Simulation of Human Colonic Fermentation

Regardless the complexity in the design, the fermentation process is performed with a faecal inoculum and a culture medium. Moreover, the compounds submitted to assay, or the non-absorbed compounds in the digesta resulting from previous digestion stages (oral, gastric, and small intestine), are incorporated into the reactor, leading to colonic fermentation. In some studies, the colonic fermentation is used to assay the effect of specific compounds (e.g., polysaccharides or oligosaccharides) on gut microbiota growth or the relative abundance of species and genera.

1. Introduction

The role of diet in human health has been repeatedly addressed and confirmed. However, elucidating the relationship between nutrition and health requires an accurate understanding of the digestion process through which foods are transformed and interact with the organism to exert their eventual biological function [1].

Along digestion, chemical and enzymatic reactions, along with mechanical forces, transform food matrices and their main macronutrients (lipids, protein, and carbohydrates) into their conforming constituents, which are absorbed by the enterocytes in the small intestine [1]. Digestion is a very efficient process, but a small fractions of food constituents still escape the hydrolytic action of enzymes and subsequent absorption, thus remaining in the lumen and passing to the colonic stage, where the colonic microbiota can further interact with them, forming what is considered to be a relevant research topic nowadays [2][3][4].

Studying the digestion process in situ presents several vicissitudes due to the issue of access to the digestive system, the impossibility of continuous monitoring, and burden for the subjects as well as ethical restrictions [5]. Animal models have been extensively applied to assess the digestion process, but the fact that animals' microbiota differ from humans' microbiota is widely accepted [6]. For these reasons, alternative methodologies to study digestion have increased, including in vitro digestion models, which enable the simulation of digestion in the laboratory [7][8][9][10]. These models allow for the possibility of sampling at any point of the process, assessing digestion of any food or nutrient and establishing its effect on the microbiota profile, or determining the products generated by the microbiota metabolism [7][11]. In vitro digestion studies address such diverse research questions as macronutrient digestibility, bioactive compounds bioaccessibility, the release of encapsulated substances, or the effect of gastrointestinal conditions on digestibility, among others.

In relation to the microbiota, these models can be useful to assess the effect of prebiotics (nutritional compounds present in foods, such as fibres), probiotics (ingestion of alive microorganisms), or symbiotics (a combination of both) on microbiota populations or the role of these populations in the production of metabolites related to diet. Other relevant fields that could benefit from applying in vitro colonic fermentation relate to microbiota transmission from mothers to infants through breastmilk, the effect of antibiotics in the area of pharmacology, or in agronomics to determine if the presence of pesticides in some foods were to be evaluated in colonic microbiota. Depending on the goals, the digestion and fermentation variables must be adapted to represent the physiological conditions to simulate [5], such as transit time, pH, digestive secretions and composition [10], or microbiota profile [12]. Compared to the standard digestion and fermentation conditions, these amendments are crucial in the context of food digestion studies in chronic disease (pancreatic insufficiency, celiac disease, etc.) , special physiological states such as pregnancy, or life stages (infancy, adulthood, elderly).

2. The Colonic Fermentation Process

The large intestine is a suitable environment (pH between 6–8 and anaerobic conditions) for the development of microorganism populations (colonic microbiota), most of which belong to the Bacteroidetes and Firmicutes phyla [13]. The main biochemical reaction occurring in the colon is fermentation, which produces a series of metabolites used by both the microorganisms and the host as a source of energy [14]. Nevertheless, it is also involved in the modulation of other body functions, such as the immune system and the brain–gut axis. The preferent substrates for the microbiota are non-digestible carbohydrates, including fibre, cellulose, hemicellulose, pectin, gums, and alginates. The colonic fermentation of these macromolecules results in short-chain fatty acid (SCFA) production, mainly acetate, butyrate, and propionate, and gas, such as hydrogen and carbon dioxide [15]. When the source of non-digestible carbohydrates is limited, the microbiota utilises protein substrates, generating sulphurated and N-nitrose compounds, ammoniac, heterocyclic amines, and branched-chain fatty acids (BCFA). On the other hand, most dietary lipids are digested and absorbed in the small intestine and do not reach the colon. However, when lipid intake is high, bile salt secretion increases, and the excess that is not absorbed along with lipids passes on to the colon and can be fermented by the microbiota, producing secondary bile salts [16]. In general terms, from the main metabolites generated by the microbiota, SCFA seem to be related to beneficial health effects, while sulphur compounds and secondary bile salts have been associated with harmful outcomes [14][15][16]. However, despite the acknowledgement of the important role that the microbiota plays in food digestion and the effect that food constituents have on promoting or reducing certain microbiota populations, many gaps in establishing explanations remain.

Even though some generalization about the species comprising the microbiota can be made [13], a wide diversity of factors (lifestyle, habitat, birth delivery mode, maternal nutritional status, or breastfeeding, among others) determine a specific microbiota profile in each individual. Eating habits and type of diet have a major impact [17] and can be modified, serving as a tool to modulate the microbiota composition. In particular, the effect of carbohydrates has been widely studied, as they are the main substrate for intestinal microorganisms [18]. Among them, non-digestible carbohydrates arrive in their native form to the colon and are fermented by the microbiota promoting their growth and activity, subsequently generating beneficial compounds for health [19]. In upper gut digestion, digestible carbohydrates are broken down into oligosaccharides, disaccharides, and monosaccharides and are absorbed before reaching the colon. Nevertheless, in high-sugar diets, they can reach the colon since the absorption capacity of the small intestine is exceeded, thus causing losses in microbial diversity [20]. Similarly, protein and lipid intake can also lead to changes in microbiota composition. Diets rich in animal-origin protein and low-digestible carbohydrates lead to the microbial production of metabolites (BCFA, N-nitroso compounds) related to the increased risk of suffering from colorectal disorders [21]. In the case of fatty acids, monosaturated fatty acids and n-3 polyunsaturated fatty acids have been associated with an increase in beneficial bacteria and the production of SCFA, while diets rich in long-chain fatty acids have the opposite effect [22]. Furthermore, a wide diversity of diets is available worldwide depending on the ecosystem and cultural, economic, or agronomic considerations. This is probably the reason behind the differences in the microbiota profile observed from distinct geographical regions [18]. In this aspect, Wilson et al. (2020) contributed a large international review to compare microbiota profiles in several regions of the globe, concluding that overall, clear differences can be found depending on the diet, even more than with genetics [23].

3. Available Colonic In Vitro Fermentation Models

As anticipated, studying colonic fermentation in vivo entails several restrictions. Thus, colonic in vitro fermentation models can be a valid and worthwhile alternative and are increasingly used in the food research field. These models emerge with methodological advantages since they allow the possibility of sampling at any point of the process, assessing digestion of any food or nutrient and establishing its effect on microbiota profile, or determining the products generated by the microbiota metabolism [7][11]. On the other hand, the limitations related to in vitro models is that they do not take into consideration the contribution of the host in digestion, and thus, they could be inherently incomplete.

For the simulation of colonic fermentation in vitro, four basic elements are required: a microbial inoculum, a culture medium, a study substrate, and a bioreactor. Depending on the complexity of the conditions of the simulation or the inoculum, different types of models are currently available and are described hereafter.

Despite of the fact that static models are useful tools to study gut microbiota-food interactions, they have limitations when it comes to mimicking gut fermentation as a dynamic process [7][11]; for that reason, great effort has been made to develop dynamic colonic methods.

Altogether, dynamic models allow for the study of the effects of food on gut microbiota in a more complex environment that simulates the in vivo conditions better than static models and allow for the assessment of longer periods of time (e.g., one or several weeks) [24][25][26]. The main research objectives when using these models have been the evaluation of the prebiotic, probiotic, and symbiotic effect of some compounds on gut microbiota: **Table 1** gathers the objectives and references of published studies reporting the applicability of dynamic colonic models in food science and technology fields over the last 10 years. As in the case for the in vitro simulation of digestion in the upper gut, most of the studies have focused on a food extract rather than a complete food matrix, probably due to the increased complexity of the experimental procedure and analytical determinations that food matrices imply.

Table 1. Applicability of dynamic in vitro colonic models in the food science and technology field from 2011 to 2021.

Dynamic Model	Main Objective and Examples	References
SHIME	**Assessing the probiotic effect of some bacteria:** -Bacteria (e.g., *Lactobacillus, Bifidobacterium,* or *Enterococcus*) carried in cheese, chocolate, cereals, sausages, and drinks. -Probiotic formulations (e.g., *Lactobacillus, Bifidobacterium,* or *Bacillus*)	[27][28][29][30][30][31][32][33][34][35]
	Assessing the prebiotic effect of some compounds: -Polyphenols (e.g., black tea or red wine grape extract, t-resveratrol, and ε-viniferin extract). -Polysaccharides (e.g., branched fructans, arabinogalactan, fructooligosaccharides, commercially available plant polysaccharide)	[36][37][38][39][40]
	Assessing the symbiotic effect (probiotic + prebiotic): -Milks and beverages fermented by different microorganisms (e.g., *Lactobacillus*) and supplemented with different compounds (e.g., passion fruit, grape pomace) -Probiotic and prebiotic formulations (e.g., *Bifidobacterium* + 3′-sialyllactose, *Lactobacillus* + red win polyphenolic extract, *Lactobacillus* + fructooligosaccharides, *Bifidobacterium* + pectins -Repressing *Escherichia coli* colonization of the gut mucus.	[25][41][42][43][44][45][46]
	Other applications assessing the effect of: -Food ingredients (e.g., Mexican "taco" from corn tortilla and black beans). -Dietary emulsifiers (e.g., polysorbate 80 and carboxymethylcellulose). -Antibiotics (e.g., amoxicillin, ciprofloxacin, and tetracycline). -Processing (e.g., fresh and pasteurized orange juice).	[47][48][49]
TIM-2	**Assessing the prebiotic effect of some compounds:** -Polyphenols (e.g., polyphenols in pre-digested mango peel, pre-digested *Hibiscus sabdariffa* calyces) -Polysaccharides: (e.g., High- and low-acetylated galactoglucomannooligosaccharides, agave fructans, xylo-oligosaccharides, pectins, chicory root pulp polysaccharides)	[50][51][52][53][54][55]
	Other applications assessing the effect of: -Supplementation with minerals (e.g., iron). -Food ingredients (e.g., two types of Mexican sauces) -Symbiotic effect (e.g., functional pasta with *Bacillus* and β-glucans).	[56][57][58]
	Assessing the probiotic effect of some bacteria -*Lactobacillus.*	[26]
SIMGI	**Assessing the prebiotic effect of some compounds:** -Polysaccharides: (e.g., apple, potato, oat and psyllium fibres, hydroxypropyl methylcellulose, and microcrystalline cellulose). -Polyphenols: (e.g., grape pomace extracts).	[59][60]
	Other applications assessing the effect of: -Food (e.g., red wine).	[61]
PolyFermS	**Assessing the prebiotic effect of some compounds:** -Polysaccharides: (e.g., fructo-oligosaccharides, β-glucan, α-galactooligosaccharide, xylo-oligosaccharide	[62]
	Other uses assessing the effect of: -Supplementation with minerals (e.g., iron).	[63]

4. Addressing Colonic In Vitro Digestion Studies

As anticipated above, when aiming to perform a colonic in vitro simulation, a series of prior considerations need to be considered. Among them, faecal inoculum collection and processing are of great importance. Furthermore, sampling points and analytical determinations are also essential and must be decided upon depending on the aim of the study.

The collection of a faecal sample (from one individual) or a faecal pool (from different individuals) is one of the first steps of an in vitro colonic simulation. Faecal donors are selected according to age, gender, body mass index, presence, or absence of illness, etc. , depending on the aim of the study. General considerations for donors include not having received antibiotics and prebiotics or probiotics at least some months before the faecal donations. The healthy individuals selected as control or reference groups must follow a standard diet and assure that they are free of any gastrointestinal disease. The faecal collection must be done as quickly as possible in anaerobic conditions, placing the sample in anaerobic tubes, jars, or chambers, or using devices that promote the preservation of anaerobiosis, such as OXOID ones.

After the collection, an individual faecal sample or faecal pool is mixed with a solution (e.g., phosphate buffered saline or sodium chloride) to obtain a faecal suspension or faecal slurry (e.g., 10% w / v or 20% w / v). Then, the suspension is manually homogenised or is homogenised using a stomacher [64][65][66][67][59]. In addition to the previous homogenisation stage, some protocols include filtering [68][69][70], or centrifugation steps [71][25][31] so that the supernatant is used as faecal inoculum in the fermentation process. In addition, other protocols include the extraction of the bacterial mass from the rest of the components of the faecal sample by means of the use of iohexol (Nycodenz ®), which is added to the supernatant of a previously homogenised faecal suspension, causing the isolation of viable cells. After centrifugation, the sample results in a concentrated phase containing the bacteria, which can be recovered and purified with sequential rinsing with sodium chloride. This bacterial extract ensures higher amounts of microbial inoculum, but on the other hand, may cause the loss of some of the species due to the additional processing [72]. Finally, some other authors add a cryoprotective agent (glycerol) to the faecal suspension so that it can be frozen for later use [73][52][72][74].

Metagenomic techniques, both 16S rRNA sequencing and shotgun metagenomics, have been widely applied to determine the composition and functionality of human microbial communities. Nevertheless, metagenomics cannot elucidate which genes are expressed. In this context, metatranscriptomic and metaproteomic methods have gained weight. On the one hand, metatranscriptomic methods, which study the transcripts produced by a microbial community, reveal knowledge related to the induced/repressed genes and active/inert/dead microorganisms. The tools used for metagenomics assessment can be adapted to be used in metatranscriptomics methods, which allow for its easy integration. On the other hand, metaproteomic methods contribute to a better understanding of microbial functionality since they study the proteins produced by a microbial community. In this case, mass spectrometry (MS) is generally performed. Finally, in terms of functionality assessment, the metabolomic approach studies the metabolic pattern of a microbial community. Both mass spectrometry (MS) and nuclear magnetic resonance (NMR) are applied, but MS is preferred over NMR because of its higher sensibility. Even though it is still challenging, the integration of metaomic data could bring promising insights in terms of the human microbiota composition, functionality, and metabolite activity [75].

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