)** ↓ Bacteroidetes, *Bifidobacterium, B. breve, B. bifidum, Parabacteroides distasonis, Faecalibacterium prausnitzii, Lactobacillus*, ↑ *Akkermansia muciniphila, Pseudomonas aeruginosa, Prevotella copri, Fusobacterium* nucleatum, *Bacteroides fragilis* **Sucralose + DSS (vs. DSS control)** ↑ Proteobacteria, Firmicutes, *Akkermansia muciniphila, Pseudomonas aeruginosa, Prevotella copri, Bilophila wadsworthia, Fusobacterium* nucleatum, *Bacteroides fragilis* ↓ *Bifidobacterium, B. breve, Parabacteroides distasonis, Faecalibacterium prausnitzii*	Sucralose aggravated DSS-induced colitis (colonic tissue damage, altered gut barrier function, inflammatory response, protease activity	(Guo *et al.*, 2021)
C57BL/6 mice Female (n=10/group)	0.1 mg/ml (5 mg/kg bw/day) in drinkin g water Standard diet 11 weeks Other groups: sucralose +metformin or fructo-oligosaccharides; neohesperidin dihydrochalcone	16S rRNA gene sequencing (caecal content)	Differences in β-diversity Changed Bacteroidetes, Firmicutes, Proteobacteria, Actinobacteria, Verrucomicrobia ↑ *Bacteroides, Clostridium*	Altered bile acid profile (DCA) and hepatic lipid metabolism. Potential microbial contribution to non-alcoholic fatty liver	(Shi *et al.*, 2021)
Human randomised, double-blind interventional study (males, n=17/group)	Sucralose: 780 mg (260 mg capsules x 3 times/day) ~75% JECFA ADI Placebo: calcium carbonate 7 days	16S rRNA (V3–V4) gene sequencing (faeces)	No microbiota changes after treatment (Different microbiota profiles - ↑ Firmicutes, ↓ Bacteroidetes – observed in (1) placebo group at baseline, correlating with higher BMI, cholesterol levels, and higher insulinaemia, and (2) regardless of treatment, in subjects with higher insulin AUC after 7 day study.	No changes in glycaemic and insulinaemic responses.	(Thomson *et al.*, 2019)
Study reported in the saccharin table					(Suez *et al.*, 2022)

Sources: See References

ANNEX III.5. SUMMARY TABLES – STEVIOL GLYCOSIDES

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Sprague-Dawley rats male (n=8/group)	> Rebaudioside A: 2–3 mg/kg bw/day (drinking water) > prebiotic 10% inulin > prebiotic + rebaudioside A 9 weeks	16S rRNA (V3–V4) gene sequencing (caecal content)	**Reb A:** ↓ Clostridiales family XIII, Ruminococcacceae, *Lactobacillus intestinalis* ↑ *Akkermansia muciniphila*, *Bacteroides goldsteinii*, *Bacteroides thetaiotaomicron* SCFA: ↑ acetate and valerate **Prebiotic groups:** ↓ α-diversity, Clostridiales family XIII, Ruminococcacceae ↑ *Bifidobacterium* and *Lactobacillus*, *Akkermansia muciniphila* SCFA: ↓ acetate, valerate, isovalerate, butyrate, isobutyrate	**Reb A:** No changes in body weight and glucose tolerance **Reb A:** Altered ↓ expression of some mesolimbic reward system genes **Prebiotic:** Altered ↑ expression of some mesolimbic reward system genes Improved body composition and insulin sensitivity. Reduced intestinal permeability	(Nettleton *et al.*, 2019)
Diet-induced obese Sprague-Dawley rats Dams, male/female offspring (n=10 group) GF mice (unknown strain), male	Obese dams: 2–3 mg/kg bw/day rebaudioside A (High fat/High sucrose diet HFSD) during gestation and nursing Dosing via drinking water Offspring post-weaning: control diet, no rebaudioside A until 18 weeks of age	qPCR specific bacteria linked to obesity (faeces) 16S rRNA (V3–V4) gene sequencing (caecal content)	**Dams:** ↑ Porphyromonadaceae, *Sporobacter* **Offspring:** ↑ Porphyromonadaceae **GF mice:** ↑ Porphyromonadaceae SCFA (dams) ↑ caecal propionate, butyrate, isobutyrate	**Dams:** Impaired Insulin sensitivity **Offspring:** Male: increased body fat (at weaning only). Female: increased body weight and body fat (at weaning only) **Dams and offspring:** Mesolimbic reward gene expression altered	(Nettleton *et al.*, 2020)
Balb/c mice male (n=5/group)	Rebaudioside A: 5 and 50 mg/kg bw/day, gavage 4 weeks	cell count, DGGE, DGGE sequencing (faeces)	Limited or no effects **High dose:** ↑ α-diversity, *Lactobacillus* species	Host not evaluated	(Li *et al.*, 2014)
In vitro – culture-specific bacteria: *Escherichia coli* 0157:H7, *Salmonella typhimurium*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Lactobacillus plantarum*, *Bifidobacterium longum*	Rebaudioside A: 0.01, 0.1, 0.5, and 1% (w/v) in media 24h	OD bacteria growth (faeces)	Limited or no effects **Dose ≥ 0.5%:** ↓ *Staphylococcus aureus* ↑ *Lactobacillus plantarum*	Host not evaluated	(Li *et al.*, 2014)
C57BL/6J mice n=10/group: 5 males, 5 females HFD (controls HFD and LFD)	Stevia powder (5 mg/kg bw/day) 10 weeks (saccharin group – 5 mg/kg bw/day – also included in this study	16S rRNA (V4) gene sequencing (faeces)	Higher impact of HFD than stevia at phyla level. Females: Differences in β-diversity ↑ *Lactococcus*	Stevia did not recover HFD effects: increased glucose levels and body weight	(Becker *et al.*, 2020)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Wistar rats (lean) 6-weeks old (n=6 mice/group)	2.5% steviol glycosides in drinking water (~1000-2000 mg/kg bw/day) fed control diet or HFD 4 weeks * This study also included other groups, including steviol glycosides and other caloric sweeteners.	16S rRNA (V3–V4) gene sequencing (faeces) Shotgun metagenomics Faecal SCFA	↓ α-diversity, Altered β-diversity (4%) **Control diet:** ↑ *Akkermansia muciniphila* ↓ *Lactococcus*, *Mucispirillum* **HFD:** ↓ gene richness Both diets: ↑ *Faecalibacterium prausnitzii*	Steviol glycosides: anti-inflammatory response (Steviol glycosides+sucrose: pro-inflammatory response) ↓ body weight	(Sanchez-Tapia *et al.*, 2020)
Mice (wild type) Strain and gender not reported (n=16 mice/group) (controls: HFD, and control chow)	Rebaudioside A: 194 mg/L (~10 mg/kg bw/day) in drinking water fed HFD [calculated based on reported obese mouse weight 40 g and ~ 2 ml daily fluid intake] 15 weeks	16S rRNA (V4) gene sequencing (faeces)	↑ ratio *Akkermansia:Bacteroides*	Ameliorates high carbohydrate/HFD or HFD-induced effects: glucose homeostasis, insulin sensitization, liver dysfunction and hepatic steatosis. Leads to less hepatic fibrosis compared to other HFD-fed groups.	(Xi *et al.*, 2020)
Kunming mice (4–5-weeks old), male (n=10/group)	Stevia extract: 200 or 400 mg/kg bw/day gavage 3 weeks	16S rRNA (V3–V4) gene sequencing Faecal content/end of study	Improved dysbiosis observed in the adenine-induced chronic kidney disease (CKD)	Improvement of CKD (high-dose)	(Mehmood *et al.*, 2020)
In vitro – Colonic simulator (GIS1) inoculated with faecal microbiota from 3 healthy children (3 replicate simulations) NO parallel control group	Stevioside ~4mg/kg bw/day 12 months	qPCR SCFA	**No change:** Prokariotes, Firmicutes, Bacteroides **Temporary increase:** Actinobacteria, *Bifidobacterium*, Enterobacteriaceae **Fluctuation:** *Lactobacillus* Alteration of SFCA and antioxidant status	n/a	(Gatea, Sârbu and Vamanu, 2021)
Study reported in the saccharin table				(Suez *et al.*, 2022)	

Sources: See References

ANNEX III.6. SUMMARY TABLES – NEOTAME

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
CD1-mice (male, n=5/group)	Neotame: 0.75 mg/kg bw/day (gavage) 4 weeks	16S rRNA (V4) gene sequencing (faeces) Faecal non-target metabolomics	Reduced α-diversity and changed -diversity ↓ Firmicutes: Ruminococcoceae, *Ruminococcus* Lachnosphiraceae, *Blautia*, *Dorea*, *Oscillospira* ↑ Bacteoidetes, *Bacteroides*, S24-7 Gene enrichment: ↑ amino acid metabolism, LPS biosynthesis, antibiotics biosynthesis and folate biosynthesis pathways ↓ carbohydrate metabolism, fatty acid and lipid metabolism and ABC transporters, butyrate pathways	No body weight change Faecal metabolome: ↓ lipid and fatty acids ↑ cholesterol	(Chi *et al.*, 2018)

Sources: See References

ANNEX III.7. SUMMARY TABLES – SUGAR ALCOHOLS

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
CD-1 mice, SPF (male, n=7/group)	5% xylitol + 0.05% daidzein in feed (control: 0.05% daidzein) 28 days	T-RFLP (caecal content)	↓ *Clostridium* XIVa, *Bacteroides*	↑ urine daidzein ↑ total faecal lipids ↓ plasma cholesterol Potential health benefits: improved bone health and decreased lipid absorption.	(Tamura, Hoshi and Hori, 2013)
C57BL/6J mice, young (male, 3-week-old) (Experiment 1: n=5/group, Experiment 2, n=6/group) FMT: C57BL/6J mice (male, 6-weeks old)	Xylitol: Experiment 1 (Control diet): 40 and 194 mg/kg bw/day in solution, 16 weeks Experiment 2 (HFD): 200 mg/kg bw/day in solution, 18 weeks	qPCR (Total bacteria, Bacteroidetes, Firmicutes) PCR-DGGE 16S rRNA (V2-V3) gene > sequencing (faeces and caecal content – only faecal microbiota reported) Caecal metabolome	Experiment 1 (high dose only): ↓ Bacteroidetes, *Clostridium* (1 species), *Barnesiella* ↑ *Clostridium* (2 species), *Faecalibaculum* Experiment 2 (HFD): ↓ Bacteroidetes, *Clostridium* (1 species), *Barnesiella* ↑ total faecal bacteria, Firmicutes, *Prevotella*, *Clostridium* (2 species), *Faecalibaculum*	No change in body weight, caecal metabolome, or expression of inflammatory markers. Lipid metabolism: Experiment 1: no alterations Experiment 2: changes in cholesterol and triglycerides due to HFD. No alterations of glucose tolerance (xylitol did not ameliorate or worsen these parameters)	(Uebanso *et al.*, 2017a)
Sprague-Dawley rats (6–8-weeks old) (male, n=10/group)	Xylitol: 1.0, 3, 10% (0.9, 3.15, 9.9 g/kg bw/day), gavage 15 days (followed by 7 days clearance period)	16S rRNA (V3–V4) gene sequencing (faeces) Colonic SCFA	Dose-dependent effects (M, H): ↓ α-diversity (high dose) End of treatment, High dose: ↑ *Bacteroides* ↓ Lachnospiraceae, *Alloprevotella*, Ruminococcaceae, Prevotellaceae. Medium dose: no significant influence. Decrease acetate, propionate, butyrate (high dose)	Dose-dependent effects (which tend to disappear at the end of clearance period) High dose: Diarrhea with inflammatory cell infiltration – no altered cytokines - and microvilli damage, weight loss Medium dose: Diarrhea, no altered pro-inflammatory markers	(Zuo *et al.*, 2021)
SPF and GF ICR mice (male, n=4/group) —- C57BL/6J (male, n=4/group)	5% sorbitol (w/w) in the drinking water, 4 days —- 5 or 10% sorbitol (w/w) in the drinking water, 4 days, after treatment with antibiotics (ampicillin, streptomycin, erythromycin or vancomycin)	16S rRNA (V3–V4) gene sequencing (faeces)	Vancomycin: ↑ *Escherichia*, *Klebsiella*, *Enterobacter*, and *Proteus* Erythromycin: ↑ *Lachnosclostridium* Capacity to degrade sorbitol: *Escherichia coli*, *Citrobacter farmeri*, *Klebsiella penumoniae* and *Enterobacter* spp.	Gut microbiota has a protective effect against sorbitol-induced osmotic diarrhea.	(Hattori *et al.*, 2021)

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MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6 mice (male, 8-weeks old, n=8/ group)	2 and 5% xylitol (w/w) in feed (~2.2 and 5.4 g/kg bw/day) 3 months	16S rRNA (V4–V5) gene sequencing (faeces) ITS2 gene sequencing Faecal metabolome (incl. SCFA)	Dose-dependent effects No change in α-diversity ↑ Bacteroidetes, Actinobacteria, *Bifidobacterium*, *Lactobacillus*, Erysipelotrichaceae, ↓ Firmicutes, Proteobacteria, *Blautia*, *Staphylococcus* ↑ Propionate, amino acid metabolism. Cross-feeding in the utilization of sorbitol (*Lactobacillus reuteri*, *Bacteroides fragilis*, *Escherichia coli*)	No changes (Body and organ weight, colon length)	(Xiang *et al.*, 2021)
In vitro 3-vessel colonic simulator (Changdao Moni simulation system – CDMN)	3% xylitol (human daily intake: ~0.27 g/kg bw) 7 days	16S rRNA (V4–V5) gene sequencing Metabolome (incl. SCFA) Metatranscriptome sequencing	↑ Lachnospiraceae; Fungus: *Trichosporon* ↓ Proteobacteria and *Escherichia-Shigella*; Fungus: *Saccharomyces* ↑ propionate in lumen; butyrate in mucosa Microbial xylitol utilization	n/a	(Xiang *et al.*, 2021)
In vitro – culture media, human faecal samples (healthy males)	5 mg/ml media xylitol 24 h (Other experimental groups given fructooligosaccharides [FOS], galactooligosaccharides [GOS], D-mannitol, D-sorbitol, or L-sorbose)	DGGE 16S rRNA gene (V3–V4) > sequencing of bands	↑ *Anaerostipes* spp. *A. hadrus* and *A. caccae (utilize xylitol and L-sorbose, responsible for* ↑ butyrate)	n/a	(Sato *et al.*, 2017)
Human randomized, double blinded, dose-response interventional study (total 40 healthy individuals)	Maltitol, maltitol + polydextrose or maltitol + resistant starch: Weeks 1–2: 22.8 g Weeks 3–4: 34.2 g Weeks 5–6: 45.6 g In chocolate	*In situ* hybridization (16S rRNA gene) with probes for specific bacteria) (faeces) Faecal SCFAs (acetate, propionate, butyrate)	All treatment groups (after 6 weeks, higher dose): ↑ Lactobacilli, *Bacteroides*, Bifidobacteria	No significant change to bowel activity or intestinal symptoms.	(Beards, Tuohy and Gibson, 2010)

Sources: See References

ANNEX III.8. SUMMARY TABLES – SWEETENER COMBINATIONS

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57Bl6 mice pregnant-Lactating females pups (male, female): gestation–weaning (d20) Controls: 11 mothers/77 pups ADI: 13 mothers/93 pups 2xADI: 8 mothers/56 pups)	**Sucralose + acesulfame K** Controls (mothers/pups) Treatment groups: Mothers (gestation-day 40 postpartum): **1xADI:** sucralose (5 mg/kg bw/d) + acesulfame K (15 mg/kg/bw/d) in rodent chow or **2xADI** Pups exposure: gestation + lactation until weaning (day 20)	16S rRNA (V3–V4) gene sequencing (faecal) Faecal and plasma metabolome	Only pups: ↑ α-diversity, firmicutes:Bacteroidetes ratio, Firmicutes, Clostridiales, Lachnospiraceae, Ruminoccocaceae ↓ Depleted Verrucomicrobia: *Akkermansia muciniphila*	Potential adverse effects on infant metabolisms after maternal exposure (pregnancy and lactation) Pups: Hepatic detoxification and metabolic alterations 2xADI group: reduced body weight and fasting glucose levels	(Olivier-Van Stichelen, Rother and Hanover, 2019)
Human randomized, double-blind crossover and controlled clinical trial. 17 Healthy Male/female Age: 18-45 Normal BMI: 20–25 fasting blood glucose < 5.7 mmol/L	Aspartame 14% Canadian ADI (40 mg/kg bw/day) (0.425 g/day) Sucralose 20% Canadian ADI (9 mg/kg bw/day) 0.136 g/day) 14d aspartame + 14d washout + 14d sucralose (8 individuals) 14d sucralose + 14d washout + 14d aspartame (9 individuals)	16S rRNA (V4) gene sequencing (faeces) Faecal SCFA	No alterations of the faecal microbiota or SCFA production.	No influence on glucose metabolism or insulin sensitivity [outcomes of this part of the study reported in (Ahmad, Friel and MacKay, 2020b)	(Ahmad, Friel and Mackay, 2020a; Ahmad, Friel and MacKay, 2020b)
CD-1 mice 3-weeks old (weaned) (n=8/group)	3 treatments: 4.1 mg/ml Splenda® (unknown sucralose content) or Svetia® (2.5% steviol glycosides, 0.6% sucralose) 41.66.mg/mL sucrose 6 or 12 weeks	Cultured microbiota of the small intestine – macroscopical and genetic identification (16S rRNA gene sequencing)	Variable results (treatment, controls): *Bacillus* species most abundant.	Changes in the immunity of the small intestine: Increased IL-6 and IL-17A Changes in lymphocyte subsets, dependent on sweetener, exposure time and sampling location	(Martínez-Carrillo *et al.*, 2019)
Monkey (Cebus apella) n=1 (no control group)	Splenda Naturals plus Stevia (erythritol + ~ 1% rebaudioside D) (6.2 mg/kg) in drinking water 2 weeks	16S rRNA gene sequencing (faeces)	Overall, study did not find negative effect of sweeteners on the gut microbiome. ↑ α-diversity Change in β-diversity No change at family/genus level	Host not evaluated	(Mahalak *et al.*, 2020)
In vitro – bioreactor with human faecal microbiota (from one individual)	Splenda Naturals plus Stevia (erythritol + ~ 1% rebaudioside D) (6.2 mg/kg) erythritol (6.2 mg/kg) Treatment period not clear (5 or 10 days?)	16S rRNA gene sequencing SCFA Bile acids (primary, secondary)	No changes in diversity and microbiota composition ↑ butyric and pentanoic acids (both treatment groups) No change in bile acids	n/a	(Mahalak *et al.*, 2020)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
In vitro (culture strains: scherichia coli, *Enterococcus caccae, Lactobacillus rhamnosus, Ruminococcus gauvreauii, Bacteroides galacturonicus,* and *Bacteroides thetaiotaomicron*	Splenda Naturals plus Stevia (erythritol + ~ 1% rebaudioside D) (25 µg/ml) erythritol (50 µg/ml) steviol (12.5, 25 and 50 µg/ml) stevioside (12.5, 25 and 50 µg/ml) reabaudioside A (12.5, 25 and 50 µg/ml) glucose 24 hours	16S rRNA gene sequencing	↑ *B. thetaiotaomicron* (steviol only)	n/a	(Mahalak *et al.*, 2020)
Wistar rats (adult males) n=9/group	0.17% commercial NNS (saccharin and sodium cyclamate) in low-fat yogurt (Control: 11.4% sucrose in yogurt 17 weeks	16S rRNA (V4) gene sequencing (faeces)	No differences in α- and β-diversities	Higher body weight and lower energy expenditure at rest.	(Falcon *et al.*, 2020; Pinto *et al.*, 2017)

Sources: See References

ANNEX III.9. SUMMARY TABLES – ARTIFICIAL SWEETENERS – EPIDEMIOLOGICAL STUDIES

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Human (Swedish) cross-sectional/ epidemiological study (1 371 participants)	Observational study (evaluate if artificial sweeteners – also added sugar – associate with gut microbiota composition)	16S rRNA (V1–V3) gene sequencing (faeces)	No associations between artificially-sweetened beverages and gut microbiota. Larger studies needed to evaluate if links exists between sweeteners and gut microbiota	Findings very modestly support the triad artificial sweeteners-gut microbiota-risk of cardiometabolic disorders.	(Ramne *et al.*, 2021)
Human observational study 100 infants	Observational study (Artificial sweeteners)	16S rRNA (V4) gene sequencing (faeces)	Microbiota clustered in four groups with different diversity and taxonomical composition, affecting different *Bacteroides* spp.	Potential impact of maternal consumption of ABS on maturation of infant gut microbiome and BMI during first year of life.	(Laforest-Lapointe *et al.*, 2021)

Sources: See References

ANNEX III.10. SUMMARY TABLES – CMC, P80

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Mouse C57BL/6 (WT, *Il-10*$^{-/-}$, *Tlr5*$^{-/-}$) Swiss Webster (WT, GF) Male, females (n variable, not clear total number of animals in control group or receiving treatment) 4-weeks old One experiment: 4-months old	Different experimental settings: **Standard:** 1% P80 or CMC, drinking water or chow, 12 weeks **Dose-response:** 0.1, 0.5, 1% P80 or CMC, drinking water, 12 weeks **Adults:** 1% P80 or CMC, drinking water, 8 weeks	qPCR (microbial load) 16S rRNA (V4) gene sequencing (faeces) Faecal SCFAs, bile acids Faecal/serum LPS, flagellin	No altered total faecal bacteria Altered microbiota (β-diversity): Affecting Bacteroidales, Clostridiales (Lachnospiraceae, Ruminococcaceae) ↓ diversity, Bacteroidales Proinflammatory microbiota (↑ LPS, flagellin) Altered SCFA and bile acid profile (WT Swiss Webster) *Il-10*$^{-/-}$: ↑ *Clostridium perfringens*, *Akkermansia muciniphila*, Proteobacteria	Hyperfagia, altered barrier function, microbiota encroachment. WT: low-grade inflammation, metabolic syndrome (glucose intolerance + ↑ adiposity) *Il-10*$^{-/-}$, *Tlr5*$^{-/-}$: colitis, inflammation GF Swiss Webster: no effects FMT GF Swiss Webster: low-grade inflammation, ↑ adiposity, dysglycaemia, microbiota encroachment,	(Chassaing *et al.*, 2015)
In vitro M-SHIME model (inoculated with faecal material from 1 human individual) *In vivo* GF, ASF and *Rag*$^{-/-}$ C57BL/6	M-SHIME: 1% P80 or CMC, 13 days 0.1, 0.5, 1% P80 or CMC, 13 days FMT (from M-SHIME) GF mice: Standard chow or HFD, 12-13 weeks	qPCR (microbial load) 16S rRNA (V4) gene sequencing metatranscriptomics SCFAs, BSCFA LPS, flagellin	**M-SHIME:** P-80: Alters microbiota composition Proinflammatory microbiota No alterations SCFAs, BCFA No clear dose–response **GF mice:** Altered α-diversity, ↑ Proteobacteria, Enterobacteriaceae, ↓ Bacteroidaceae Proinflammatory microbiota **ASF mice:** no alteration of the ASF population	**GF mice:** Low grade inflammation Microbiota encroachment Indication of metabolic syndrome. Exacerbation of HFD effects **ASF mice:** No intestinal inflammation nor metabolic syndrome nor microbiota encroachment	(Chassaing *et al.*, 2017)
Interventional human trial 16 healthy subjects: control (n=9), CMC group (n=7)	15 g CMC/day for 11 days No CMC in 3 pre-trial days Same Western-style diet for all participants	qPCR 16S rRNA gene sequencing Shotgun metagenomics (faeces) Faecal metabolomics LPS, flagellin	Microbiota composition alterations – not At phylum or Order levels –: ↓ *Faecalibacterium prausnitzii*, *Ruminococcus* spp. ↑ *Roseburia* sp. and Lachnospiraceae Altered microbial pathways (genes) ↓ some SCFAs and amino acids during treatment No differences in LPS or flagellin levels	No impact on host (body weight, pro-inflammatory cytokines, glucose homeostasis, Lipocalin 2). Microbiota encroachment in two CMC-treated individuals	(Chassaing *et al.*, 2021)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Mouse C57BL/6 and Swiss Webster (WT and GF) 4-weeks old (n=5–8, not clear how many for controls or treatment groups)	1% P80 or CMC, drinking water, 13 weeks followed by i.p. AOM (10 mg/kg) to induce colon cancer + 2x 7-day 2.5% DSS separated by 14-day recovery.	qPCR (microbial load) 16S rRNA (V4) gene sequencing LPS, flagellin	Microbiota tested only in C57BL/6 mice before carcinogenesis induction: All groups cluster Both CMC, P80 ↓ α-diversity, Firmicutes: Clostridiales, *Lactobacillus* ↑ Bacteroidetes, Bacteroidales No changes: γ-Proteobacteria, Enterobacteriaceae, *Escherichia coli*, or colibactin-related gene	↑ inflammation markers (lipocalin 2, chemokine gene expression), cell proliferation Limited phenotype transfer to GF mice after FMT After inducing carcinogenesis: ↑ tumour number Overall, microbiota disturbances causing low-grade inflammation can promote colon cancer	(Viennois *et al.*, 2017)
Mouse C57BL/6 (WT or APC^{min}) 7-weeks old (n=2–11, not clear how many for controls or treatment groups)	1% P80 or CMC, drinking water, 15 weeks	16S rRNA (V4) gene sequencing (faeces) Faecal LPS, flagellin	P80 or CMC treatment groups: **WT C57BL/6:** ↓ Actinobacteria. Other changes are gender specific **APC^{min}:** ↓ Clostridia ↑ Proteobacteria (males only)	Limited inflammation ↑ tumour number size (P80 and CMC-treated APC^{min}) in the small intestine	(Viennois and Chassaing, 2021)
Mouse C57BL/6 (ASF, GF, IL-$10^{-/-}$) 6-weeks old (gender not specified for ASF and GF) (n=4–5, taken from figures) All mice (control and treatment) colonized with adherent-invasive *E. coli* (AIEC) (via drinking water for 1 week)	1% P80 or CMC, drinking water, 12 weeks model of colitis-associated cancer: 1% P80 or CMC, drinking water (4 weeks) followed by i.p. AOM (10 mg/kg) to induce colon cancer + 2x 7-day 2.5% DSS separated by 14-day recovery In vitro: 1, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016 (units not specified but assumed as %)	16S rRNA (V4) gene sequencing (faeces) Faecal LPS, flagellin	**Experiment with ASF C57BL/6 mice (many reported with data from day 56, not at the end of study):** P80: shift ASF consortium (β-diversity), loss of Clostridiaceae P80, CMC: Encroachment of AIEC in the colonic inner mucus layer *In vitro*: CMC ↑ transcription of virulence factors and adherence capacity (dose-dependent)	CMC (with differences) increases susceptibility to intestinal inflammation by promoting virulence and adherence capacity of the pathobiont AIEC. CMC: increased number and size of colonic tumours	(Viennois *et al.*, 2020)
Mouse C57BL/6, males and females 3-weeks old (n=5–6/group)	1% P80 or CMC, drinking water, 12 weeks	16S rRNA (V4) gene sequencing (faeces)	Gender and emulsifier dependent effects on the microbiota composition.	Gender and emulsifier-dependent effects on intestinal inflammation and behaviours	(Holder *et al.*, 2019)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
In vitro MiniBioReactor Array (MBRA) (inoculated with faecal material from one human individual) (triplicate experiment: n=3)	0.1% of 20 emulsifiers (6 days treatment + 3 days clearance): Sodium carboxymethylcellulose (CMC, E466), polysorbate 80 (P80, E433), soy lecithin (E322), sunflower lecithin (E322), propylene glycol alginate (E405), agar agar (E406), iota carrageenan (E407), kappa carrageenan (E407), lambda carrageenan (E407), locus bean gum (E410), guar gum (E412), gum arabic (E414), xantham gum (E415), diacetyl tartaric acid ester of mono- and diglycerides (DATEM, E472e), hydroxypropyl methyl cellulose (HPMC, E464), sorbitan monostearate (E491), mono- and diglycerides (E471), glyceryl stearate (E471), glyceryl oleate (E471) and maltodextrin (E1400)	16S rRNA (V4) gene sequencing Faecal LPS, flagellin metatranscriptomics	Effects depend on the emulsifier, generally reduced diversity. 72h of treatment: ↓ Lactobacillales (*Streptococcus*), ↓ *Faecalibacterium* (P80, iota carrageenan, agar agar, and DATEM) ↑ *Bacteroides* (kappa and lambda carrageenans, DATEM and glyceryl stearate) Carrageenans (especially kappa carrageenan) and gums (especially guar gum), glycerol stearate > most disturbances: microbial load and composition and the expression of pro-inflammatory compounds, flagellin in particular, and other genes soy lecithin and mono- and diglycerides > no effects	n/a	(Naimi *et al.*, 2021)
GF 129SvEv IL-10$^{-/-}$ mice Male, 7.5–10-weeks old (n=7–8/group) Humanized with pooled stools from three individuals diagnosed with inflammatory bowel disease	1% P80 or CMC, drinking water, 4 weeks	Shotgun metagenomics (caecal content)	P80: no/limited effects CMC: limited changes: ↓ Caudoviricetes (bacteriophages)	P80: no differences from control CMC: ↑ large intestine histologic inflammation scores and inflammatory biomarkers	(Rousta *et al.*, 2021)
C57BL/6 mice (gender, age not specified) n=10/group (?)	1% P80/kg bw, gavage 4 weeks	16S rRNA (V1-V4) gene sequencing qPCR (*Clostridium* XIV) LPS, flagellin SCFAs	↑ Gram positive, Porphyromonadaceae, *Campylobacter jejuni*, *Helicobacter* ↓ *Bacteroides* ↓ SCFA ↑ LPS, flagellin, deoxycholic acid Bacterial encroachment	↑ adiposity, Altered glucose homeostasis Low-grade inflammation with increased intestinal permeability Hepatic dysfunction	(Singh, Wheildon and Ishikawa, 2016)
C57BL/6 mice Male (age not specified) n=5 (?) *In vitro* characterization of Enterobacteria	1% P80, drinking water, 1% P80 + 5 mg/kg bw indomethacin (non-steroideal anti-inflammatory drug) i.p. (last 2 days) 8 weeks	16S rRNA (V4) gene sequencing (leal and caecal content) PCR (16S rRNA gene) and terminal restriction fragment length polymorphism	P80: ↓ α-diversity in small intestine (not in colon) β-diversity differences (small and large intestine) P80 and P80+indomethacin: ↑ γ-Proteobacteria (*Proteus mirabilis* identified in vitro) P80: ↓ *Bacteroides*	P80 exacerbates indomethacin-induced ileitis and increased IL-1β (mitigated with antibiotics) P80 alone (no indomethacin) does not cause ileitis	(Furuhashi *et al.*, 2020)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6 mice Male, 8-weeks old n=12/group (power 80%)	1% P80, 200 µl gavage (~100 mg/kg bw day) 7 days before radiation treatment	16S rRNA (V4) gene sequencing (feces) Faecal SCFAs	**Before radiation:** ↓ α-diversity (# species), *Allobaculum*, *Lactobacillus* **After radiation (P80 group):** ↓ α-diversity (# species) ↑ Bacteroidetes, *Rikenella*, *Lactobacillus*, *Roseburia*, and *Anaerotruncus*. ↓ *Parasutterella*, *Akkermansia* SCFA: ↓ butyrate, ↑ propionate **Butyrate supplementation** recovered effects of P80-radiation on microbiota composition (however, study lacked a control not exposed to P80 or radiation)	P80 exacerbated radiation-induced gastrointestinal toxicity (shorter colons, epithelial ↑ damage, expression of proinflammatory markers, ↓ expression of intestinal integrity markers) Improvement after butyrate supplementation	(Li *et al.*, 2020b)
C57BL/6 mice F0 Male and female offspring (F1): n=19 (7 evaluated at W3, 5 at W8. Remaining mice assessed at ~W9 for DSS-induced colitis) For FMT: 8-weeks old C57BL/6 (treated with antibiotic cocktail)	Mothers (F0) 1% P80, drinking water, 3 weeks prior mating until weaning. Pups (F1) never exposed to P80 directly, evaluated at W3, W8. DSS-induced colitis in W8 mice: 2% DSS in water for 5 days. FMT: faecal material from F1 at W3	16S rRNA (V3–V4) gene sequencing (faeces)	No between-group differences in α-diversity. But differences within group between W3 and W8 Differences in β-diversity between groups. W3 and W8: ↑ Proteobacteria, Desulfovibrionaceae, and Helicobacteraceae W3: ↑ *Bacteroides, Helicobacter* ↓ *Alloprevotella*, *Clostridium* XIVa, and *Alistipes* W8: ↑ Actinobacteria, Erysipelotrichia	No changes in body weight Evaluated only at W3 and in FMT mice: Perturbed intestinal development, disrupted intestinal barrier and low-grade inflammation (no signs of microscopic inflammation) Increased colitis severity Overall conclusion: Maternal exposure could induce microbial dysbiosis and promote colitis susceptibility in adulthood.	(Jin *et al.*, 2021)
In vitro batch fermentation Non-pooled faecal material from 10 human individuals (eight omnivores, one vegetarian, one vegan)	0.005, 0.05, 0.5% (m/v) CMC, P80, soy lecithin, sophorolipids and rhamnolipids 48-h	16S rRNA (V4) gene sequencing SCFA flagellin	Donor-, emulsifier- and dose-dependent effects: sophorolipids, rhamnolipids > soy lecithin. CMC, P80: No/limited effects ↓ Diversity, *Faecalibacterium, Prevotella* ↑ *Escherichia/Shigella, Bacteroides* Changes in SCFA profiles No changes in flagellin levels	n/a	(Miclotte *et al.*, 2020)

Notes: GF: germ-free; WT: wild-type; ASF: Altered Schaedler Flora (ASF); FMT: Faecal microbiota transplant.

Sources: See References

ANNEX III.11. SUMMARY TABLES – MONOGLYCEROL OF FATTY ACIDS

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6 mice Male, age not specified. (n=10/group)	150 mg/kg glycerol monolaurate (GML) in food, 8 weeks [calculated as 22–26 mg/kg bw/day] Of note: animals in treatment group consumed more food than control	16S rRNA (V4) gene sequencing (faeces)	Altered β-diversity, no change in α-diversity ↓ Verrucomicrobia, *Akkermansia muciniphila*, *Lupinus luteus** (plant species reported as microbiome member) ↑ *Roseburia, Turicibacter, Escherichia coli* and *Bradyrhyzobium* ten pathways enriched, mostly amino acids and lipid/fatty acid metabolism Authors refer to these changes as dysbiosis	Metabolic syndrome and low-grade inflammation (based on authors' interpretation of limited significant findings/parameters)	(Jiang *et al.*, 2018)
C57BL/6 mice Male, 6-weeks old (n=15/group)	150, 300, 450 mg/kg glycerol monolaurate in HFD, 10 weeks [doses calculated approx. as 22, 44, 66 mg/kg bw/day, respectively] Two controls fed HFD or standard rodent chow	16S rRNA (V4) gene sequencing (faeces) Circulating LPS	β-diversity in treatment groups differed from controls, no change in α-diversity GML improved HFD-induced changes: Dose-response effect (higher at 450 mg GML/kg) ↑ Verrucomicrobia, *Akkermansia, Bifidobacterium, Lactobacillus, Bacteroides uniformis* ↓ *Lactococcus, Flexispira, Escherichia coli*	Dose-response amelioration of HFD-induced metabolic changes (higher at 450 mg GML/kg): e.g. improvement of lipid metabolism, reduced serum LPS and TNF-α mid and high dose GML groups showed significantly reduced intestinal effects due to HFD	(Zhao *et al.*, 2019)
C57BL/6 mice Male, 4–5-weeks old (n=6–10/group)	400, 800, 1 200 mg/kg glycerol monolaurate (GML) in standard feed, 4 months [theoretical estimated exposure 60, 120, 180 mg/kg bw/day; not accounted for current feed intake, or body weight]	16S rRNA (V3–V4) gene sequencing (faeces) Faecal SCFAs	Dose-response effects. Authors identified changes as promotion of beneficial microbiota taxa. β-diversity in treatment groups differed from control. ↑ α-diversity (600 and 1 200 mg/kg) **All groups:** ↓ *Tenericutes* (Anaerosplasmataceae), *Anaeroplasma*, Desulfovibrionaceae ↓ faecal acetic acid **1 200 mg/kg:** ↑ Proteobacteria (mainly Sutterellaceae), *Clostridium* XIVa, *Oscillibacter* **800 and 1200 mg/kg:** ↓ Baceroidaceae, Erysipelotrichaceae 400 and 800 mg/kg: ↑ Porphyromonadaceae, *Barnesiella* ↓ Total faecal SCFA	No physiological changes (absence of systemic inflammation and dysfunction of glucose and lipid metabolism) (↑ circulatory TGF-β1 and IL-22 in 1 600 mg/kg group)	(Mo *et al.*, 2019)
C57BL/6J mice Male, 6-weeks old (n=15/group)	1 600 mg/kg glycerol monolaurate (GML) in HFD, 16 weeks [theoretical estimated exposure 240 mg/kg bw/day] Controls: LFD or HFD 2nd experiment with antibiotics: HFD and HFD-GLM groups given an antibiotic cocktail (1g/L each metronidazole, ampicillin, neomycin, and 0.5 g/L vancomycin) in the drinking water for the 16 weeks	16S rRNA (V3–V4) gene sequencing (faeces)	β-diversity differed between all groups. GML microbiota more similar to LFD control than HFD Compared to HFD control, GML: ↑ α-diversity, Verrucomicrobia, *Bifidobacterium, Allobaculum, Streptococcus* ↓ *Dorea, Bacteroides, Eggerthella, Parabacteroides* *Bifidobacterium pseudolongum* showed in correlations between microbiota, hepatic transcriptomics and serum metabolomics. After antibiotic treatment: No differences between HFD and HFD-GML groups	GML prevented the development of features of obesity (weight gain, adiposity, endotoxemia, inflammation, altered lipid metabolism and glucose homeostasis)	(Zhao *et al.*, 2020)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6 mice Male, 5-weeks old (n=15/group)	1 600 mg/kg glycerol monolaurate (GML) in HFD, 16 weeks (theoretical estimated exposure 240 mg/kg bw/day) (Other treatment groups: 1 169 mg/kg lauric acid [LA], 1 243 mg/kg lauric triglyceride [GTL]) Controls: LFD or HFD	16S rRNA (V3–V4) gene sequencing (faeces)	Compared to HFD control, GML: ↓ α-diversity β-diversity: diet-dependent clustering Compared to HFD control, all treatment groups: ↓ Proteobacteria, *Desulfovibrio, Oscillospira, Turicibacter, Mucispirillum*, AF12 and *Parabacteroides* ↑ *Allobaculum, Bifidobacterium, Bacteroides, Streptococcus, Ruminococcus, Lactococcus, Sutterella*	GML prevented the development of features of obesity: hyperlipidemia, alterations of glucose homeostasis and systemic inflammation	(Zhao *et al.*, 2022)
C57BL/6 mice Male, 4–5 weeks-old (n=12/group)	150, 1 600 mg/kg glycerol monocaprylate (GMC) in standard feed, 4 months 22 weeks	16S rRNA (V3–V4) gene sequencing (faeces) Faecal SCFA	↑ α-diversity, Treatments differed from control in β-diversity. Dose-dependent microbiota composition: **Low dose:** ↑ Firmicutes, Lactobacillaceae, Bacilli ↓ S24-7 ↑ total SCFAs, acetic acid, propionic acid **High dose:** ↑ Clostridiales, Lachnospiraceae, *Ruminococcus, Turicibacter, Prevotella* ↓ Erysipelotrichaceae ↑ propionic acid, isobutyric acid, isovaleric acid	Limited or no metabolic and inflammatory alterations	(Zhang, Feng and Zhao, 2021)
In vitro Pooled human faecal microbiota from 12 healthy individuals Culture media: brain heart infusion broth and chemically-defined medium	0.025% each emulsifier: glycerol monoacetate, glycerol monostearate, glycerol monooleate, propylene glycol monostearate, or sodium stearoyl lactylate (SSL)	16S rRNA (V3–V4) gene sequencing SCFA	sodium stearoyl lactylate: Similar microbiota effects in both culture media: ↓ α-diversity, butyrate-producing Clostridia: Clostridiaceae, Ruminococcaceae and Lachnospiraceae, e.g. *Dorea, Anaerostipes, Faecalibacterium, Coprococcus, Flavonifractor* and *Pseudoflavonifractor. Bifidobacterium.* ↑ Bacteroidaceae (*Bacteroides*), Enterobacteriaceae (*Escherichia*), *Desulfovibrio* ↓ Butyrate, ↑ Propionate ↑ LPS, Flagellin (pro-inflammatory potential of the microbiota) Other emulsifiers (limited evaluation): media dependent microbiota changes	n/a	(Elmén *et al.*, 2020)

Notes: GML: glycerol monolaureate; GMC: glycerol monocaprylate; HFD: high-fat diet; LFD: low-fat diet.

* Authors reported *Lupinus luteus* as idenfied gut microbiome member. However, *L. luteus* is a plant species (yellow lupin).

Sources: See References

ANNEX III.12. SUMMARY TABLES – CARRAGEENAN

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
In vitro: batch culture with human faecal microbiota from 8 healthy individuals —- *In vivo*: GF Kunming mice 3-weeks-old male and female (+8 week to complete colonization before treatment) (n=6/group)	*In vitro* (volume of culture medium not specified) 48h: κ-carrageenan polysaccharide (KCP, 450 kDa): 1 g mild-acid-degraded κ-carrageenan (SKCO, 100 kDa): 5 g —- κ-carrageenan oligosaccharide (KCO, 4.5 kDa): 8 g —— *In vivo* experiment (4 groups): 5% KCO 5% KCO with KCO-degrading bacteria. Degrading bacteria Control	PCR-DGGE (16S rRNA V3 gene) Faecal SCFA	*In vitro:* KCP and SKCO not degraded by human faecal microbiota *Bacteroides xylanisolvens* primary KCO-degrader. *Escherichia coli:* cross-feeder. ↑ butyrate and propionate —- *In vivo:* higher degradation of KCO in large intestine.	No histological damage in small intestine Inflammatory response in colon, higher in rectum in all treatment groups but stronger in group treated with KCO+bacteria (*B. xylanisolvens, E. coli*)	(Yin *et al.*, 2021)
C57BL/6J 6-weeks old (n=6/group)	3 treatment groups: 20 mg/L κ-, ι- or λ-carrageenan in drinking water, 6 weeks	16S rRNA (V3–V4) gene sequencing (colonic content)	Richness and α-diversity estimators: ↑ λ- and ι- carrageenan (richness not altered in λ-carrageenan), ↓ κ-form All carrageenan groups: ↓ Bacteroidetes, Verrucomicrobia (*A. muciniphila*) ↑ Firmicutes. κ-carrageenan: ↑ Helicobacteraceae, Lactobacillaceae, Clostridiales, Peptococcaceae, Bacteroidales S24-7, Bacteroidaceae. λ- and ι- carrageenan: ↑ *Alistipes*, Lachnospiraceae ↓ Helicobacteraceae	All carrageenans: Colitis (negatively correlated with ↓ *Akkermansia muciniphila* Positively correlated with ↑ Tenericutes and Firmicutes)	(Shang *et al.*, 2017)
C57BL/6J 4-weeks old n=7 (HFD) n=9 (LFD)	Study using native κ-CGN: HFD+5% κ-CGN in feed HFD+0.5% κ-CGN in water LFD+5% κ-CGN in feed LFD+0.5% κ-CGN in water 6-week treatment	16S rRNA (V3–V4) gene sequencing (faeces)	Microbiota shifts (β-diversity) HFD+0.5% κ-CGN in water: ↑ *Bacteroides acidifaciens*, *Alistipes finegoldii* and *Burkholderiales bacterium* LFD+0.5% κ-CGN in water: ↑ *Akkermansia muciniphila*	Colitis (in mice given HFD+0.5% κ-CGN in water)	(Mi *et al.*, 2020)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6 (conventional and germ-free) Male, 6-weeks old (n=8/group)	**Experiment 1** (conventional mice): 1.7, 8.3, 41.7 mg/kg λ-carrageenan gavage, 90 days + 7 days washout. Half of animals infected with 109 CFU *Citrobacter rodentium* (effects observed 7 days later) **Experiment 2** (germ-free mice). Same as experiment 1 but using high dose λ-carrageenan only **Experiment 3** (FMT from control and high dose λ-carrageenan groups from experiment 1 in germ-free animals). Same periods as above. Half of animals infected too	16S rRNA (V3–V4) gene sequencing (faeces) Faecal LPS Faecal SCFA	Samples from high dose λ-CGN and control groups experiments 1 and 3: Microbiota in λ-CGN groups different from controls: α-diversity ↑ Proteobacteria, *Akkermansia*, *Bacteroides fragilis*, *Ruminococcus gnavus*, *Desulfovibrio*, *Anaerotrucus*, *Bilophila wadsworthia, Clostridium Leptum* ↓ Firmicutes, Verrucomicrobia, *Bacteroides thetaiotaomicron*, *Faecalibacterium*, *Bifidobacterium*, *Blautia*, *Roseburia* ↑ faecal LPS ↓ faecal SCFA	λ-CGN increased severity of *Citrobacter rodentium*-induced colitis in conventional mice (reproduced in transplanted GF mice). Mucus layer thinning and reduced distance between bacteria and epithelium. λ-CGN treatment in GF mice did not differ from control. No effects in non-infected mice.	(Wu *et al.*, 2021)
C57BL/6 (conventional and germ-free) Male, 6-weeks old (n=8/group)	**Experiment 1** (conventional mice): 1.7, 8.3, 41.7 mg/kg κ-CGN gavage, 90 days + 7 days washout. Half of animals infected with 109 CFU *Citrobacter rodentium* (effects observed 7 days later) **Experiment 2** (FMT from control and κ-CGN groups from experiment 1 in germ-free animals). Same periods as above. Half of animals infected too	16S rRNA (V3–V4) gene sequencing (faeces) Metagenomics Faecal SCFA	Dose-response κ-CGN effects: High-dose (conventional and transplanted mice) ↑ richness, Bacteroidetes, Ruminococcaceae_unclassified and *Bacteroides* ↓ Proteobacteria, *Akkermansia*, *Bifidobacterium*, Lachnospiraceae, *Faecalibacterium*, *Mucispirillium*, [*Ruminococcus*]_torques_group, *Ruminiclostridium*_5. Gut microbiota partially recovered after supplementation with probiotics treatment (*Bifidobacterium longum* and *Faecalibacterium prausnitzii*) ↓ faecal SCFA, especially butyric and valeric acids	No significant inflammatory symptoms (in the absence of *Citrobacter rodentium*). High dose κ-CGN effects: ↑ severity of *Citrobacter rodentium*-induced colitis in conventional mice (reproduced in transplanted GF mice) Mucus layer thinning and reduced distance between bacteria and epithelium. ↑ genes: mucosal polysaccharide binding proteins, mucin degrading enzymes. Colitis and gut barrier function partially recovered with probiotics supplementation (*Bifidobacterium longum* and *Faecalibacterium prausnitzii*)	(Wu *et al.*, 2022)
In vitro Fermenter (inoculated with pooled human faecal microbiota from four healthy individuals) followed by incubation of supernantants in HT29 cell cultures	Fermenter: 1% w/v degraded κ-CGN for 3 (KO3) or 6h (KO6) 0, 6 , 12, 24, 48, 72 h (1 fermentation vessel per time and per group) HT29 cell culture: Fermenter filtered supernatants (50, 100 and 200 µl/ml) for 24 h	16S rRNA (V3–V4) gene sequencing SCFA production	KO3 and KO6: ↑ Prevotellaceae, Veillonellaceae, Bifidobacteriaceae, *Prevotella*, *Megamonas*, *Bifidobacterium* ↓ Enterobacteriaceae, Desulfovibrionaceae, Ruminococcaceae, Lachnospiraceae, Bacteroidaceae, Porphyromonadaceae, *Parabacteroides, Escherichia-Shigella, Desulfovibrio* KO3: ↑ *Streptococcus Lactobacillus* KO6: ↑ *Megaspharea*	HT29 cell culture (both treatments): No toxic effect Proinflammatory effect of κ-CGN oligosaccharides: ↑ IL-1β and TNF-α, sIgA and mucin 2	(Sun *et al.*, 2019)

Note: CGN: Carrageenan.

Sources: See References

ANNEX III.13. SUMMARY TABLES – XANTHAM GUM, MALTODEXTRIN, LECITHIN

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Mouse, male (age and strain not specified) (n=10/group)	Xanthan gum (XG) or low molecular weight XG (LMW-XG): 0.1 mg daily gavage 28 days	16S rRNA (V3–V4) gene sequencing (caecal content) Caecal SCFAs	LMW-XG: ↑ α-diversity, Firmicutes ↓ Bacteroidetes XG and specially LMW-XG: ↑ Total SCFA, acetate, propionate and butyrate	(caco-2 cell culture: no toxicity) ↑ body weight No other host parameters were evaluated	(Sun *et al.*, 2022)

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Balb/c (6–7-weeks old, gender not specified) (n=6-12/group)	1, 3 or 5% maltodextrin (MDX), 5% propylene glycol, or 5 g/L animal gelatin In drinking 45 days (Induction of colitis with DSS or indomethacine) – 5% MDX for 10 weeks	16S rRNA (V4) gene sequencing (colon biopsy: mucosa-associated microbiota)	No effect on mucosal-associated microbiota	5% MDX-35 days: Exacerbation of intestinal inflammation in colitis model 5% MDX-10 weeks: low-grade inflammation and high fasting blood glucose levels (healthy animals)	(Laudisi *et al.*, 2019)
Swiss mice 4-weeks old, males (n=12/group)	10% soybean lecithin (97 mg/kg bw/day) 1, 3 or 10% rapeseed lecithin (10, 29 or 97 mg/kg bw/day, respectively) 5 days in feed, following by one time gavage of same lecithin concentrations (3, 10 or 33 mg/kg bw/day)	Real time-PCR (primer specific for Bacteroidetes, Firmicutes, Bifidobacteria, *Escherichia coli, Akkermasia muciniphila, Clostridium coccoides, Clostridium leptum* group, Lactic acid bacteria and *Faecalibacterium prausnitzii.* (faeces)	Both lecithin and all doses: ↑ *Clostridium leptum*	No changes in hepatic lipid metabolism or related gene expression. High dose rapeseed lecithin: ↑ postprandial abundance α-linolenic acid, beneficial changes in bile acid profile	(Robert *et al.*, 2021)
In vitro batch fermentation Non-pooled faecal material from ten human individuals (eight omnivores, one vegetarian, one vegan)	soy lecithin, sophorolipids and rhamnolipids	Described in CMC/P80 tables			(Miclotte *et al.*, 2020)

MDX: maltodextrin; DSS: dextran sodium sulfate

Sources: See References

ANNEX III.14. SUMMARY TABLES – PRESERVATIVES

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Bacteria culture (source: stools from three healthy adult individuals)	1 µg to 100 mg/ml sodium benzoate, sodium nitrite, potassium sorbate and their combination. 6–10 h for aerobic strains 2–3 days for anaerobic strains	Bacteria culture (*Escherichia coli, Enterococcus faecalis, Lactobacillus paracasei, Bifidobacterium longun, Bacteroides coprocola, Helicobacter hepaticus, Bacteroides thethaiotaomicron* and *Clostridium tyrobutyricum*) and serial dilutions (dose–response curves)	Different susceptibilities (IC50): Most sensitive: *Bacteroides coprocola* Most resistant: *Enterococcus faecalis* Sodium nitrite and its combinations: most potent of all tested preservatives	n/a	(Hrncirova *et al.*, 2019)
C57BL/6J mice Male, 5-weeks old (n=8/group=	0.1% benzoic acid (BA), 0.3% potassium sorbate (PS), 0.05% sodium nitrite (SN) (0.019, 0.049, and 0.007 mg/kg bw, respectively) in feed 12 weeks	16S rRNA (V4) gene sequencing (faeces)	No gut dysbiosis, no negative effects on beneficial bacteria. PS > BA: ↓ α-diversity: Distinct microbial signatures (β-diversity, taxonomical composition) All preservatives: ↓ Proteobacteria, Erysipelotrichae, *Sarcina* and ↑ Actinobacteria, *Lactobacillus* and *Blautia* BA: ↑ *Bacteroides*, ↓ *Ruminococcus* SN: ↑ Verrucomicrobia, *Turicibacter* and *Akkermansia*	↓ tight-junctions gene expression (ileal samples)	(Nagpal, Indugu and Singh, 2021)

Sources: See References

ANNEX III.15. SUMMARY TABLES – CURDLAN

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Experiment 1 C57BL/6 mice Female, 10–14-weeks old (n=5–10/group) **Experiment 2** *In vitro*: i-Screen platform (with ileal efflux medium) (pooled faecal microbiota from 6 adult humans)	**Experiment 1** 1 mg/day curdlan, gavage, 14 days Followed by colitis induction with DSS for 7 days + 2-day clearance. **Experiment 2** 1, 2 or 4 mg/ml curdlan 24 h fermentation	16S rRNA (V3–V4) gene sequencing ITS1 gene sequencing (colonic content)	**Experiment 1** Healthy mice: no differences in α- or β-diversity In colitis model: diversity changes driven by DSS, slightly improved by curdlan (bacteriome only). Curdlan decreased fungal richness. Curdlan (independent from DSS treatment): ↑ *Bifidobacterium* (*B. choerinum*), Lachnospiraceae; ↓ *Blautia* **Experiment 2** Dose–response effect: ↑ α-diversity, clustering β-diversity ↑ *Bifidobacterium* (different species from those found in the mouse colon), *Blautia*, Lachnospiraceae; ↓ *Bacteroides*	Curdlan: Improved some signs of inflammation in colitis model (ulceration and crypt loss). Modulation of macrophage innate immune response. [many null effects not considered in discussion]	(Rahman *et al.*, 2021)

Sources: See References

ANNEX III.16. SUMMARY TABLES – COLOURS

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
R23FR mice (C57BL/6 background) FR, *IL-22*$^{-/-}$, CD45.1, *Rag1*$^{-/-}$, *Ifng*$^{-/-}$, germ-free *Rag1*$^{-/-}$, germ-free R23FR, *Ifng*$^{-/-}$ *Rag1*$^{-/-}$	Allura Red AC (Red 40, E-129) Erythrosine (Red 3, E-127) Sunset yellow FCF (Yellow 6, E-110) Brilliant Blue FCF (Blue 1, E-133) 0.025% w/v in drinking water, or 0.25 g/kg in rodent feed (Allura Red only) 3 weeks with 7-day clearance in between treatment weeks	16S rRNA gene sequencing (faeces)	*Bacteroides ovatus* and *Enterococcus faecalis* reduce Allura Red and Sunset yellow	Colitis in mice overexpressing IL-23 (induced by ANSA-Na, product of microbial reduction of Allura Red and Sunset Yellow)	(He *et al.*, 2021)

Note: ANSA-Na: metabolite 1-amino-2-naphthol-6-sulfonate sodium salt.

Sources: See References

ANNEX III.17. SUMMARY TABLES – TITANIUM DIOXIDE

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
In vitro Colon bioreactor model Inoculated with faecal microbiota from one vegetarian female.	~03-07 mg/kg bw/day food-(Ø122±48 nm) or industrial (Ø21 nm)-grade (P25) TiO_2 5 days	16S rRNA (V1–V2) gene sequencing	Food-grade TiO_2: Unlike control, Proteobacteria remain the most abundant phyla throughout the study.	n/a	(Waller, Chen and Walker, 2017)
In vitro chemostat bioreactor inoculated with standardized stool-derived microbial ecosystem therapeutic (MET-1) (n=1, no replicate simulations)	100 and 250 ppm food-grade TiO_2 from two different vendors: E171-1 (17% nanoparticles) and E171-6a (21% nanoparticles) 48h incubation	16S rRNA gene sequencing	No/limited effects. E171-1-High dose: ↓ *Bacteroides ovatus*	n/a	(Dudefoi *et al.*, 2017)
Sprague-Dawley rats Male, 3-weeks old (n=6/group)	2, 10 and 50 mg/kg bw/day TiO_2 NP (Ø29±9 nm), gavage 30 days	16S rRNA (V3-V5) gene sequencing (faeces) Fecal SCFA	No changes in total observed species, α- and β-diversities. High dose (Days 14 & 28): ↑ *Lactobacillus gasseri* (this information from text does not match figures: High dose does not affect *L. gasseri*) No clear trends for the same dose between days 14 and 28. No changes in SCFA	High dose: Alteration colonic epithelium. Oxidative stress and proinflammatory activity	(Chen *et al.*, 2019a)
Sprague-Dawley rats Male, 3-weeks old (n=6/group)	2, 10 and 50 mg/kg bw/day TiO_2 NP (Ø29±9 nm), gavage 90 days	16S rRNA (V3-V5) gene sequencing (faeces) Fecal SCFA, LPS	↑ diversity Phylum abundance not affected but ↓ ratio Firmicutes:Bacteroidetes Medium dose: ↑ *Lactobacillus reuteri* and ↓ *Romboutsia* ↑ Faecal LPS and no changes in SCFA	Slight hepatotoxicity, Altered hepatic metabolome (energy and oxidative metabolism) Oxidative stress Proinflammatory response	(Chen *et al.*, 2019b)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6J mice Female, 3-weeks old (n=5–6/group)	0.1% TiO_2: Three groups based on particle size: 10, 50 and 100 nm) in standard rodent chow. 3 months	16S rRNA (V3–V4) gene sequencing (faeces)	Limited changes (α-diversity and total bacteria abundance not affected) 10 and 50 nm TiO_2: ↑ Bacteroidetes ↓ Actinobacteria, *Bifidobacterium, Lactobacillus, L. jonsonii*	Unaltered inflammation biomarker (lipocalin-2) Aggravation of DSS-induced chronic inflammation. Potential to develop low-grade intestinal inflammation and immune imbalance	(Mu *et al.*, 2019)
Sprague-Dawley rats Pregnant females, 12-weeks old (n=4/group)	5 mg/kg bw/day TiO_2 NP (Ø ~21 nm), gavage (suspended in 0.5% methylcellulose) 12 days: gestation days (GD): 5–18	16S rRNA (V3–V4) gene sequencing Faecal microbiota checked at day 0 GD 10 (mid-term pregnancy) and GD 17	No changes in α-diversity Mid-term pregnancy: ↓ Clostridiales End pregnancy (GD 17): ↓ Dehalobacteriaceae	Fasting glucose slightly increased during pregnancy (significant in treatment group at mid-term), not sufficient to induce gestational diabetes. (However, authors also conclude that it may result in adverse effects in pregnant females and offspring, e.g. risk of obesity and abnormal glucose tolerance)	(Mao *et al.*, 2019)
C57BL/6J mice Male, 7-weeks old n=15/group (different tests used different sample sizes	150 mg/kg (*not clear whether per kg food or per kg bw/day*) TiO_2 NP (Ø 21 nm), gavage 30 days	16S rRNA (V3) gene sequencing (faeces)	↓ α-diversity and differences (clustering) in β-diversity Shifts in microbiota composition	No changes in body weight No pathological alterations or inflammation in brain and small intestine. No effects in learning and memory activities. Abnormal locomotor activity (open field test)	(Zhang *et al.*, 2020)
C57BL/6J mice Pregnant females, 8 week-old n=10 dams Offspring number in study not specified (variable mouse number per test	Dams: 150 mg/kg TiO_2 NP (Ø 21 nm), gavage between GD 8 and 21 Offspring: not fed with TiO_2 NP	16S rRNA (V3) gene sequencing (faeces)	No changes in diversity Mothers: no effects (GD21) Offspring: effects only at PD49: ↓ Bacteroidota (or Bacteroidetes), Cyanobacteria ↑ Campylobacterota	Mothers: no effects Offspring (PD49): Neurobehavioral impairments, pathological alterations in intestine and cerebral cortex. ↑ intestinal immune response	(Su *et al.*, 2021)
C57BL/6J mice Pregnant females, 8 week-old n=10 dams	Dams: 150 mg/kg TiO_2 NP (Ø 21 nm), gavage between GD 8 and 21	16S rRNA (V3) gene sequencing (faeces)	α-diversity not altered β-diversity – groups clustered differently ↓ Verrucomicrobiota, Desulfobacterota Altered: Bacilli, Clostridia, Verrucomicrobiae, α-Proteobacteria	Persistent neurobehavioral impairments. Brain alterations (integrity of hippocampus and cerebral cortex, neurobehavioral impairment) Altered gut-brain axis (↓ enteric neuronal receptors, expression gut-derived neurotransmitters and gut-brain peptides) Small intestinal alterations (integrity and barrier function, ↓ digestive enzymes)	(Yang *et al.*, 2022b)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6 mice Male, 8-weeks old n=28/group (of which 10 for microbiota studies) MFT: antibiotic-treated C57BL/6 (n=15 donors and 12 recipients/group)	1 mg/kg bw/day Anatase TiO_2 NP (Ø 25, 50 or 80 nm), gavage 7 days	16S rRNA (V4) gene sequencing (faeces)	25 nm TiO_2: Distinct microbiota differences in distal colon compared to control ↓ *Bifidobacterium* (remains reduced after MFT), *Dorea*, *Sutterella*, *Rikenella* Inulin supplementation avoids *Bifidobacterium* reduction	25 nm TiO_2: Disruption of the intestinal barrier (mucus layer, expression levels of tight junction biomarkers) MFT: ↓ thickness mucus layer Inulin supplementation prevents intestinal epithelial damage	(Li *et al.*, 2019)
C57BL/6J mice Male, 6 week-old n=not specified (n varied with test)	Food grade TiO_2 (Ø 202 nm): 2, 10 or 50 mg/kg bw/day in drinking water 3 weeks	16S rRNA (V3–V4) gene sequencing (faeces, small intestine content) SCFA, TMA (serum)	**Faecal microbiota:** No changes in α-diversity. β-diversity: clustering of treatment groups Dose–response effect in microbiota composition: All doses: ↑ *Lactobacillus*, *Allobaculum* Mid- and high doses: ↓ *Adlercreutzia* and Unclassified Clostridiaceae Highest dose: ↑ *Parabacteroides* **Small intestine:** No changes in diversity and composition Highest dose: ↓ SCFA, ↓ choline, ↑ TMA (also mid-dose) In vitro biofilm formation (mid-high-dose)	Dose-dependent alterations Epithelial function: ↓ *Muc2* gene expression. ↑ expression β-defensin (other antimicrobial peptides not affected) No change in expression of tight junction markers Colonic immune and inflammatory response: ↑ macrophages, CD8+ T cells, T17 cells, expression pro-inflammatory cytokines (IL-17A, IL-5, TNF-α)	(Pinget *et al.*, 2019)
C57BL/6 mice Male, 6 week-old n=15 (different number of mice used for different experiments; n=5 in microbiota studies)	0.1% (~150 mg/kg bw/day) food-grade TiO_2 (Ø E171, 112 ± 34 nm, 44% < 100 nm) or TiO_2 NP (33 ± 14 nm, 100% < 100 nm) in chow (LFD or HFD) 8 weeks	16S rRNA (V3–V4) gene sequencing (faeces) Caecal SCFA	Most alterations due to HFD TiO_2 NP: ↓ *Bifidobacterium*, *Allobaculum* (LFD) Synergistic effect TiO_2 NP and HFD ↓ SCFA: Butyric (all treatment groups), Acetic (E171, both diets); valeric and isovaleric (LFD)	TiO_2 NP induced intestinal inflammatory response. Effects aggravated by HFD. Effects reproduced after MFT	(Cao *et al.*, 2020)
Kunming mice Male, 5-weeks old (n=9-10/group)	20 mg/kg bw/day TiO_2 NP (Ø 25.2 nm) by gavage + 30% fructose in drinking water Other groups: control, 30% fructose 8 weeks	16S rRNA (V3–V4) gene sequencing (faeces)	Most effects due to fructose only. TiO_2 NPs augmented specific fructose-induced alterations: ↑ Firmicutes, Proteobacteria, Desulfovibrionaceae, Clostridia ↓ Bacteroidetes ↑ serum and faecal LPS	TiO_2 NPs augmented specific fructose-induced alterations: Hepatic pro-inflammatory alterations and oxidative stress. Colonic barrier damage and pro-inflammatory activity. Some hepatic and colonic alterations reproduced after FMT from TiO_2+fructose group in antibiotic-treated mice	(Zhao *et al.*, 2021)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
ICR mice Freshly weaned, male (n=22) FTM: normal male ICR mice (n=8/group)	Food-grade micro (Ø 0.25 µm) or nano (Ø 20 nm) TiO_2: 10 or 40 mg/kg bw/day Oral gavage, 28 days (Groups of six mice sacrificed at days 8, 15 or 29) FMT (enema) from 40 mg/kg bw donor groups. Microbiota tested at days 4, 7, 14, 28	16S rRNA (V3–V4) gene sequencing (faeces)	β-diversity clustered by treatment Microbiota composition: disturbances that fluctuate over time	Effects seem dependent on the dose- and particle size (more evident in smaller particles) Disruption of mucosa structure and barrier integrity, with signs of inflammatory activity. ↑ faecal and serum LPS. Similar finding observed in transplanted mice with faecal material (enemas) from treated donors. Alterations of several gut and host co-metabolites related to energy and fat metabolic pathways. No TiO_2 accumulation in intestinal cells	(Yan *et al.*, 2022)
$APOE^{-/-}$ C57BL/6J mice Female, 5-weeks old (n=6/group)	40 mg/kg bw/day food-grade TiO_2 (E171, Ø 120 ± 47 nm, ~36% < 100 nm) gavage Diet: normal chow (NCD) or 1% choline-supplemented western diet (HCD) 4 months	16S rRNA (V4) gene sequencing (faeces)	α-diversity: ↑ (Shannon index) in E171+NCD β-diversity showed clustering by treatment E171+HCD: ↑ Firmicutes, *Clostridium* XIVa, *Eubacterium*, *Prevotella*, Lachnospiraceae	E171+ HCD: Promoted the development and aggravation of atherosclerosis lesions induced by the HCD diet. ↑ TMAO, TMA (TMAO precursors) and TMA lyases	(Zhu *et al.*, 2022)
C57BL/6J mice Male, 3-weeks old (n=6/group)	100 mg/kg bw/day TiO_2 NP (Ø 10–30 nm) alone or combined with 5 or 50 mg/kg bw/day bisphenol A (BPA) (these BPA doses also tested alone without TiO_2) Gavage, 13 weeks	16S rRNA (V3–V4) gene sequencing (faeces) Caecal SCFA Faecal metabolome	TiO_2 groups: ↓ α-diversity TiO_2: ↑ Bacteroidetes, ↓ Firmicutes, but reverted by BPA in a dose-dependent manner. Altered genera: TM7, *Lactobacillus*, *Oscillospira*, *Odoribacter*. TiO_2 groups: ↓ Total SCFA (propionic and butyric acids)	Combined exposure: Altered faecal metabolome (amino acid, carbohydrate and purine metabolism)	(Yang *et al.*, 2022a)
Sprague-Dawley rats Male and female (n=10/group)	(a) 10, 100 or 1 000 mg/kg bw/day TiO_2 NP (Ø 40.9 ± 9.6 nm), gavage, 90 days (b) 1 000 mg/kg bw/day TiO_2 NP, gavage, 45 days (mid-term) (c) 1 000 mg/kg bw/day TiO_2 NP, gavage, 90 days (mid-term) + 28-day recovery	16S rRNA (V4) gene sequencing (faeces)	High dose (1 000 mg/kg bw/day): No changes in α- and β-diversities Microbiota composition: No changes at phylum level Males: ↑ *Bacteroides*, *Eubacterium* Females: ↑ *Oscillibacter*	No adverse effects (numerous parameters evaluated for multi-organic function and histology)	(Lin *et al.*, 2023)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
In vitro human gut simulator (HGS) Inoculated with distal colonic microbiota from three healthy male humans (two replicates)	100 mg/day TiO_2 NP (Ø ~25 nm) or Ag NP (Ø ~30–50 nm) 7 days + 7-day clearance	16S rRNA (V4) gene sequencing SCFA	↓ microbial density (lower with Ag NP), recovered in clearance period (slower in Ag NP group) TiO_2: limited direct impact on the microbiota (diversity, composition, functional metagenome, SCFA production)	n/a	(Agans *et al.*, 2019)
C57BL/6J Male, female, 5-10 weeks old (n=10/group)	1% TiO_2 NP (Ø ~26 nm) ~2 000 mg/kg bw/day, in feed, 28 days 0.2% Ag NP (Ø ~40 nm, PVP-stabilized) ~400 mg/kg bw/day, in feed, 28 days 1% SiO_2 NP (Ø ~13 nm) ~2 000 mg/kg bw/day, in feed, 21 days 1% SiO_2 NP (Ø ~35 nm) ~2 000 mg/kg bw/day, in feed, 21 days	16S rRNA (V4) gene sequencing (faeces)	No major effects on gut microbiome No changes in α- or β-diversities SiO_2 NP: ↓ Actinobacteria Gender dependencies: Ag NP: ↑ *Roseburia* (females), Tenericutes (males)	Absence of macroscopic pathologies	(Bredeck *et al.*, 2021)

Note: Ø: average diameter.

Sources: See References

ANNEX III.18. SUMMARY TABLES – SILVER

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
Wistar rats Male, female, 4-weeks old (n=6–10/group)	Ag NP (Ø ~14 nm, PVP-stabilized): 2.25, 4.5, 9.0 mg/kg bw/day Ag acetate: 14 mg/kg bw/day Gavage, 28 days	qPCR: Firmicutes, Bacteroidetes Silver-resistance genes (*sil*RS, *sil*CBA, *sil*E, *sil*P) (caecal content)	No alterations (Firmicutes, Bacteroidetes) No effect in the expression levels of silver resistance genes	No pathological changes Sporadic differences in haematological and plasma biochemistry parameters Ag acetate: ↑ plasma ALP, ↓ urea in plasma, body weight, thymus weigh	(Hadrup *et al.*, 2012)
Sprague-Dawley rats Male, female, 7-weeks old (n=10/sex/dose)	Ag NP (Ø 10, 75, 110 nm, citrate-stabilized): 9, 18, 36 mg/kg bw/day Ag acetate: 100, 200, 400 mg/kg bw/day Gavage, 13 weeks	Real-time PCR (Firmicutes, Bacteroidetes, *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, Enterobacteriaceae) (Ileal content)	Ileal *Lactobacillus* culture: increased antimicrobial activity with decreasing Ag NP size No change in total bacteria (CFU) Size, dose and gender-dependent alterations at phylum and genus levels. Ag acetate: Loss of *Bifidobacterium* and ↑ Enterobacteria family (size and dose-dependent, with stronger effect in females)	Ag acetate: rats moribund (high-dose) or had severe gastroenteritis (mid-dose) Ag NP: ↓ expression of markers related to gut functional immunity. Effects: dose- and size-dependent (especially low dose and smaller particle size), and gender differences. No or little effect on gene expression by Ag acetate and high dose Ag NP. Potential health effects of observed changes would require further investigation	(Williams *et al.*, 2015)

continues

MODEL	TREATMENT	MB METHODS	MB	HOST	REFERENCES
C57BL/6NCrl mice Male, 10–12 weeks old (n=6/group)	10 mg/kg bw/day Ag NP (Ø 20 or 110 nm, PVP-stabilized) 10 mg/kg bw/day Ag NP (Ø 20 or 110 nm, citrate-stabilized) Gavage, 28 days Two controls: no test compound and Ag ions (Ag acetate)	16S rRNA (V3-V5) gene sequencing (caecal content)	No effects	Host not evaluated	(Wilding *et al.*, 2016)
C57BL/6 mice Female, age not specified. (n=5/group)	Ag NP (Ø ~55 nm, PVP-stabilized): ~0.009, 0.071 or 0.679 mg/kg bw/day (based on food consumption) in feed, 28 days	16S rRNA (V4) gene sequencing (pool from ileal, caecal, colonic content)	Dose-dependent gut microbiota disturbances: α-diversity: No effect on richness but ↓ evenness Differences in β-diversity (treatment vs control) ↓ Bacteroidetes, Odoribacteraceae, Bacteroidaceae, S24-7 family ↑ Firmicutes, Lactobacillaceae, Lachnospiraceae Effects diminished with feed age (4- and 8-months storage, increasing Ag sulfidation: ↓ bioavailability Ag ions	No effects (body weight, intestinal damage or structural alterations, C-Reactive protein)	(van den Brule *et al.*, 2016)
In vitro: Fermenter (medium mimicking high fat-high protein diet) inoculated with pooled human faecal microbiota from four healthy individuals. (three replicates) Followed by evaluation of medium supernatants in caco-2 cell culture	1 mg/ml Ag NP (Ø ~14 nm citrate stabilized), alone or in combination with probiotic *Bacillus subtilis* 24 h	16S rRNA (V3–V4) gene sequencing Targeted fluorescent in-situ hybridization (FISH) SCFA	No changes (diversity, core microbiota composition, SCFA) No cytotoxicity or genotoxicity AgNP: ↓ *Faecalibacterium prausnitzii, Clostridium coccoides/Eubacterium rectales* Changed predicted microbial function of 4 gene categories	n/a	(Cattò *et al.*, 2019)
In vitro (with human faecal microbiota from two healthy individuals, run separately): Static fermentation vessel SIMGI® computer-controlled simulator of the gastrointestinal system (five compartments: stomach, small intestine, and ascendent, transverse and descendent colon)	11 µg/mL, Ag NPs (Ø ~4–6 nm, PEG-stabilized) 7.6 µg/mL, Ag NPs (Ø ~3–5 nm, GSH-stabilized) Static vessel: 48 hours SIMGI® model: 48 hours + 8-day wash out period	Plate counting and qPCR (total aerobes, total anaerobes, Enterobacteriaceae, *Clostridium* spp., lactic acid bacteria and Enterococcus spp.)	No significant changes in microbial composition or metabolic activity (i.e. proteolytic activity)	n/a	(Cueva *et al.*, 2019)

Notes: Ø: average diameter; NP: nanoparticle; LFD: low-fat diet; HFD: high-fat diet; HCD: choline-supplemented western diet; MFT: Microbiota faecal transplant; TMA: trimethylamine; GD: gestational day; PD: post-delivery day; BPA: bisphenol A; TMA: trimethylamine; TMAO: trimethylamine-N-oxide.

Sources: See References

ANNEX IV. GUIDELINES AND BEST PRACTICES

Although some of the following references are assigned to one category, some of them address different categorical topics. Note that some documents are based on consensus and others are based on the experience of individual scientists or research groups.

STUDY DESIGN

PREPARE: guidelines for planning animal research and testing. Laboratory Animals (Smith *et al.*, 2018)

Guidelines for Transparency on Gut Microbiome Studies in Essential and Experimental Hypertension (Marques *et al.*, 2019)

The gut microbiome of laboratory mice: considerations and best practices for translational research (Ericsson and Franklin, 2021)

EXPERIMENTAL PROTOCOLS AND ANALYTICAL METHODOLOGIES

Best practices for analysing microbiomes (Knight *et al.*, 2018)

Current challenges and best-practice protocols for microbiome analysis (Bharti and Grimm, 2021)

Measuring the microbiome: best practices for developing and benchmarking microbiomics methods (Bokulich *et al.*, 2020)

The madness of microbiome: attempting to find consensus "best practice" for 16S microbiome studies (Pollock *et al.*, 2018)

Optimizing methods and dodging pitfalls in microbiome research (Kim *et al.*, 2017)

Comparative pathologists: ultimate control freaks seeking validation! (La Perle, 2019)

Protocols for the gut microbiota transplantation for colonization of germ-free mice (Choo and Rogers, 2021b)

Procedures for fecal microbiota transplantation in murine microbiome studies (Bokoliya *et al.*, 2021)

Investigating causality with fecal microbiota transplantation in rodents: applications, recommendations and pitfalls (Gheorghe *et al.*, 2021)

Guidelines for Transparency on Gut Microbiome Studies in Essential and Experimental Hypertension (Marques *et al.*, 2019)

Metabolic phenotyping guidelines: assessing glucose homeostasis in rodent models (Bowe *et al.*, 2014)

INTERPRETATION AND REPORTING

The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research (Percie du Sert *et al.*, 2020)

Reporting guidelines for human microbiome research: the STORMS checklist (Mirzayi *et al.*, 2021)

Guidelines for reporting on animal fecal transplantation (GRAFT) studies: recommendations from a systematic review of murine transplantation protocols (Secombe *et al.*, 2021)

Metabolic phenotyping guidelines: assessing glucose homeostasis in rodent models (Bowe *et al.*, 2014)

Principles for Valid Histopathologic Scoring in Research (Gibson-Corley, Olivier and Meyerholz, 2013)

Use of Severity Grades to Characterize Histopathologic Changes (Schafer *et al.*, 2018)

OECD Omics Reporting Framework (OORF): Guidance on reporting elements for the regulatory use of omics data from laboratory-based toxicology studies https://one.oecd.org/document/ENV/CBC/MONO(2023)41/en/pdf

VALIDATION

Validation and standardization of DNA extraction and library construction methods for metagenomics-based human fecal microbiome measurements (Tourlousse *et al.*, 2021)

Standards and Guidelines for Validating Next-Generation Sequencing Bioinformatics Pipelines: A Joint Recommendation of the Association for Molecular Pathology and the College of American Pathologists (Roy *et al.*, 2018)

OTHER RESOURCES

Norecopa: A global knowledge base of resources for improving animal research and testing. (Smith, 2023) Norway's National Consensus Platform for the advancement of "the 3 Rs" (Replacement, Reduction, Refinement) in connection with animal experiments. https://norecopa.no/ (Accessed on March 1, 2024)

OECD Series on Principles of Good Laboratory Practice (GLP) and Compliance Monitoring https://www.oecd.org/env/ehs/testing/oecdseriesonprinciplesofgoodlaboratorypracticeglpandcompliancemonitoring.htm (Accessed on 21 March 2024)

Series on Testing and Assessment / Adopted Guidance and Review Documents https://www.oecd.org/env/ehs/testing/seriesontestingandassessmentadoptedguidanceandreviewdocuments.htm (Accessed on 21 March 2024)

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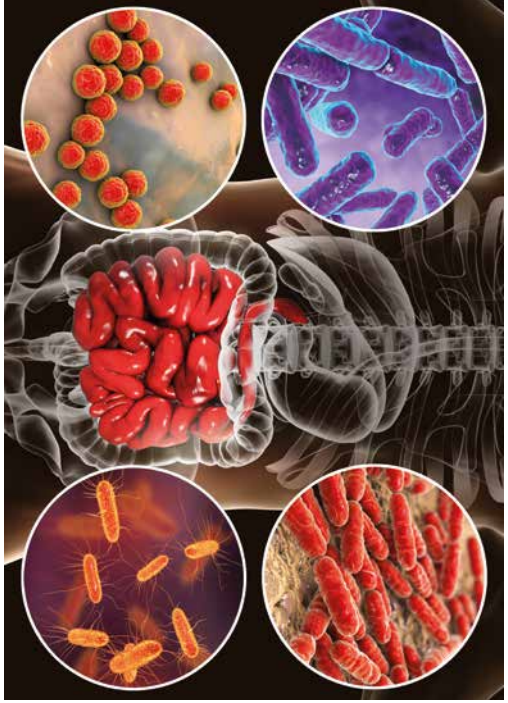

STATE OF RESEARCH ON THE INTERACTIONS BETWEEN FOOD ADDITIVES, THE GUT MICROBIOME AND THE HOST

A FOOD SAFETY PERSPECTIVE

A scientific literature review was conducted with a focus on food safety to critically assess the current research on the impact of selected food additives on, and their interactions with the gut microbiome, as well as the resulting implications for host health. The review's main goals were to:

- gather and assess the quantity, quality, and reliability of scientific information;
- identify research limitations, knowledge gaps, and related needs; and
- explore the applicability of microbiome data in food safety chemical risk assessments.

This review concludes on recommendations to guide and improve microbiome science in risk assessment. Through this work, the Agrifood Systems and Food Safety Division contributes to the FAO's role of improving food safety by encouraging high-quality research to produce robust data, promoting effective scientific communication, and investigating how knowledge regarding gut microbiome-food additive interactions could potentially support the modernization of food safety chemical risk assessments.

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