In vitro batch fermentation protocol to study the contribution of food to gut microbiota composition and functionality

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Editor Summary: This Extension of a previous in vitro digestion protocol provides a subsequent in vitro batch fermentation stage that is carried out afterwards to enable the effect of food on the gut microbiome to be investigated.

Tweet: *In vitro* batch fermentation carried out after in vitro digestion enables the effect of food on the gut microbiome to be investigated.

Related Links

Key references using this protocol.

Pérez-Burillo, S. et al. *Food Chem.* 239, 1253–1262 (2018). DOI: 10.1016/j.foodchem.2017.07.024

Pérez-Burillo, S. *et al. Food Chem.* 279, 252–259 (2019). DOI: 10.1016/j.foodchem.2018.11.137

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Abstract

Knowledge about the effect of foods on gut microbiota composition and functionality is expanding. To isolate the effect of single foods and/or single nutrients (i.e. fibre, polyphenols), this protocol describes an *in vitro* batch fermentation procedure to be carried out after an *in vitro* gastrointestinal digestion. Therefore, this is an extension of the previous protocol described by Brodkorb et al. (2019) about *in vitro* digestion. The protocol uses an oligotrophic fermentation medium with peptone, and a high concentration of fecal inocula from human fecal samples both to provide the microbiota and as the main source of nutrients for the bacteria. This protocol is recommended for screening work to be performed when many food samples are to be studied. It has been successfully used to study gut microbiota fermentation of different foodstuffs giving insights into their functionality, community structure, or ability to degrade particular substances, which can contribute to the development of personalized nutrition strategies. To carry out this procedure there is no need for a specific level of expertise. The protocol takes between 4 – 6 hours to prepare fermentation tubes and 20 hours of incubation.

Keywords

In vitro fermentation, gut microbiota, food, nutrients, batch fermentation, in vitro digestion, microbiome, gut microbiome, microbiota, digestion, personalized nutrition.

Introduction

During recent times, the gut microbiota has become a major focus of interest in the study of human health. Gut microbes are closely related to human health[1], and have been linked to important conditions such as inflammatory bowel disease, immune system disorders, obesity, or even autism spectrum disorders[2]. Gut microbiota can be disturbed by many different factors such as age, antibiotics, exercise, but diet, and specifically food components, are most probably the main drivers causing changes in gut microbiota behavior[3]. It has been extensively demonstrated how the gut of human populations with different dietary patterns is colonized by different microbial communities, which in turn reflects on people's health[4, 5, 6]. Therefore, many efforts have been put into developing different strategies to study the gut microbial community and its functionality such as *in vitro* models (including static batch fermentations ad continuous systems), animal models, and human clinical/observational studies[7]. Each one of them has their own limitations and advantages/disadvantages. Although human studies provide the information with the highest physiological relevance, *in vitro* models are still essential to test specific foods or food components and for initial screenings[7].

Development of the protocol

There are essentially two types of *in vitro* fermentation models to study gut microbiota: those based on batch fermentations and those based on continuous systems. The latter are closer to physiological conditions than batch fermentations[7]. Moreover, they allow for a better representation of the gut microbial communities and to keep them stable for longer periods of time, even several weeks, and mimic the conditions of the different portions of the colon in an automatized manner[7]. A widely used continuous system is the Simulator of Human Intestinal Microbial Ecosystem (SHIME®)[8]. This is a complex and expensive system comprised of 5 stages, two of them to mimic gastrointestinal digestion and three of them to mimic colonic fermentation. There are also continuous systems that are less complicated but still comprised of three vessels to mimic colonic conditions and automated to control pH and to pump the

contents from one vessel to another[9]. These are large systems that need their own room and specialized operators, which in turn make them sometimes inappropriate for certain laboratories. Miniaturized systems have been developed to overcome this problem[10]. Regardless of their size or complexity, they all work in a similar way and try to mimic the different portions of the colon, controlling pH and nutrients. Their main disadvantage is that only one sample can be studied at once and the experiment usually takes at least a month since they first stabilize microbial communities for 1-2 weeks and then add the component subject to study and keep the experiment running for another 1-2 weeks[9].

On the other hand, batch fermentations, generally carried out in test tubes, allow many samples to be studied at once and within a short period of time, usually 24-48 hours[11, 12]. Therefore, these models become convenient when the objective is to make an initial screening of many foodstuffs or food components[13]. However, they are less physiologically relevant, and bacterial waste products will eventually accumulate, hence the need for a shorter experimental duration[7]. Still, both *in vitro* approaches could complement each other, using batch fermentations for initial screening of foods or food components and continuous systems for the selected ones, according to the investigator's needs.

Many different *in vitro* batch fermentations have been used, thus there is a need to propose a common methodology, since a variation in the conditions (fecal material origin, fecal slurry concentration, incubation time, culture media composition, etc.) would affect microbial communities and their metabolism (**Table 1**). The first issue that arises is whether to perform a prior *in vitro* digestion; as **Table 1** shows, some researchers perform the *in vitro* digestion, whilst others do not. This will translate into the presence or absence of enzymes during colonic fermentation (they are proteins, so they can be used by microbes) and bile salts (which are also transformed by microbes). In addition, in case of actual foodstuffs, *in vitro* digestion would break down their structure, making nutrients more accessible for microbes, which will not be achieved if prior digestion has not been performed. Additionally, when a previous digestion is performed, it is carried out using different protocols across experiments, which

translates into different amounts of digestion components available for colonic fermentation. Another main difference that can be observed in **Table 1** is the fermentation medium. There are two main types - a rich nutrient medium or a minimum medium. A rich medium could be more appropriate for continuous systems since they look to stabilize the microbial community before adding the substance under study. For batch fermentations, a minimum medium can help highlight the metabolism of the substance under study and quickly identify the resulting metabolites or involved bacteria with results being easier to interpret. Afterwards, substances selected by the investigator could be tested in a continuous system with a rich medium over several weeks. Moreover, even among those who use the same type of medium, concentrations usually vary among experiments and nutrients are not always the same (**Table 1**). In fact, Mould et al. (2005) proposed a simplified medium comprised only of a buffering solution, cysteine, a sulfur source and several minerals[14]. Finally, fecal material source and fecal slurry concentration vary across experiments, which will affect the results.

Therefore, a need for standardized conditions has been identified and the authors propose an *in vitro* protocol to simulate colonic fermentation based on batch culture and using human feces as the source of gut microbes coupled with a prior *in vitro* digestion. This prior phase will not be discussed here since the authors propose the use of the INFOGEST digestion protocol already published in this journal[15]. The proposed *in vitro* digestion-fermentation protocol has been successfully used by the authors: to test antioxidant capacity after fermenting chicken, whole grain bread, lentils, orange, tomato, yoghurt, and peanuts[16]; and to study how microbial communities and their functionality are affected by chicken, bread, pepper, chickpeas, and banana subjected to different cooking methods[17], roasted and green coffee[18], salami with different potential prebiotic agents added[19], mannooligosaccharides extracted from spent coffee grounds[20], and melanoidins extracted from different food sources (coffee, black and pilsner beer, breakfast cereal, bread crust, biscuits, chocolate, balsamic vinegar and sweet wine)[21]. Other authors also

used this protocol to study the modulatory effect of polyphenols and sesquiterpene lactones from artichoke heads[22] or Chlorella spp.[23] on gut microbiota composition and functionality.

Overview of the procedure

The procedure is summarized in **Figure 1.** It can be divided into seven basic stages (see Experimental Design for further information): 1. fecal material collection; 2. preparation of the equipment and reagents; 3. setup of the *in vitro* digestion samples to be fermented; 4. preparation of the fecal slurry; 5. fermentation; 6. Sampling; and 7. Sample processing.

Fecal material should be collected from volunteers (always at least four to pool together the feces and minimize inter-individual variation[5]) under conditions dependent on the aim of the experiments; if the aim is to investigate regular microbiota, feces should come from healthy individuals not taking antibiotics, normal body mass index, etc.; however, if the aim is to investigate microbiota from a particular illness, then the feces should be collected from volunteers suffering such a condition. Collection should always be done in sterile containers specifically made for that purpose and wearing gloves to avoid any contamination. Fecal material should ideally be collected the same morning when the experiment is going to be carried out; however, they could be stored at 4 ºC for 24 h or frozen at -80 ºC with glycerol to protect microbes from ice crystals, microbial communities should be stable for 2 months.

Preparation of the materials and reagents involves firstly the preparation of the fermentation medium with peptone and resazurin, oxygen removal by bubbling nitrogen through it and autoclaving it. Cysteine and sodium sulfide (reductive solution) are added afterwards to avoid losing cysteine during the thermal treatment. Phosphate buffer for fecal slurry preparation is also made and autoclaved. Different materials to be used during the experiment are also autoclaved: milli-Q water, pipette tips, and lab spoons.

The fecal slurry is prepared at 32% feces (wt/vol) in phosphate buffer. Each fermentation tube carries 7.5 mL of medium, 2 mL of fecal slurry and 0.5 g of substrate sample from *in vitro* digestion. Tubes are kept at 37 ºC with oscillating shaking at 20 rpm for 20 hours. Right after, microbial activity is stopped by placing the tubes on ice. Experimental conditions will be described in the section "Experimental conditions". A typical fermentation example with quantities and volumes added is described in **Table 2.**

Sampling and sample storage will vary depending on the analysis to be performed. Therefore, an appropriate sampling strategy should be considered before carrying out the experiment (see Experimental design, Stage 6 and **Table 3** for details). And finally, samples are processed to assess substrate degradation, for metabolomics analysis (to measure the presence of certain metabolites and study gut microbial functionality), or for 16S RNA sequencing or shotgun metagenomics analysis to reveal gut microbial community structure.

Advantages/limitations

Batch *in vitro* fermentations are the simplest methodology to simulate colonic fermentation. On one hand, this methodology allows as many substrate samples as the investigator wants to be studied, either actual food, cooked or raw, or specific food components such as dietary fibers or phenolic compounds. Secondly, it allows to investigators to study the behavior of healthy or altered gut microbiota against the same substrate at the same time, which could help formulate initial hypotheses and plan future experiments. Furthermore, by exposing gut microbes to certain compounds, batch fermentations can help elucidate the metabolic routes involved and the intermediate metabolites appearing. Therefore, this could in turn give clues on how to drive microbial metabolism towards a specific goal (i.e.: production of a particular beneficial metabolite or favor the growth of a beneficial bacteria), getting a step closer to gut microbiota modulation via diet. Moreover, batch fermentation experiments are much less time consuming

than *in vitro* continuous systems or animal or human studies. In addition, whereas continuous systems usually require a big space for the vessels to simulate the different portions of the colon, batch fermentations do not. The costs of batch experiments are lower since they do not require certain equipment such pH controlling systems or pumps to move the fermentation medium from one vessel to the next, or the additional costs that involve working with animal models or human volunteers. Additionally, another advantage over animal models is that animals' gut microbiota is different to that of human beings (even if gnobiotic animals are used). Therefore, in an animals' gut microbiota, some human gut bacteria species would be missing, and there would be other species only present in animals' guts, so bacterial interactions could be different, as could the effect of a specific food or food component.

However, batch *in vitro* fermentations also have some limitations. The main issue is that this methodology is the farthest from physiological conditions, and hence, results should not be considered definitive. pH is not controlled during fermentation and therefore it will change during the process due to the acidic metabolites generated. As batch fermentations are not continuous systems, the accumulation of microbial waste products cannot be controlled, and bacterial growth could be potentially affected. Moreover, due to the larger number of test tubes usually used in batch fermentations, which involves more pipetting and more manipulation of the samples, microbial contamination is probably more likely to happen in this kind of experiment.

Nonetheless, batch fermentations are still essential to study different foods or food components, and to make initial screenings which would be otherwise unfeasible due to the time required in the case of continuous systems or the costs for animal and human studies. Furthermore, batch fermentations are essential to unravel microbial metabolism of specific nutrients and therefore to understand the effects of incorporating certain components to human diet.

Applications

One of the main applications of *in vitro* batch fermentations is to study microbial degradation of specific dietary compounds to uncover which metabolites are released and hence, how the host health could be affected. Polyphenols have been some of the most studied compounds in this sense (see **Table 1**). Some phytochemicals, especially phenolic compounds, are only partially absorbed in the small intestine, therefore reaching the large intestine[24]. Thus, *in vitro* batch fermentations become essential to rapidly test how these compounds are metabolized by gut microbes. Most dietary polyphenols are metabolized in the colon by gut microbes. This metabolism is usually mandatory for their absorption and it can modify or modulate their actual biological activity[25]. Even though human enzymes will not breakdown the phytochemical structure, prior *in vitro* digestion is still recommended since pH changes during digestion and salts could chemically alter the phytochemical structure. Additionally, the enzymes and bile salts (which can be used by gut microbes) will be present. Therefore, prior *in vitro* digestion will make the experiment more physiologically relevant even though phytochemicals are not digested as carbohydrates, proteins or fats would be. Phenolic compounds have been associated with different beneficial health effects such as antioxidant, anti-inflammatory, neuroprotective, and cancer chemoprotective effects[24]. Most polyphenols are present in foods as glycosides, other are polymeric molecules (anthocyanins, ellagitannins) which are poorly active and must be converted into their aglycones or to monomers[25]. The first metabolism steps usually follow a specific pathway and for their complete degradation a consortium of microbes is needed. However, it is also important to take into account inter-individual variability, which can lead to different outcomes (different metabolites and/or physiological effects). Inter-individual variation refers to the fact that different individuals can harbor different microbes, leading to variations in dietary response. Some dietary components require specific bacterial species (commonly known as keystone species) to be metabolized and hence, if not present, these compounds would remain intact. Therefore, what is beneficial for one person, could be less positive or even useless for another.

There are two examples widely studied in the field of phenolic compounds: metabolism of the soy isoflavone daidzein, which can be metabolized following two different pathways depending on the gut microbes of the host[25]. The other example is the metabolism of ellagitannins, which in most of the population leads to urolithin (3,8-Dihydroxy-urolithin, commonly known as urolithin A and/or 3-Hydroxy-urolithin, commonly known as urolithin B) production, whereas in a smaller percentage no urolithins are produced, and, therefore, no beneficial effect from ellagitannins consumption is produced[26]. Although there is still much to unravel in relation to inter-individual variability, *in vitro* fermentations can help identify the potential keystone species needed for the metabolism of specific dietary components, which is a first step towards predicting whether a specific person will be able to benefit (and how) from consuming a specific food component.

Many studies have been carried out to investigate phenolic metabolism by gut microbes: Saura-Calixto et al.[27] studied microbial degradation of proanthocyanidins, discovering several phenolic acids as metabolites, which were also detected in plasma; these authors suggested that microbial degradation of proanthocyanidins would result in absorbable metabolites with potential health effects[27]; Ludwig et al.[11] studied the catabolism of coffee chlorogenic acids by gut microbes, which allowed them to detect the pathways involved and the main metabolites derived from such phenolic compounds. These authors also demonstrated that chlorogenic acid metabolism was influenced by inter-individual gut microbiota variation. Other phenolic compounds studied were flavan-3-ols[28], rutin[29], flavonols, flavones, flavanones and phenolic acids[30], quinic acid[31], or anthocyanins[32]. Until now, phenolic metabolism by gut microbes has been extensively studied and most of the current knowledge is summarized in Selma et al.[24], Serra et al.[30], Marín et al.[33], Stevens and Maier[34], Rowland et al.[25], among others.

Batch fermentations have also been of great help for mapping microbial metabolic pathways. This information has been successfully used to predict gut microbial metabolic outcomes after certain conditions, such a specific diet[35]. In addition, batch fermentations have been also used to study the effect

that specific foods could have on gut microbiota community structure and its functionality, which is usually measured through short chain fatty acids (SCFA) production analysis. Their generation and relative abundance are considered health biomarkers. Individual SCFA have been linked to several health benefits which have been already reviewed[1,36]. In a previous paper, we studied how green and roasted coffee could affect microbial composition and functionality, allowing us also to observe that each type of coffee affected gut microbiota differently[18]. In another study, we investigated the effects of chicken, chickpeas, bread, banana and pepper on gut microbiota and its functionality, observing how each type of food promoted the growth of specific bacteria[17], probably due to the different composition of each food. In the same study, we also observed how the cooking method applied also modified the way gut microbes metabolized such food, resulting in somewhat different microbial communities and functionalities. Other research projects have studied how meats[37], legumes and insects[38] cooked differently affected gut microbiota. Moreover, not only microbial communities and their functionality can be studied, but also other biological activities such as inflammatory or antioxidant capacity after fermentation of specific foods[16].

Harmful enzymatic activity of the gut microbiota is another example of an application that has been reported. This involves a set of bacterial enzymes involved in metabolism of different substances that have as output potentially harmful metabolites[39]. Among them, beta-glucosidase, beta-glucuronidase, tryptophanase, urease, azoreductase, or nitroreductase are found. They are involved in creating aromatic amines, aglycones, secondary bile acids, hydrogen sulphide or oxygen species[40].
Moreover, an important field in which batch fermentations are essential is the search for prebiotic compounds[41]. Accordingly, many compounds have been submitted to *in vitro* colonic fermentation to study their potential prebiotic effect: manooligosaccharides from spent coffee grounds[20], melanoidins from different food sources[21], exopolysaccharides[2], maltopolysaccharides[42], inulin, galacto- and xylooligosaccharides[43]. All these studies would help screening for prebiotic ingredients, which could be

added to certain foods in order to formulate functional foods. Once the novel prebiotic food has been formulated, it could be submitted to *in vitro* colonic fermentation to study its potential as prebiotic food. In this sense, in a previous work, our research group designed a prebiotic salami in which several potential prebiotic ingredients were tested[19]. As result, we selected the best prebiotic ingredient and the improved salami was tested in a human intervention[44]. This is a clear example of how batch fermentations are essential to make initial screenings, making future interventions easier.

Finally, batch fermentations still have a critical contribution in projects focused on achieving personalized nutrition according to a person's gut microbiota. Current attempts to modulate gut microbiota via diet are usually based on genome-based metabolic reconstructions[45]. However, though these reconstructions are incredibly informative and useful[46], they can only tell so much: carrying a gene does not mean that it is going to be expressed. They cannot account for gene expression in response to environmental changes such as pH or competition for a substrate[47]. Moreover, they usually misrepresent ecological interactions, overestimating mutually beneficial ones[47]. Therefore, batch fermentations become essential as an intermediate step to work out bacterial roles and gene expression in response to specific substrates.

Alternative methods

Alternative methods to batch *in vitro* fermentations consist of scaling up the model to continuous systems (with or without immobilized feces), animal models and human trials. Continuous systems are usually comprised of three vessels mimicking the environment in the proximal, transverse and distal colon by pH, temperature, and medium flow rate control[7,9]. The control of these parameters allows investigators to achieve a steady microbial composition as well as a steady metabolite concentration[48]. Therefore, conditions are closer to those occurring in the human colon, making experiments more physiologically relevant. A widely used continuous system is SHIME®[8], which also includes two previous compartments

to mimic gastric and intestinal digestion. However, they present some disadvantages: only one substrate (food or a specific molecule) can be tested at once, which would make it impossible to perform initial screenings, test different foods or investigate metabolic pathways of different molecules; they require a large space to set up all the compartments and additional equipment to control the different parameters; the inoculation is usually performed through a liquid fecal suspension, which usually leads to a rapid washout of less competitive bacteria, limiting the operation time to less than 4 weeks[48]. To overcome these limitations, several systems have been developed. Wiese et al.[10] developed a continuous system called CoMiniGut, which works with volumes as low as 5 mL with controlled conditions, solving the space limitations. On the other hand, there are also systems with immobilized feces where gut microbes are suspended within a porous polysaccharide matrix, overcoming the problem of using liquid fecal suspensions, demonstrating an operation time of up to 71 days[48]. Still, these two systems cannot handle many samples at once, and hence, batch fermentations are irreplaceable when the aim is to investigate different foods or molecules. Plus, continuous systems are much more expensive and time consuming. Therefore, batch cultures could be used for initial screenings and continuous systems to further research one or few selected compounds.

Secondly, animal models (specially gnobiotic mice) are also used to investigate gut microbiota. However, the data obtained could be put into question due to the physiological differences between animals and humans[49]. Human trials/interventions are the 'gold standard', although they are expensive and are limited by social and ethical issues[50]. Therefore, we feel that the best approach is to combine *in vitro* and *in vivo* models[48]. First, batch culture for initial trials and screenings, then scale up to continuous systems and/ finally animal/human models.

Experimental design

Prior in vitro gastrointestinal digestion

Foods do not reach the colon in the same state as they are eaten. During digestion, food integrity is compromised and vegetable-animal cells are broken down, releasing their contents. Moreover, starch, proteins and fats are hydrolyzed into smaller molecules. Therefore, to mimic gut microbes' action on foods or molecules, an *in vitro* gastrointestinal digestion should be previously performed. We recommend the use of the protocol described by Brodkorb et al.[15]. Hence, we use as fermentation substrate the non-digested residue left after the intestinal digestion phase of the Brodkorb et al INFOGEST protocol[15]. It has however been estimated that on average, 10% of the potentially absorbable content of the small intestine is actually not absorbed and does reach the large intestine. Therefore, to further mimic what reaches the colon, we add 10% (vol/vol) of the intestinal soluble fraction, along with the non-digested solid residue, as fermentation substrate. If the fermentation is not going to be performed the same day as the digestion, the solid residue should be stored at -20 ºC or below along with aliquots of the intestinal soluble fraction enough to add a 10% to all the fermentation samples. A typical fermentation example with quantities and volumes added is described in **Table 2.**

Stage 1: Fecal material collection (Step 1)

Fecal material should be collected from human volunteers rather than from animals since, as stated before, gut microbiota varies among human and animals[42]. Moreover, to overcome inter-individual variability[5], it has been recommended to collect and pool feces from several volunteers, at least four[41]. Pooling will ensure that keystone microbes are not missing, which could result in compounds not being metabolized. One example is *Ruminococcus bromii,* which is needed for resistant starch degradation and if it is missing, resistant starch will not be degraded. Further examples can be found in the case of polyphenols; daidzein can be metabolized following two different pathways depending on the gut microbes of the host[25], and ellagitannins which are metabolized by some microbes which are not present in a small percentage of the general population[26]. However, it is important to note that if the aim of the

experiment is to study a specific microbial community (i.e. an individual's microbial community), then the pooling strategy would not be ideal, since the objective would be, for instance, to check whether that individual is able to metabolize ellagitannins or not. To use the protocol to test the effect of an individual food on different fecal samples (i.e. feces from different donors, not pooled, just assessed individually) feces would need to be weighed in individual tubes as soon as they are received in the laboratory. and frozen, before being defrosted on the day of the fermentation and mixed with the digested food to be tested. Although we plan to do this in future, it is not something that we have tested as yet, and thus is outside the scope of this protocol.

When the aim is to investigate how gut microbes from healthy people are affected by specific foodstuffs or molecules, feces should be collected from healthy volunteers, who are not overweight (body mass index within normal range)[51], with no antibiotic treatment in the last 6 months[52], not suffering any intestinal conditions[53], belonging to the same age range, following similar diets, and exposed to similar environments[54]. Batch fermentations could be also used to study gut microbiota from people suffering from different conditions: colorectal cancer, inflammatory diseases, obesity, celiac disease, etc. Fecal material must be always collected in sterile conditions: sterile container, using gloves and with help of a disposable sterile spoon. Another option is the use of stool collectors (i.e.: FECOTAINER, AT Medical BV, The Netherlands). Therefore, proper instructions should be provided to the volunteers. Fecal material collection should be planned in advance to make sure that feces are available on the desired day to perform the experiment.

When possible, fecal samples should be collected and used for inocula preparation within 1 hour after collection, keeping them at 4 ºC or on ice[55]. This would avoid significant changes in the metabolic profile[55] and bacterial taxa abundance[56]. However, this is not always possible. Keeping the stool samples at 4 ºC or room temperature for longer periods of time would affect metabolic and bacterial abundance profiles[56]. And, although freezing the fecal material will compromise cell viability[55], adding glycerol as

cryoprotectant can help preserve bacteria. Therefore, as most times all fecal material needed will not be collected within 1 hour before the experiment, we think the best option is to freeze it as standard procedure. This could happen, for example, when fecal material from at least four volunteers is needed and one of them was not able to provide it due to physiological (or other) reasons. Still, the fecal material collected should be from that morning. Once received in the laboratory, it has to be mixed with 20% (wt/vol) glycerol in proportion 50:50 and frozen at -80 ºC. Transportation to the laboratory can be done in a cooler bag if the distance is short (i.e. same city), always ensuring that the container is properly closed. If the distance is larger, transportation should be performed on dry ice.

Stage 2: Equipment and reagent preparation (Steps 2 and 3)

In this stage, all the equipment and reagents to be used are autoclaved: pipette tips (1 mL, 10 mL), lab spoons, and tubes. It is also important to autoclave milli-Q water to make up for the volume of medium and buffer lost during autoclaving due to evaporation.

In relation to medium preparation, there are two main options: phosphate-buffered saline (oligotrophic) and basal culture medium (eutrophic) (see **Table 1**). Oligotrophic fermentations are inoculated with a higher concentration of fecal inocula (5-30%, wt/vol) as a source of both nutrients and microbes, whereas eutrophic fermentations are inoculated with a lower fecal inocula concentration (around 1%, wt/vol) into a basal culture[54,57]. Typical eutrophic medium is composed of peptone water, yeast extract, bile salts, cysteine, vitamin K, hemin and several salts, whereas typical oligotrophic medium is only composed of several salts and cysteine[57]. Some authors, however, add peptone water in addition to the salts mix in oligotrophic mediums (see **Table 1**), as a source of additional nitrogen (N). Nevertheless, it has been suggested that additional N is not usually needed since it is provided in enough quantities by the fermentation substrate and the inocula[14]. According to Long et al.[57], the microbial communities resulting from eutrophic mediums are similar to those observed in animal and human

studies receiving high-fat/high-protein diets. These microbial communities would be characterized by high abundance of *Escherichia/Shigella* and low of *Faecalibacterium* (as opposed to those diets characterized by high plant consumption), an increase of bile-tolerant bacteria and a decrease of SCFA producing genera. On the other hand, the oligotrophic medium lead to a community similar to those observed in feces of humans on a normal or calorie-restricted diet. In this protocol we propose the use of a 32% (wt/vol) inocula, as it has been used in our previous works with success, as well as by other authors[11,17-21,28,29] along with an oligotrophic medium with peptone.

This stage should be carried out the day before performing the experiment, so all equipment is ready and buffers and media have cooled down enough to be used. Cysteine is sensitive to temperature, so it has to be added to the medium after the medium has been autoclaved. As cysteine cannot be autoclaved but still needs to be sterile, a filtration step could be performed after its addition.

Stage 3: Setting up samples from in vitro digestion for in vitro fermentation (Steps 4 and 5)

First, it is important to run a blank in parallel to control for the effect of the fermentation medium on gut microbial behavior. This tube will have an equivalent volume of milli-Q water added instead of the actual sample (i.e. food or food component). This control tube will be called control tube A. Additionally, another control tube for *in vitro* fermentation will be needed (control tube B). This tube will carry the same components as control tube A and sample tubes but instead of the actual sample or milli-Q water (as in tube A), it will carry the solid residue from the control tube coming from *in vitro* digestion. This is important since the latter will contain the salts and enzymes used for the digestion, which could be used by gut microbes (see Control tubes section).

As sample, the solid residue (not digested fraction) from the previous *in vitro* digestion will be used. In order to collect this solid residue, *in vitro* digestion tubes have to be centrifuged at 4ºC and 4000 x g for 10 minutes. The supernatant can be stored for analysis or discarded depending on the needs of the

experiment, as described previously[15]. We recommend to ferment 0.5g of solid residue as we have observed that it ensures enough sample for bacteria to ferment[16]. Additionally, because it is known that (on average) 10% of the supposedly absorbable fraction in the large intestine is actually not absorbed, we also add the corresponding volume of the *in vitro* digestion supernatant (absorbable fraction reaching the large intestine). This volume is calculated as follows: we first calculate 10% (vol/vol) of the supernatant, which corresponds to the total amount of solid residue (not digested fraction) available from that digestion reaction. As we will only ferment 0.5g of solid residue, we then calculate the proportional volume of supernatant to add. Example: if the *in vitro* digested reaction had 3g of solid residue and 37 mL of supernatant; 10% of supernatant is 3.7 mL, which would correspond to 3g of solid residue. So, to ferment 0.5g of solid residue, we would add 0.5*3.7/3 = 0.62 mL of supernatant.

Stage 4: Fecal slurry preparation (Step 6)

Here we use a 32% (wt/vol) inocula (fecal slurry) in phosphate buffer adjusted to pH 7.0 with HCl 0.1M (instructions for preparation in Reagent setup). With 32% (wt/vol) of feces we ensure an appropriate bacterial cell density, and the inocula can function as both a source of microbes and a source of nutrients for the microbes, instead of using a more complex medium. To prepare the inocula, if the feces were frozen, they first have to be thawed at room temperature. Feces manipulation has to be performed in an anaerobic chamber (80% N_2, 10% CO_2 and 10% H_2). Once thawed, glycerol has to be removed by centrifuging at 4000 x g for 10 min at 4 ºC, keeping the pellet and discarding the supernatant. The fecal pellet is resuspended in phosphate buffer 0.1M pH 7.0 at a concentration of 32% fecal pellet:phosphate buffer (wt/vol). Right after, once the feces have been mixed with the phosphate buffer, they have to be properly homogenized using a vortex for 1 minute. Secondly, the fecal suspension should be centrifuged to remove larger particles (550 x g for 5 min at room temperature). Since this centrifugation is only to remove large particles, we now keep the supernatant which is where bacterial cells are. This supernatant

is the fecal inocula. The fecal inocula is then added to each fermentation tube at 20% (vol/vol) in relation to the fermentation medium and substrate sample to be tested (Step 7). This concentration was tested before[16], observing how with 20% (wt/vol) of inocula a higher degradation of the substrate was achieved than with 10% (wt/vol), which resulted in a higher antioxidant capacity release**.** We recommend preparing 1.5 or 2x the inocula volume needed. For instance: for 10 fermentation tubes, the inocula volume needed would be 20 mL (2 mL each, if final tube volume is 10 mL, 20% vol/vol). However, the inocula volume to be prepared should be 30-40 mL, as after centrifuging the fecal suspension, some volume will remain in the sedimented pellet and therefore will not be accessible, thus some extra volume should be prepared. During this stage, it is important to take samples of the fecal slurry to determine baseline conditions, which will be needed for subsequent data analysis. Baseline sampling should be performed according to the aim of the experiment and subsequent analysis methods that will be used (metabolomics, 16S rRNA or amplicon sequencing, shotgun metagenomics, metatranscriptomics, etc.). Some examples of further analysis usually performed and initial baseline sampling strategies are as follows:

- Metabolomics analysis: the aim of these experiments will be to measure the presence of certain metabolites. Typical metabolites measured as result of *in vitro* fermentation are SCFA. In this case, it is enough to take 1 mL of the fecal slurry into a tube and store it at -80 ºC (for no longer than a month since some molecules, such as some polyphenols, can breakdown after prolonged storage).

- 16S rRNA amplicon sequencing or shotgun metagenomics analysis: here it is especially important to keep sterile conditions to avoid contamination with environmental bacteria, or bacteria from the researcher. It is enough to take 1 mL of the fecal slurry. Right after, the tubes have to be centrifuged (16000 x g, 2 min) to remove the supernatant and keep the bacterial pellet. The tubes have to be stored at -80 ºC as quickly as possible. Samples stored at -80ºC are stable for 3-6 months.

- Metatranscriptomics analysis: the bacterial pellet has to be stored in RNAlater for RNA preservation and kept at -80 ºC for up to a month.

- Measure activity of potentially harmful microbial enzymes (beta-glucosidase, beta-glucuronidase, tryptophanase, urease): obtain the bacterial pellet as for 16S rRNA amplicon sequencing or shotgun metagenomics and store it at -80 ºC for up to 3 months.

Stage 5: Fermentation (Steps 7-9)

The fermentation involves mixing the following different components into sterile tubes: medium, inocula (fecal slurry), sample from *in vitro* digestion (substrate), and 10% (vol/vol) of the digestion supernatant volume. This process should be performed under anaerobic conditions in an anaerobic workstation (80% N_2, 10% CO_2 and 10% H_2). The amount of substrate and volume of fecal slurry were previously tested[16]. Once the different components have been added to the tube, N_2 is bubbled for 1 minute and tubes are placed at 37 ºC with oscillation at 20 rpm for 20 hours. The final volume inside the fermentation tube is 10 mL plus the 10% of the volume of the *in vitro* digestion supernatant, so a 15 mL tube would be sufficient. However, due to gas production during fermentation a bigger headspace is needed and hence, 50 mL tubes are recommended.

Stage 6: Sampling and storage (Step 10)

Once incubation has finished, to stop microbial fermentation the tubes are submerged in ice for 15 minutes and aliquots are taken as needed at the end of the 15 minutes and keeping the tubes in the ice (see **Table 3**). Additionally, samples can be taken during fermentation at desired timepoints if, for instance, the researcher wants to study the production of a certain metabolite over time. In this case, tubes must not be submerged in ice and anaerobic as well as aseptic conditions must be kept while sampling. Sampling should be performed according to the aim of the experiment and subsequent analysis (metabolomics, 16S rRNA amplicon sequencing or shotgun metagenomics, metatranscriptomics, etc.),

as described above for baseline sampling (see Experimental design, Stage 4). Further information about the sampling procedure can be found in **Table 3.**

Stage 7: Sample Processing (Step 11)

Since the water content of the sample will change during the prior *in vitro* digestion (digestion will usually increase water content of the sample, resulting in a solid residue with a lower percentage of solid matter than the original food), a critical point is to measure the water content of the original food sample (if it is food), the undigested solid residue, and the unfermented solid residue (if any). By calculating the water content, we will know the solid matter submitted to digestion and fermentation and what is left after fermenting, which will allow us to go back in the calculations and determine the amount of actual digested and fermented food. Water content measurements are useful to express results (for instance, metabolite concentration) per unit of mass of the original food and secondly, to know the amount of digested food used by gut bacteria. Water content of original food or undigested solid residue can be measured by weighing 1g of each and heating to 100 ºC on a stove for 2 hours, or longer (samples with high water content could need up to three hours). Water content can be calculated by determining the weight loss (see Step 11A). However, to measure the water content of the unfermented residue, we recommend lyophilization, which is more expensive since this equipment is not available in all laboratories, but may be necessary as there will be very little residue after sampling.

Sample processing will depend on the analysis to be performed afterwards. As described in Anticipated results section, some possible goals of *in vitro* fermentation are to:

- Investigate substrate degradation. This data will give information about how much of a given food has been degraded by the gut microbial populations, see Step 11A for detailed information on how to proceed.

- Investigate metabolism of phytochemicals and gut microbial functionality. Here we will rely on metabolomics analysis. Some typical metabolites measured as result of *in vitro* fermentation are SCFA and polyphenols. Microbial metabolization of phenolic compounds has been extensively studied. Since most phenolic compounds are not absorbed in the small intestine and reach the colon, the data obtained using in vitro fermentation will enable study of how they are metabolized by the gut microbiota and which metabolites are available for absorption in the large intestine. SCFA are the main metabolites resulting from microbial fermentation of food, especially fiber, and have proven to be beneficial for human health, and their production from given foods is important to understand for dietary advice purposes. Prior to metabolomics analysis, sampled tubes are centrifuged and the supernatant is treated as described in Step 11B.

- Investigate gut microbial community structure. Depending on the technology used here - 16S rRNA amplicon sequencing or shotgun metagenomics analysis (enabling analysis of the whole bacterial genome) - the data set will provide phylogenetic information up to genus or species, respectively. The first scenario is the most commonly used since it is cheaper and these experiments are usually initial screenings, with the aim to select specific conditions for further exploration. However, 16S RNA data also has its downside as has been reported when screening for prebiotics[57]. Sometimes, prebiotics only have an effect on certain species of the same genus which 16S RNA is not usually able to detect, since the species that don't change mask the affected ones. There are however bioinformatic approaches that can estimate up to species level. Regardless of the approach, such investigations generate a huge amount of data, which makes its analysis difficult. Therefore, a proper methodology to obtain meaningful and valid conclusions has to be performed. Paliy and Shankar[58] reviewed the main statistical approaches available to give meaning to genomic data.

This data will give information about the different bacteria present in the gut. Many of them have been linked to either beneficial or detrimental effects on human health. Thus, investigating which

microbes can be favored or inhibited by given foods could prove essential for dietary advice. For 16S rRNA amplicon sequencing or shotgun metagenomics analysis, sample preparation is described in Step 11C.

Control tubes

Control tubes are used to control for the effect of anything that is not the sample (i.e. food or food component) being studied. The following control tubes should be prepared:

- Control tube from *in vitro* digestion. Its preparation is thoroughly described in the *in vitro* digestion protocol[15]. This control tube is important because it contains all salts and enzymes, which can be used by gut microbes.

- Control tube for *in vitro* fermentation A. This tube should carry milli-Q water instead of the substrate sample (i.e. food or food component), in an equivalent volume. Therefore, 7.5 mL of fermentation medium, 2 mL of 32% inocula (fecal slurry) and 0.5 mL of milli-Q water. This tube will allow users to control for the effect of the fermentation medium on the gut microbiota.

- Control tube for *in vitro* fermentation B. This tube will carry the solid residue from control tube from *in vitro* digestion (0.5g) and 7.5 mL of fermentation medium and 2 mL of 32% inocula (fecal slurry). This tube will allow users to control for the effect of the enzymes and salts used in the *in vitro* digestion on the gut microbiota.

Throughput

One of the main advantages of this protocol is being able to test many different foodstuffs or food components within the same experiment. Therefore, is a perfect screening tool to search for relations between bacteria and food. Examples of the numbers of food samples we tested in some of our studies are as follows: In ref. 59 we tested 127 vegetable samples x 3 (triplicates), performed during seven days

(around 50 fermentations per day); In ref. 17 we tested 15 foodstuff samples x 3 (triplicates); in ref. 18, 6 coffee samples x 3 (triplicates); in ref. 19, 5 salami samples x 3 (triplicates); in ref. 20, 12 coffee samples x 3 (triplicates); and in ref. 21, 10 melanoidin samples x 3 (triplicates).

Reproducibility

Reproducibility was tested by repeating this protocol on 6 consecutive days to ferment lentils using fecal material from 4 celiac, 4 obese and 4 lean adults. The fecal material from the 4 individuals with each condition was pooled together to obtain three fecal slurry samples, one from celiac condition (n_{celiac}=4) one from obese (n_{obese}=4) and one from lean (n_{lean}=4). The *in vitro* fermentation protocol was run for each fecal slurry sample (i.e. three fermentations) and repeated 6 times on consecutive days. Each day, aliquots were taken and the gut microbial community was investigated via 16S rRNA sequencing. We obtained the relative abundance (% of each bacteria with respect to the total community) of each gut bacteria, at the genus level, present in the microbial community (i.e. we learned which bacteria were present and in which proportions). In order to assess whether these gut microbial communities significantly differed from day to day, dissimilarity between samples was calculated according to Bray-Curtis method and results were graphically depicted in PCoA. This approach allowed us to calculate similarity or dissimilarity between samples (i.e. lean, obese and celiac subjects) according to a set of variables (i.e. each bacteria genus forming the microbial population). Basically, this depicts how different or similar microbial communities from each day are. As depicted in **figure 2**, samples (dots) are grouped together according the different individuals with very little distance between dots belonging to the same group. This indicates that microbial communities are very similar regardless of the day the aliquot was taken and no significant ($p < 0.05$) differences between days was found. On the other hand, the large

distance observed between groups, demonstrates how different microbial communities from lean, obese and celiac people can be. This protocol is able to reflect such differences.

Materials

Biological materials

- Fecal material ! CAUTION: Use proper personal protective equipment and work in an anaerobic chamber while handling feces. When possible, use sterile plastic material that can be discarded afterwards. These materials used for feces handling and inocula preparation must be discarded into a biological hazards container. Material not to be discarded should be washed with bleach (10-20%) and/or autoclaved. CAUTION: Ethical approval to work with human feces has to be obtained from the Ethics Committee of the interested institution. A fecal sample volunteer information sheet and collection consent has to be prepared.

Reagents

- Ultrapure type I water, generated by a Milli-Q system or similar
- Sodium phosphate monobasic dihydrate (Merck, cat. no. 567550)
- NaOH (Merck, cat. no. 9141) ! CAUTION: NaOH is corrosive and causes severe skin burns and eye damage. Use proper personal protective equipment.
- HCl (J. T. Baker, cat. no. 6081) ! CAUTION: HCl is corrosive, causes burns and is irritating to the respiratory system. Use proper personal protective equipment and work in a fume hood while handling it.
- Peptone (Sigma-Aldrich, cat. no. T7293)
- L-cysteine (Sigma-Aldrich, cat. no. 168149)
- Resazurin sodium salt (Sigma-Aldrich, cat. no. 199303)

- Sodium sulfide hydrate (Sigma-Aldrich, cat. no. 14738)

Equipment

- Standard laboratory centrifuge suitable for 50 mL tubes (Sigma 2-16KL 5710350, Sigma).
- Standard laboratory centrifuge suitable for Eppendorf tubes (Labnet Spectrafuge 24D LA-C2400, Labnet).
- Standard laboratory pH meter (Laqua-PH1100 3200674407, Horiba Scientific)
- Standard laboratory vortex (Select Vortexer SBS100-2, Select Bioproducts)
- Milli-Q water system (Synergy UV F2PA71772C, Merck)
- Fecal collection kits (Fisherbrand Commode Specimen Collection System 02-544-208, Fisher Scientific, part of Thermo Fisher Scientific)
- Gas installation (nitrogen, carbon dioxide and hydrogen)
- Anaerobic chamber (Whitley A25 Workstation, Don Whitley Scientific)
- Oscillator able to hold 50 mL tubes (IKA Rocker 2D digital 0004003000, IKA)
- Incubator large enough as for the oscillator to fit inside (Universal Incubator Memmert UF75, Memmert)
- -80 ºC freezer (Lab Care Plus ULF50086, Infrico Medcare)
- Basic benchtop laboratory freeze dryer (LyoQuest -85ºC 58201, Telstar)
- Eppendorf tubes (1.5 and 2 mL) (Deltalab ref.: 200400P (1.5 mL) and 4092.6N (2 mL))
- 50 mL centrifuge tubes (Deltalab, ref.: 429926)
- Micropipettes and tips (Gilson P1000 F167550, sterile tips Neptune BT1000.95)
- Glass bakers and volumetric flasks.
- Precision Balance (Radwag PS4500.X2, Radwag Balances and Scales, WL-212-0134)

- Analytical Balance (Radwag AS 82/220.R2, Radwag Balances and Scales, WL-104-1051)
- Magnetic stirrer (SinerLab MS-H-Pro+ SN74915, SinerLab)

Reagent setup

- Phosphate buffer solution: prepare the phosphate buffer at 0.1 M concentration and adjust the pH to 7.0 with 1M HCl. Dissolve the reagent in less volume of milli-Q water than the final volume, adjust the pH and then, make up to the final volume with water. Phosphate buffer can be stored for up to a month at room temperature (always checking for salt precipitation) though pH has to be checked prior use, corrected if needed, and the solution autoclaved. Sterile Milli-Q water to make up for the volume loss during autoclaving has to be added.
- Peptone solution. Prepare the peptone solution by dissolving 15 g of peptone in almost a liter of milli-Q water, adjust the pH to 7.0 and then make up to 1 L with water. Make freshly before use. Autoclave before use. Volume lost during autoclaving will have to be compensated with sterile milli-Q water. **CRITICAL**: if the volume is not corrected, the concentration of nutrients will vary and experiments will not be reproducible. In addition, it is critical to avoid contamination afterwards.
- Reductive solution. Prepare the reductive solution by dissolving 312 mg of cysteine and 312 mg of sodium sulphide in 2 mL of 1M NaOH and make up the volume to 50 mL with milli-Q water. Make freshly before use. **CRITICAL STEP**: cysteine is sensitive to thermal treatment so it cannot be autoclaved. Instead, reductive solution has to be prepared in sterile conditions and under anaerobic environment (80% N_2, 10% CO_2 and 10% H_2).
- Resazurin solution. Prepare resazurin solution at 0.1% (w/v). To prepare this solution, weigh 1 mg of resazurin and dissolve it in 1 mL of milli-Q water. Only 1.25 mL of resazurin solution is

needed for each liter of fermentation medium, so usually it is enough with 2 mL of resazurin. Make freshly before use. Autoclave before use. Volume lost during autoclaving will have to be compensated with sterile milli-Q water. **CRITICAL**: if the volume is not corrected, the concentration of nutrients will vary and experiments will not be reproducible. In addition, it is critical to avoid contamination afterwards.

- Final fermentation medium: mix 1 liter of peptone solution with 50 mL of reductive solution and 1.25 mL of resazurin for each liter of fermentation medium. **CRITICAL STEP**: this has to be carried out under anaerobic and sterile conditions to avoid contamination.

Procedure

In vitro fermentation. Timing: 5-7 hours to prepare the equipment, reagents and tubes before incubating plus 20 hours of incubation and 1 hour for sampling. Timing is estimated for 50 fermentation tubes.

1 *Fecal material collection.* Fecal material must be always collected using sterile conditions: sterile container, using gloves and with help of a disposable sterile spoon. Another option is the use of stool collectors (i.e.: FECOTAINER, AT Medical BV, The Netherlands). Once collected, fecal material can be stored by the volunteer in their home refrigerator and transported to the laboratory in a cooler bag within 24 hours. Upon arriving at the laboratory, mix the feces with a water:glycerol solution (20% vol/vol) and store at -80 ºC. For detailed instructions see Experimental Design, Stage 1.

2 *Reagent setup.* Prepare reagents, including phosphate buffer 0.1M pH 7 for fecal slurry preparation and fermentation media. Reagents should be prepared the day before the experiment as well as autoclaved. For detailed instructions on preparing the different reagents, see

Experimental design, Stage 2 and Reagent Setup section. **CRITICAL STEP**: Note that cysteine cannot be autoclaved as it is heat sensitive.

3 *Equipment setup.* Autoclave all utensils that will be used. This should be performed the day before the experiment keeping utensils wrapped in foil after autoclaving. For detailed instructions see Experimental design, Stage 2 and Equipment section.

4 *Fermentation Setup.* Weigh the substrate samples into their corresponding 50 mL tubes. The amount of sample to be used is 0.5 g of the solid residue left after centrifuging the *in vitro* digestion reaction. Homogenization of the solid residue is needed, which can be done with a stainless steel sterile spatula **? TROUBLESHOOTING; CRITICAL STEP**: It is mandatory to weigh the empty tube first in order to calculate the remaining solid residue after fermentation.

5 Add 10% of the *in vitro* digestion supernatant (see Experimental Design, Stage 3 for how to calculate the amount).

6 Using the fecal sample from Step 1, prepare the inocula at 32% (wt/vol) feces/phosphate buffer 0.1M at pH 7.0. Right after centrifuging, we recommend to move the inocula with care to other vessel, so large particles remain in the centrifuged tube. Take baseline samples from the fecal inocula. See Experimental Design, Stage 4 for further information. **CRITICAL STEP**: It is especially important to keep conditions as sterile and anaerobic as possible to avoid contamination and death of strictly anaerobic bacteria. **CRITICAL STEP**: Do not centrifuge above 550 xg in order to avoid sedimentation of bacterial cells.

7 Under anaerobic (80% N_2, 10% CO_2 and 10% H_2) and aseptic conditions, add 7.5 mL of the fermentation medium (from Step 2) and 2 mL of fecal inocula (from Step 6) to each fermentation tube containing the weighed sample and supernatant (from Step 5). Bubble nitrogen for 1 min to remove any oxygen that might have entered the tube. Also set up control tubes as described in the Experimental Design. **? TROUBLESHOOTING**

8 Note down the final volume added to the tube. **CRITICAL STEP**: needed for future calculations.

9 *Fermentation.* Incubate the tubes for 20 hours at 37 ºC under oscillation at 20 rpm. **? TROUBLESHOOTING**

10 *Sampling.* To stop fermentation, submerge the tubes in ice for 15 minutes, then quickly agitate the tube and take aliquots, keeping the tubes in ice. Additionally, samples can be taken during fermentation at desired timepoints if, for instance, the researcher wants to study the production of a certain metabolite over time. In this case, tubes must not be submerged in ice and anaerobic as well as aseptic conditions must be kept while sampling. Sampling should be performed, depending on the analysis, as described in **Table 3** (and see Experimental Design, Stage 6 for further details)**.** We recommend to take 2 mL for each desired further experiment and store in two separate 1 mL tubes at -80 ºC until analysis, that way there is no need to open and defrost the same sample more than once. **CRITICAL STEP**: Note that such volumes are valid for the types of analyses described in this protocol, but other analyses may require larger volumes. **CRITICAL STEP**: For sampling it is essential to firstly agitate the tube to ensure that bacterial communities or compounds of interest have not sedimented during their time in ice.

11 *Sampling processing*. Sampling processing should be performed, depending on the analysis, as follows. Some examples of results previously obtained using this protocol are described in **Anticipated Results** section. These were obtained according to the following procedures – use option A to assess substrate degradation (see **Table 5**), option B for metabolite analysis, or option C for shotgun metagenomics and 16S rRNA analysis (and see Experimental Design, Stage 7 for further details):

.A **Substrate degradation. (for 50 samples, 4h if using a stove, 45h if using lyophilization).**

i Perform dry matter calculation on samples of the original substrate (food) submitted to in vitro digestion, the solid residue from *in vitro* digestion (used as the fermentation input sample), and

the unfermented residue left after *in vitro* fermentation. To calculate the water content of samples, firstly weigh 1g (this is not a fixed amount) of the given food tested, the solid residue obtained after *in vitro* digestion (fermentation input) and the solid residue left after the *in vitro* fermentation (unfermented residue).

ii Remove water content by heating on a stove at 100 ºC for 2 hours or by lyophilization. If the latter option is chosen, samples have to be first frozen at -80 ºC. Once frozen, they can be introduced into the lyophilizer (quickly to prevent samples from thawing) and lyophilized following the instructions of the manufacturer. Lyophilization of 1g of sample will take about 6 hours (see Experimental Design, Stage 7 for further information).

iii Calculate the water content of each sample as follows: Weight before drying - weight after drying = water content.

iv Calculate the dry matter of each sample as follows: g of sample - (g of sample* % water content/100)

v Use the dry matter to calculate how much food has been degraded, as follows:

food degraded during *in vitro* digestion (%): (dry matter of food submitted to *in vitro* digestion (g) – dry matter of solid residue after *in vitro* digestion (g))*100/ dry matter of food submitted to *in vitro* digestion (g). See Table 5, Digested % column.

food degraded during *in vitro* fermentation (%): (dry matter of sample submitted to *in vitro* fermentation (g) – dry matter of solid residue after *in vitro* fermentation (g))*100/ dry matter of sample submitted to *in vitro* digestion (g). See Table 5, Fermented % column.

food not degraded (final solid residue after *in vitro* fermentation, (%): (dry matter of solid residue after *in vitro* fermentation (g))*100)/ dry matter of food submitted to *in vitro* digestion (g) . See Table 5, Final solid residue % column.

vi Using this data, express results per unit of food digested or fermented.

.B Metabolite analysis (for 50 samples, phenolic analysis takes 2 days for extraction and 2 days for HPLC analysis. For SCFA, 1 day to prepare the samples and 1 day for HPLC analysis).

CRITICAL: Sample processing can be very different depending on the metabolites under study. In the examples shown in the Anticipated results, we specifically describe phenolic compounds and short chain fatty acids (SCFA).

i *Phenolic extraction.* For phenolic compounds, firstly extract with an organic solvent, one commonly used is diethyl ether. Although the process is described in references 18 and 21, the extraction procedure is as follows: Firstly, mix 1 mL of fermentation liquid with 1 mL of diethyl ether (relation 50:50, vol/vol) and store at 4ºC for 24 hours in darkness.

ii - Recover the organic fraction and put into another clean 10 mL tube. CRITICAL STEP: The organic fraction will be the one beneath the aqueous phase.

iii Again add 1 mL of diethyl ether to the fermentation liquid (second extraction), mix carefully and manually to avoid foam, and recover the organic fraction into the same 10 mL tube.

iv Repeat one more time, to obtain 3 mL of organic solvent with dissolved polyphenols.

v Evaporate the solvent (diethyl ether) in a rotatory evaporator with water bath (at 30ºC) and resuspend the polyphenols in 1 mL of milli-Q water:methanol 50:50 (vol/vol).

vi *Phenolic HPLC analysis.* Once the extraction is complete, perform identification and quantification of the compounds via HPLC-UV or, ideally, HPLC-MS as described in refs [19, 22].

vii *SCFA analysis.* To analyse SCFA, centrifuge samples (16000 xg, 2 min) and filter through a 0.22 µm nylon filter. Right after, SCFA can be identified by HPLC-UV or HPLC-RI as described in refs [17, 18].

.C **Shotgun metagenomics and 16S rRNA analysis (for 50 samples, 1 week to extract and amplify DNA, 1 week for sequencing and analysis, though shotgun metagenomics bioinformatic analysis will take longer).**

i Extract DNA following the instructions of the extraction kit company.

ii Amplify DNA via PCR, as described in ref. [18], for example.

iii Pool DNA and sequence following the instructions of the sequencing machine manufacturer.

iv For 16S rRNA analysis, assign DNA reads to specific taxonomies via different bioinformatic tools, as described in refs [17-21]. For shotgun metagenomics, sequenced gene fragments have to be assembled into complete genomes before taxonomy annotation which requires further bioinformatic processing as described in ref [60].

Timing (estimated for 50 fermentation tubes)

Steps 2-3. Reagent preparation and autoclaving material: 2-3 hours.

Steps 4-5. Weigh the samples into fermentation tubes: highly dependent on the number of samples, for 50 samples, 1.5 - 2 hours.

Step 6. Prepare the fecal slurry (inocula): highly dependent on the number of samples. For 50 samples, 1 hour.

Steps 7-8. Adding components to fermentation tubes: highly dependent on the number of samples. For 50 samples, 0.5 hour.

Step 9. Incubation: 20 hours

Step 10. Cooling, sampling and storage: 15 minutes for cooling. Sampling is highly dependent on the number of aliquots to be taken. For three aliquots per sample, 1 hour.

Step 11. Sample Processing:

Option A: Substrate degradation: 4 hours if using a stove and 45 hours if using lyophilization.

Option B: Metabolite analysis: for phenolic analysis, 30-32 hours for extraction and 48 hours for identification and quantification. For SCFA, 4 hours for extraction and 24 hours for identification and quantification.

Option C: Shotgun Metagenomics and 16S RNA analysis: two weeks. However, this timing is highly dependent on the laboratory resources, specifically on whether the laboratory owns an automated DNA extraction system, which would reduce the timing by approximately one week.

Troubleshooting

See **Table 4** for troubleshooting advice.

Anticipated results

Substrate degradation

In vitro fermentation can be used to study microbial degradation of a substrate of interest[16]. These data could provide information about microbial capabilities to use specific foodstuffs. In a previous experiment, we studied the fermentability of several foodstuffs (see **Table 5**, adapted from ref. 16).

Reproducibility was also tested, showing an inter-day variation coefficient of 5.18% and an intra-day variation coefficient of 5.26%.

Study of phytochemicals and microbial metabolic pathways

We studied phenolic compounds released from green and roasted coffee[18] and melanoidins[21] after microbial fermentation as well as their metabolites. Coffee brew fermentation yielded much higher amounts of 4'-Hydroxy-3'-methoxycinnamic acid (ferulic acid), 4-Hydroxybenzoic acid and 4'-Hydroxyphenyl-acetic acid than found in coffee brew before fermentation, all related to chlorogenic acid microbial degradation[18]. In another study, the fermentation of melanoidins yielded high concentrations of different benzoic acids (C_6-C_1), related to phenolic degradation[25]. For fermented coffee and chocolate melanoidins, high concentrations of Benzene-1,2,3-triol (pyrogallol) were found. The latter is a flavan-3-ol metabolite[24], and flavan-3-ol is known to be incorporated into the structure of coffee and chocolate melanoidins[61].

The data obtained in the previously mentioned experiments is not only useful to understand how different molecules are metabolized by gut microbes, but also to be able to predict the metabolic behavior of such bacteria in different contexts by building metabolic networks and constraint-based modeling[46,62]. In a previous study, this approach was applied to unravel the metabolic changes that occur during the first year of life in the gut microbiota of a cohort of Spanish infants[35]. This study showed how the introduction of solid food generated a different signature of metabolites released by gut microbes which was validated through experimental data.

Gut microbial functionality

Gut microbial functionality is most commonly measured through SCFA (mainly acetate, propionate and butyrate) production since they are the main microbial fermentation products[1]. According to epidemiological data, high fiber-low fat and low meat diets result in a higher SCFA production than those with low fiber consumption[6,63]. Therefore, foods with higher fiber content or added fiber should in theory increase SCFA production. This was demonstrated in previous batch fermentations experiments: in one of them, mannoligosaccharides increased SCFA in a dose dependent manner[20]; in another one, the addition of different fibers to salami increased SCFA production, especially butyrate production[19], which agrees with data found *in vivo*[6], and also with the results found of a human intervention where the same salami was tested[44]. It is expected to find higher concentrations of SCFA after fermenting fiber rich foodstuffs, an expectation that was confirmed in a previous experiment where fermentations of pepper, bread, banana or chickpeas showed higher SCFA concentrations than with chicken[17]. If the experiment also involves microbial analysis (16S rRNA amplicon sequencing or shotgun metagenomics), it is expected to find some correlations with SCFA-producing bacteria[17]., such as *Ruminococcus* or *Bifidobacterium*, which are major acetate producing genera, *Faecalibacterium* and *Eubacterium*, the top butyrate producers, and *Roseburia* or *Blautia* that are the main propionate producers[1].

Other fermentation metabolites, mainly fumarate, succinate and lactate, involved in cross-feeding mechanisms, can also be assessed. However, they are usually found in very low concentrations since they are used by different bacteria; for example, lactate can be used to produce propionate and butyrate[25]. Regardless, in certain circumstances they can be useful; for instance, lactate is abnormally high in patients with ulcerative colitis[64].

Acetate, propionate and butyrate are the main metabolites from carbohydrate degradation. However, gut microbes have also an important proteolytic activity, which yields different compounds such as peptides, amino acids, branched chain fatty acids or amonia[25]. Some of the protein-derived metabolites are negatively associated with host health: such as trimethylamine, ammonia or hydrogen

sulfide[4]. According to Shankar et al.[4], a typical western diet, rich in animal products and refined cereals, is characterized by a predominance of proteolytic microbial communities, whereas populations with fiber rich diets are characterized by bacteria responsible for carbohydrate degradation. Therefore, the measurement of both SCFA and protein metabolites could give a preliminary view of the metabolic preferences of gut microbes and indicate how the microbial functionality can be shifted depending on the substrate given.

Specific health conditions may also affect gut microbial composition and, hence, their functionality. For instance, SCFA concentration in feces of obese/overweight people and celiac patients is higher than in lean and healthy people[63,65]. We tested this protocol using fecal material from lean (n=4), celiac (n=4) and obese (n=4) people to ferment lentils (**Figure 3)**. The results obtained with this protocol are in accordance with those described by Fernandes et al.[63] and Nistal et al.[65] who observed how acetate, propionate, and butyrate concentration were higher in obese[63] and celiac[65] people than in lean or healthy people. However, our results showed how differences were only statistically significant ($p < 0.05$) in the case of butyrate production between groups and in propionate production between lean and celiac people. It has been reported that the gut microbiota associated with obese people is able to scavenge higher amounts of energy from substrates, hence higher production of SCFA[63]. In the case of celiac people, the differences between this group and lean people are not as clear. These results show how the protocol is able to reflect the SCFA production ability of the original feces. In this sense, the *in vitro* batch fermentation protocol described here has been successfully used in several studies to measure SCFA production [17-21].

Gut microbial community structure

One of the main outcomes of *in vitro* fermentations is data regarding gut microbial community structure. *In vitro* batch fermentations are especially useful to explore how gut microbes can use different foodstuffs or molecules.

Two typical data sets obtained are those in which the aim is to identify differences in microbial community structure after fermenting different foodstuffs, and those in which the aim is to study the effects of potential prebiotic agents. In a previous research project, we investigated the effect that different foodstuffs (chicken, chickpeas, pepper, bread and banana) could have on gut microbial structure[17]. Through multivariable analysis, we found a clear difference between the communities exposed to the different foods (**Figure 4)**, with the structure of the communities given protein-rich foods closer to one another, starchy foods also closer to each other, and the fiber rich food (pepper) separated from the rest. Interesting information regarding SCFA producing genera was also found, which was backed by existing literature, as *Ruminococcus* was found in higher abundance in communities given starchy foodstuffs[66], and a higher abundance of butyrate producing genera were observed with higher fiber content foods [6]. We also investigated the potential use of food melanoidins as prebiotic agents[21]. In this regard, we found that biscuit melanoidins stimulated the growth of *Faecalibacterium* (a butyrate producer) whereas others (such as bread crust melanoidins) stimulated the growth of *Bifidobacterium.*

References

1. Flint, H. J., Duncan, S. H., Scott, K. P. & Louis, P. Links between diet, gut microbiota composition and gut metabolism. *Proceedings of the Nutrition Society* **74**, 13–22 (2015).
2. Salazar, N. *et al.* Exopolysaccharides produced by Bifidobacterium longum IPLA E44 and Bifidobacterium animalis subsp. lactis IPLA R1 modify the composition and metabolic activity of human faecal microbiota in pH-controlled batch cultures. *International Journal of Food Microbiology* **135**, 260–267 (2009).

3. Power, S. E., O'Toole, P. W., Stanton, C., Ross, R. P. & Fitzgerald, G. F. Intestinal microbiota, diet and health. *British Journal of Nutrition* **111**, 387–402 (2014).
4. Shankar, V. *et al.* Differences in Gut Metabolites and Microbial Composition and Functions between Egyptian and U.S. Children Are Consistent with Their Diets. *mSystems* **2**, e00169-16 (2017).
5. David, L. A. *et al.* Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**, 559–563 (2013).
6. De Filippo, C. *et al.* Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci U S A* **107**, 14691–14696 (2010).
7. Venema, K. & van den Abbeele, P. Experimental models of the gut microbiome. *Best Practice & Research Clinical Gastroenterology* **27**, 115–126 (2013).
8. Molly, K., Vande Woestyne, M. & Verstraete, W. Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. *Appl Microbiol Biotechnol* **39**, 254–258 (1993).
9. Agans, R. *et al.* Dietary Fatty Acids Sustain the Growth of the Human Gut Microbiota. *Appl. Environ. Microbiol.* **84**, e01525-18 (2018).
10. Wiese, M. *et al.* CoMiniGut—a small volume *in vitro* colon model for the screening of gut microbial fermentation processes. *PeerJ* **6**, e4268 (2018).
11. Ludwig, I. A., Paz de Peña, M., Concepción, C. & Alan, C. Catabolism of coffee chlorogenic acids by human colonic microbiota: Colonic Catabolism of Coffee Chlorogenic Acids. *BioFactors* **39**, 623–632 (2013).
12. Coles, L. T., Moughan, P. J. & Darragh, A. J. In vitro digestion and fermentation methods, including gas production techniques, as applied to nutritive evaluation of foods in the hindgut of humans and other simple-stomached animals. *Animal Feed Science and Technology* **123–124**, 421–444 (2005).

13. Wang, M. *et al.* In vitro colonic fermentation of dietary fibers: Fermentation rate, short-chain fatty acid production and changes in microbiota. *Trends in Food Science & Technology* **88**, 1–9 (2019).
14. Mould, F. L., Morgan, R., Kliem, K. E. & Krystallidou, E. A review and simplification of the in vitro incubation medium. *Animal Feed Science and Technology* **123–124**, 155–172 (2005).
15. Brodkorb, A. *et al.* INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nat Protoc* **14**, 991–1014 (2019).
16. Pérez-Burillo, S., Rufián-Henares, J. A. & Pastoriza, S. Towards an improved global antioxidant response method (GAR+): Physiological-resembling in vitro digestion-fermentation method. *Food Chemistry* **239**, 1253–1262 (2018).
17. Pérez-Burillo, S. *et al.* Effect of Food Thermal Processing on the Composition of the Gut Microbiota. *J. Agric. Food Chem.* **66**, 11500–11509 (2018).
18. Pérez-Burillo, S. *et al.* Effect of in vitro digestion-fermentation on green and roasted coffee bioactivity: The role of the gut microbiota. *Food Chemistry* **279**, 252–259 (2019).
19. Pérez-Burillo, S. *et al.* Potential probiotic salami with dietary fiber modulates antioxidant capacity, short chain fatty acid production and gut microbiota community structure. *LWT* **105**, 355-362 (2019).
20. Pérez-Burillo, S. *et al.* Spent Coffee Grounds Extract, Rich in Mannooligosaccharides, Promotes a Healthier Gut Microbial Community in a Dose-Dependent Manner. *J. Agric. Food Chem.* **67**, 2500–2509 (2019).
21. Pérez-Burillo, S., Rajakaruna, S., Pastoriza, S., Paliy, O. & Rufián-Henares, J. A. Bioactivity of food melanoidins is mediated by gut microbiota. *Food Chemistry* **316**, 126309 (2020).
22. Rocchetti, G. *et al.* Transformation of polyphenols found in pigmented gluten-free flours during in vitro large intestinal fermentation. *Food Chemistry* **298**, 125068 (2019).

23. Jin, J. B. *et al.* Supplementation with Chlorella vulgaris, Chlorella protothecoides, and Schizochytrium sp. increases propionate-producing bacteria in in vitro human gut fermentation. *J Sci Food Agric* **100**, 2938–2945 (2020).
24. Selma, M. V., Espín, J. C. & Tomás-Barberán, F. A. Interaction between Phenolics and Gut Microbiota: Role in Human Health. *Journal of Agricultural and Food Chemistry* **57**, 6485–6501 (2009).
25. Rowland, I. *et al.* Gut microbiota functions: metabolism of nutrients and other food components. *Eur J Nutr* **57**, 1–24 (2018).
26. Tomas-Barberan, F. *et al.* In vitro transformation of chlorogenic acid by human gut microbiota. *Molecular Nutrition & Food Research* **58**, 1122–1131 (2014).
27. Saura-Calixto, F. *et al.* Proanthocyanidin metabolites associated with dietary fibre from in vitro colonic fermentation and proanthocyanidin metabolites in human plasma. *Molecular Nutrition & Food Research* **54**, 939–946 (2010).
28. Roowi, S. *et al.* Green Tea Flavan-3-ols: Colonic Degradation and Urinary Excretion of Catabolites by Humans. *J. Agric. Food Chem.* **58**, 1296–1304 (2010).
29. Jaganath, I. B., Mullen, W., Lean, M. E. J., Edwards, C. A. & Crozier, A. In vitro catabolism of rutin by human fecal bacteria and the antioxidant capacity of its catabolites. *Free Radical Biology and Medicine* **47**, 1180–1189 (2009).
30. Serra, A. *et al.* Metabolic pathways of the colonic metabolism of flavonoids (flavonols, flavones and flavanones) and phenolic acids. *Food Chemistry* **130**, 383–393 (2012).
31. Pinta, M. N. *et al.* In Vitro Gut Metabolism of [U-13C]-Quinic Acid, The Other Hydrolysis Product of Chlorogenic Acid. *Molecular Nutrition & Food Research* **62**, 1800396 (2018).
32. Hidalgo, M. *et al.* Metabolism of Anthocyanins by Human Gut Microflora and Their Influence on Gut Bacterial Growth. *J. Agric. Food Chem.* **60**, 3882–3890 (2012).

33. Marín, L., Miguélez, E. M., Villar, C. J. & Lombó, F. Bioavailability of Dietary Polyphenols and Gut Microbiota Metabolism: Antimicrobial Properties. *Biomed Res Int* **2015**, (2015).
34. Stevens, J. F. & Maier, C. S. The chemistry of gut microbial metabolism of polyphenols. *Phytochemistry Reviews* **15**, 425–444 (2016).
35. Fuertes, Á. *et al.* Adaptation of the human gut microbiota metabolic network during the first year after birth. *Front. Microbiol.* **10**, 848 (2019).
36. Ríos-Covián, D. *et al.* Intestinal Short Chain Fatty Acids and their Link with Diet and Human Health. *Front Microbiol* **7**, 185 (2016).
37. Shen, Q., Chen, Y. A. & Tuohy, K. M. A comparative in vitro investigation into the effects of cooked meats on the human faecal microbiota. *Anaerobe* **16**, 572–577 (2010).
38. Poelaert, C. *et al.* Cooking Has Variable Effects on the Fermentability in the Large Intestine of the Fraction of Meats, Grain Legumes, and Insects That Is Resistant to Digestion in the Small Intestine in an in Vitro Model of the Pig's Gastrointestinal Tract. *Journal of Agricultural and Food Chemistry* **65**, 435–444 (2017).
39. Lee, D. K. *et al.* The combination of mixed lactic acid bacteria and dietary fiber lowers serum cholesterol levels and fecal harmful enzyme activities in rats. *Arch. Pharm. Res.* **34**, 23–29 (2011).
40. Molska, M. & Regula, J. Potential Mechanisms of Probiotics Action in the Prevention and Treatment of Colorectal Cancer. *Nutrients* **11**, 2453 (2019).
41. Pham, V. T. & Mohajeri, M. H. The application of in vitro human intestinal models on the screening and development of pre- and probiotics. *Benef Microbes* **9**, 725–742 (2018).
42. Gu, F. *et al.* In Vitro Fermentation Behavior of Isomalto/Malto-Polysaccharides Using Human Fecal Inoculum Indicates Prebiotic Potential. *Molecular Nutrition & Food Research* **62**, e1800232 (2018).

43. Fehlbaum, S. *et al.* In Vitro Fermentation of Selected Prebiotics and Their Effects on the Composition and Activity of the Adult Gut Microbiota. *International Journal of Molecular Sciences* **19**, 3097 (2018).

44. Pérez-Burillo, S. *et al.* Potential probiotic salami with dietary fiber modulates metabolism and gut microbiota in a human intervention study. *Journal of Functional Foods* **66**, 103790 (2020).

45. Kolodziejczyk, A. A., Zheng, D. & Elinav, E. Diet–microbiota interactions and personalized nutrition. *Nature Reviews Microbiology* **17**, 742–753 (2019).

46. Magnúsdóttir, S. *et al.* Generation of genome-scale metabolic reconstructions for 773 members of the human gut microbiota. *Nature Biotechnology* **35**, 81–89 (2017).

47. Coyte, K. Z. & Rakoff-Nahoum, S. Understanding Competition and Cooperation within the Mammalian Gut Microbiome. *Current Biology* **29**, R538–R544 (2019).

48. Payne, A. N., Zihler, A., Chassard, C. & Lacroix, C. Advances and perspectives in in vitro human gut fermentation modeling. *Trends in Biotechnology* **30**, 17–25 (2012).

49. Cohen, S. M. Human relevance of animal carcinogenicity studies. *Regul Toxicol Pharmacol* **21**, 75–80; discussion 81-86 (1995).

50. Eckburg, P. B. *et al.* Diversity of the human intestinal microbial flora. *science* **308**, 1635–1638 (2005).

51. Ley, R. E. *et al.* Obesity alters gut microbial ecology. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 11070–11075 (2005).

52. Belkaid, Y. & Hand, T. W. Role of the Microbiota in Immunity and Inflammation. *Cell* **157**, 121–141 (2014).

53. Shankar, V. *et al.* The networks of human gut microbe-metabolite associations are different between health and irritable bowel syndrome. *ISME J* **9**, 1899–1903 (2015).

54. Aguirre, M. & Venema, K. Challenges in simulating the human gut for understanding the role of the microbiota in obesity. *Beneficial Microbes* **8**, 31–53 (2017).

55. Gratton, J. *et al.* Optimized Sample Handling Strategy for Metabolic Profiling of Human Feces. *Anal. Chem.* **88**, 4661–4668 (2016).

56. Gorzelak, M. A. *et al.* Methods for Improving Human Gut Microbiome Data by Reducing Variability through Sample Processing and Storage of Stool. *PLOS ONE* **10**, e0134802 (2015).

57. Long, W. *et al.* Differential responses of gut microbiota to the same prebiotic formula in oligotrophic and eutrophic batch fermentation systems. *Sci Rep* **5**, 1–11 (2015).

58. Paliy, O. & Shankar, V. Application of multivariate statistical techniques in microbial ecology. *Mol Ecol* **25**, 1032–1057 (2016).

59. Pérez-Burillo, S., Rufián-Henares, J. A. & Pastoriza, S. Effect of home cooking on the antioxidant capacity of vegetables: Relationship with Maillard reaction indicators. *Food Research International* **121**, 514-523 (2019).

60. Quince, C., Walker, A. W., Simpson, J. T., Loman, N. J. & Segata, N. Shotgun metagenomics, from sampling to analysis. *Nature Biotechnology* **35**, 833–844 (2017).

61. Morales, F. J., Somoza, V. & Fogliano, V. Physiological relevance of dietary melanoidins. *Amino Acids* **42**, 1097–1109 (2012).

62. Bauer, E. & Thiele, I. From Network Analysis to Functional Metabolic Modeling of the Human Gut Microbiota. *mSystems* **3**, 00209-17 (2018).

63. Fernandes, J., Su, W., Rahat-Rozenbloom, S., Wolever, T. M. S. & Comelli, E. M. Adiposity, gut microbiota and faecal short chain fatty acids are linked in adult humans. *Nutr & Diabetes* **4**, e121–e121 (2014).

64. Bjerrum, J. T. *et al.* Metabonomics of human fecal extracts characterize ulcerative colitis, Crohn's disease and healthy individuals. *Metabolomics* **11**, 122–133 (2015).

65. Nistal, E. *et al.* Differences in faecal bacteria populations and faecal bacteria metabolism in healthy adults and celiac disease patients. *Biochimie* **94**, 1724–1729 (2012).
66. Ze, X., Duncan, S. H., Louis, P. & Flint, H. J. Ruminococcus bromii is a keystone species for the degradation of resistant starch in the human colon. *ISME J* **6**, 1535–1543 (2012).
67. Zhou, L., Xie, M., Yang, F. & Liu, J. Antioxidant activity of high purity blueberry anthocyanins and the effects on human intestinal microbiota. *LWT* **117**, 108621 (2020).
68. Gonçalves, G. A. *et al.* Effects of in vitro gastrointestinal digestion and colonic fermentation on a rosemary (Rosmarinus officinalis L) extract rich in rosmarinic acid. *Food Chemistry* **271**, 393–400 (2019).
69. Chen, Y., Chang, S. K. C., Zhang, Y., Hsu, C.-Y. & Nannapaneni, R. Gut microbiota and short chain fatty acid composition as affected by legume type and processing methods as assessed by simulated in vitro digestion assays. *Food Chemistry* **312**, 126040 (2020).
70. del Hierro, J. N. *et al.* In Vitro Colonic Fermentation of Saponin-Rich Extracts from Quinoa, Lentil, and Fenugreek. Effect on Sapogenins Yield and Human Gut Microbiota. *J. Agric. Food Chem.* **68**, 106–116 (2020).
71. Zhang, X. *et al.* Phytochemical Profile, Bioactivity, and Prebiotic Potential of Bound Phenolics Released from Rice Bran Dietary Fiber during in Vitro Gastrointestinal Digestion and Colonic Fermentation. *J. Agric. Food Chem.* **67**, 12796–12805 (2019).
72. Wang, M. *et al.* Purified fraction of polysaccharides from Fuzhuan brick tea modulates the composition and metabolism of gut microbiota in anaerobic fermentation in vitro. *International Journal of Biological Macromolecules* **140**, 858–870 (2019).
73. Rui, Y. *et al.* Simulated digestion and fermentation in vitro by human gut microbiota of intra- and extra-cellular polysaccharides from Aspergillus cristatus. *LWT* **116**, 108508 (2019).

74. Chen, L. *et al.* Simulated digestion and fermentation in vitro by human gut microbiota of polysaccharides from Helicteres angustifolia L. *International Journal of Biological Macromolecules* **141**, 1065–1071 (2019).

75. Huang, F. *et al.* Structural characterization and in vitro gastrointestinal digestion and fermentation of litchi polysaccharide. *International Journal of Biological Macromolecules* **140**, 965–972 (2019).

1

2 **Author contributions statements**

3 Sergio P., S.M., and J.A.R.H. wrote the manuscript. B.N.P., A.V.M., D.H.N., A.L.M. and Silvia P.
4 contributed to the writing of the manuscript. Sergio P., S.M., B.N.P., A.V.M., D.H.N. and A.L.M.
5 contributed to formal analysis and investigation; Sergio P. developed the methodology. Silvia P. and
6 J.A.R.H. supervised the work. J.A.R.H. obtained founding and coordinates the EU project
7 Stance4Health.

8

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11

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16

17 **Competing interests**

18 The authors declare that they have no known competing financial interests or personal relationships that
19 could have appeared to influence the work reported in this paper.

20

Data availability statement

The data shown in Figure 4 is available from the supporting primary research paper previously published by Perez-Burillo et al.[17]. The data presented in Figs. 2&3 were generated for this protocol. The source data underlying Figs. 2&3 are provided as Source Data files with this protocol.

25

26 **Table 1.** Examples of batch fermentations found in literature from other research groups.

Prior *In vitro Digestion*	Medium	Food/Food component	Feces origin	Inocula	Time	Reference
No	2.0 g/L of peptone 2.0 g/L of yeast extract 0.02 g/L of hemin 0.5 g/L of cysteine 0.5 g/L of bile salts 2.0 mL/L of Tween 80 10 uL/L of vitamin K1 Several micro- and macrominerals	Blueberry anthocyanins	Fecal pool from 3 healthy volunteers	10 % feces/physiological saline solution with cysteine (w/v)	12-24 hours	Zhou et al.[67]
Yes	Carbonate-phosphate solution with glucose (0.8%)	Rosemary extract	Fecal pool from 5 Wistar rats	10 % feces/Culture medium (w/v)	24 hours	Gonçalves et al.[68]
Yes	37 g/L Brain Heart Infusion medium 0.25 g/L cysteine 4 g/L Na2CO3	Pinto bean and soybean	Fecal pool from three healthy pigs	33 % feces/Culture medium (w/v)	24 hours	Chen et al.[69]
No	2.0 g/L of peptone 2.0 g/L of yeast extract 0.02 g/L of hemin 0.5 g/L of cysteine 0.5 g/L of bile salts 2.0 mL/L of Tween 80 10 uL/L of vitamin K1 Several micro- and macrominerals	Saponin rich extracts from quinoa, lentils and fenugreek	Fecal pool from 3 healthy volunteers	10 % feces/Phosphate buffer (w/v)	72 hours (samples taken every 24 h)	del Hierro et al.[70]
Yes, no oral phase	2.5 g/L of peptone 2.5 g/L of tryptone 2.25 g/L of yeast extract 1 g/L of pectin 2 g/L of mucin 1.5 g/L of casein 1 g/L of arabinogalactan 0.5 g/L of guar 0.5 g/L of xylan	Bound phenolics from rice bran fiber	Fecal pool from 4 healthy volunteers	10 % feces/D-PBS buffer (w/v)	48 hours (samples taken at different time points)	Zhang et al.[71]

	0.4 g/L of cysteine 0.2 g/L of bile salts 0.5 mL of Tween 80					
No	2.0 g/L of peptone 2.0 g/L of yeast extract 0.02 g/L of hemin 0.5 g/L of cysteine 0.5 g/L of bile salts 2.0 mL/L of Tween 80 10 uL/L of vitamin K1 Several micro- and macrominerals	Purified polysaccharides from tea	Fecal pool from 4 healthy volunteers	Feces mixed with physiological saline buffer + cysteine No information given about ratio feces:buffer	36 hours (samples taken at different time points)	Wang et al.[72]
Yes	2.0 g/L of peptone 2.0 g/L of yeast extract 0.02 g/L of hemin 0.5 g/L of cysteine 0.5 g/L of bile salts 2.0 mL/L of Tween 80 10 uL/L of vitamin K1 Several micro- and macrominerals	Intra and extra-cellular polysaccharides from *Aspergillus cristatus*	Fecal pool from 4 healthy volunteers	10 % feces/physiological saline solution with cysteine (w/v)	24 hours	Rui et al.[73] (Same research group as Wang et al.[72])
Yes	4.0 g/L of yeast extract 2.0 g/L of peptone 0.46 g/L of cysteine 0.02 g/L of hemin 0.5 g/L of bile salts 2.0 mL/L of Tween 80 10 uL/L of vitamin K1	Polysaccharides from *Helicteres angustifolia L*	Fecal pool from 4 healthy volunteers	10 % feces/PBS (w/v)	24 hours	Chen et al.[74] (Same research group as Rui et al.[73])
Yes, no oral phase	Carbohydrate free medium 0.8 g/L of cysteine 0.4 g/L of bile salts 1 mL/L of Tween 80	Polysaccharide from litchi	Fecal pool from 4 healthy volunteers	12% feces/preculture medium (tryptone, glucose, maltose and yeast) Preculture time 12 hours	24 hours (samples taken at different time points)	Huang et al.[75]
Yes, no oral phase	Buffer solution	Polyphenols of flour	Fecal pool from 5 healthy pigs	5% feces/buffer (w/v)	24 hours (samples taken at different time points)	Rocchetti et al.[22]

Yes, 23 hours	Tryptone Resazurin Several micro- and macrominerals	Proanthocyanidins from carob pod	Fecal pool from5 Wistar rats	10% feces/medium (w/v)	24 hours	Saura-Calixto et al.[27]
No	10 g/L of tryptone 0.312 g/L of cysteine 0.312 g/L of sodium sulfide Several micro- and macrominerals	Coffee chlorogenic acids	Fecal samples individually tested from three healthy volunteers	32% feces/phosphate buffer (w/v)	6 hours (samples taken at different time points)	Ludwig et al.[11]
No	10 g/L of tryptone 0.312 g/L of cysteine 0.312 g/L of sodium sulfide Several micro- and macrominerals	Green tea flavan-3-ols	Fecal samples individually tested from five volunteers with ileostomy	32% feces/phosphate buffer (w/v)	48 hours (samples taken at different time points)	Roowi et al.[28]
No	5 g/L of tryptone 0.312 g/L of cysteine 0.312 g/L of sodium sulfide Several micro- and macrominerals	Rutin	Fecal samples individually tested from three healthy volunteers	32% feces/phosphate buffer (w/v)	48 hours (samples taken at different time points)	Jaganath et al.[29]

27

Table 2. Example of an *in vitro* fermentation reaction setup

	Solid residue, g	**Supernatant volume, mL**
***In vitro* digestion**	3	37
	Component	**Quantity**
***In vitro* fermentation**	Digestion solid residue/chemical of interest, g	0.5
	10% of digestion supernatant, mL	0.5*3.7/3 = 0.62
	Inocula (32% w/v; feces/phosphate buffer pH 7), mL	2
	Medium (peptone 15g/L + 50 mL of reductive solution/L of peptone), mL	7.5
	Bubble nitrogen	1 minute
	Incubation	Oscillation at 20rpm, 20 hours, 37 ºC

Table 3. Sampling timing and conditions

Application	Objective	Which samples to take/measurements to make, and when	Sampling procedure
Metabolomics	Measurement of different metabolites	Sample fecal inoculum* Weigh blank tube before incubation* Weigh sample tube before incubation* Weigh blank tube after incubation* Weigh sample tube after incubation* Take samples every X hours during incubation	Pipette 1 mL for each analysis foreseen and store it at -80 ºC.
16S rRNA amplicon sequencing or shotgun metagenomics	Explore gut microbial community structure	Sample Fecal inoculum* Weigh blank tube after incubation* Weigh sample tube after incubation* Take samples every X hours during incubation	Take 1 mL and right after centrifuge the tubes (16000 xg, 2 min at 4 ºC) to remove and discard the supernatant and keep the bacterial pellet. The tubes have to be stored at -80 ºC as quickly as possible.
Metatranscriptomics	Explore gut microbial gene expression	Sample fecal inoculum* Weigh blank tube after incubation* Weigh sample tube after incubation* Take samples every X hours during incubation	Obtain bacterial pellet as for 16S rRNA amplicon sequencing and store it in RNAlater to preserve RNA at -80 ºC.
Bacterial enzymes activity (beta-glucosidase, beta-glucuronidase, tryptophanase, urease)	Check the activity of potentially harmful enzymes	Sample fecal inoculum* Weigh blank tube after incubation* Weigh sample tube after incubation* Take samples every X hours during incubation	As for 16S amplicon sequencing or shotgun metagenomic

* denotes mandatory samplings

Table 4. Troubleshooting advice.

Step	Problem	Possible reason	Possible solution
3	Solid residue from *in vitro* digestion is often wet, viscous and hard to grab, even after centrifugation. This can result in taking a non-homogenous sample.	During *in vitro* digestion, substrate sample's structure is broken down and it incorporates water from the medium	Essential to carefully homogenize the solid residue from the *in vitro* digestion before adding it to the *in vitro* fermentation tube. This will ensure a homogenous and representative sample is taken. Homogenization can be done with a stainless-steel sterile spatula.
7	Difficulties pipetting due to large particles from fecal material that clog the tips	Due to the slow centrifugation in Step 6 (550 x g) some large particles from the fecal material could resuspend eventually if pipetting and adding the inocula to the different fermentation tubes takes too long.	Right after centrifuging, we recommend to move the inocula with care to other vessel, so large particles remain in the centrifuged tube.
9	Fermentation tubes break during the process	Due to gas accumulation during fermentation	We recommend using 50mL tubes rather than 15mL so there is more space for the gas and ensure no content spilling.

Table 5. Fermentability of different foodstuffs. Adapted from ref. [16].

	Initial amount, g	*In vitro* digestion		*In vitro* fermentation		
		Digested %*	**Non-digested %***	**Fermented %***	**Non-Fermented %***	**Final solid residue, %***
Whole grain bread	5.00	55.07	44.93	65.54	34.46	15.48
Lentils	5.00	60.48	39.52	55.32	44.68	17.66
Orange	5.00	36.83	63.17	65.05	34.95	22.08
Tomato	5.00	59.74	40.26	72.46	27.54	11.09
Yoghurt	5.00	86.34	13.66	76.39	23.61	3.23
Peanuts	5.00	4.19	95.81	26.88	73.12	70.06

* Calculations were performed as explained in Step 11A. of the Procedure.

FIGURES

Figure 1. *In vitro* fermentation process.

Figure 2. Reproducibility assessment. Principal Coordinates Analysis with Bray-Curtis dissimilarity distance of microbial genus abundance obtained after repeating the protocol on 6 consecutive days to *in vitro* ferment lentils using fecal material from lean (n=4), celiac (n=4) and obese (n=4) adults. Each dot represents the gut microbial community obtained that day from lentil fermentation. Each group clustering together indicates that microbial communities were very similar across days. Source Data provided in Supplementary information.

Figure 3. Microbial functionality affected by specific health conditions. Short chain fatty acids (acetate, butyrate and propionate) production after *in vitro* fermentation of lentils with feces from lean, celiac and obese people (n = 12; celiac n = 4, lean n = 4, obese n = 4). Statistical differences were calculated by means of one-way ANOVA using lean as the reference group. Significance labels: ns: not significant; **: $p < 0.01$; ***: $p < 0.001$. Source Data provided in Supplementary information.

Figure 4. Differences in gut microbial community structure after fermenting different foodstuffs. PCoA ordination analysis of genus abundance among all profiled samples (n = 15; chicken n = 4, pepper n = 3, banana n = 3, bread n = 3, chickpeas n = 2, as previously published by Perez-Burillo et al.[17]). Phylogenetic weighted UniFrac distance was used to calculate the sample dissimilarity matrix. Samples (dots) closer together indicate similarity among those samples. Samples farther away indicate dissimilarity among those samples. Reproduced with permission from ref. 17.

Figure 1.

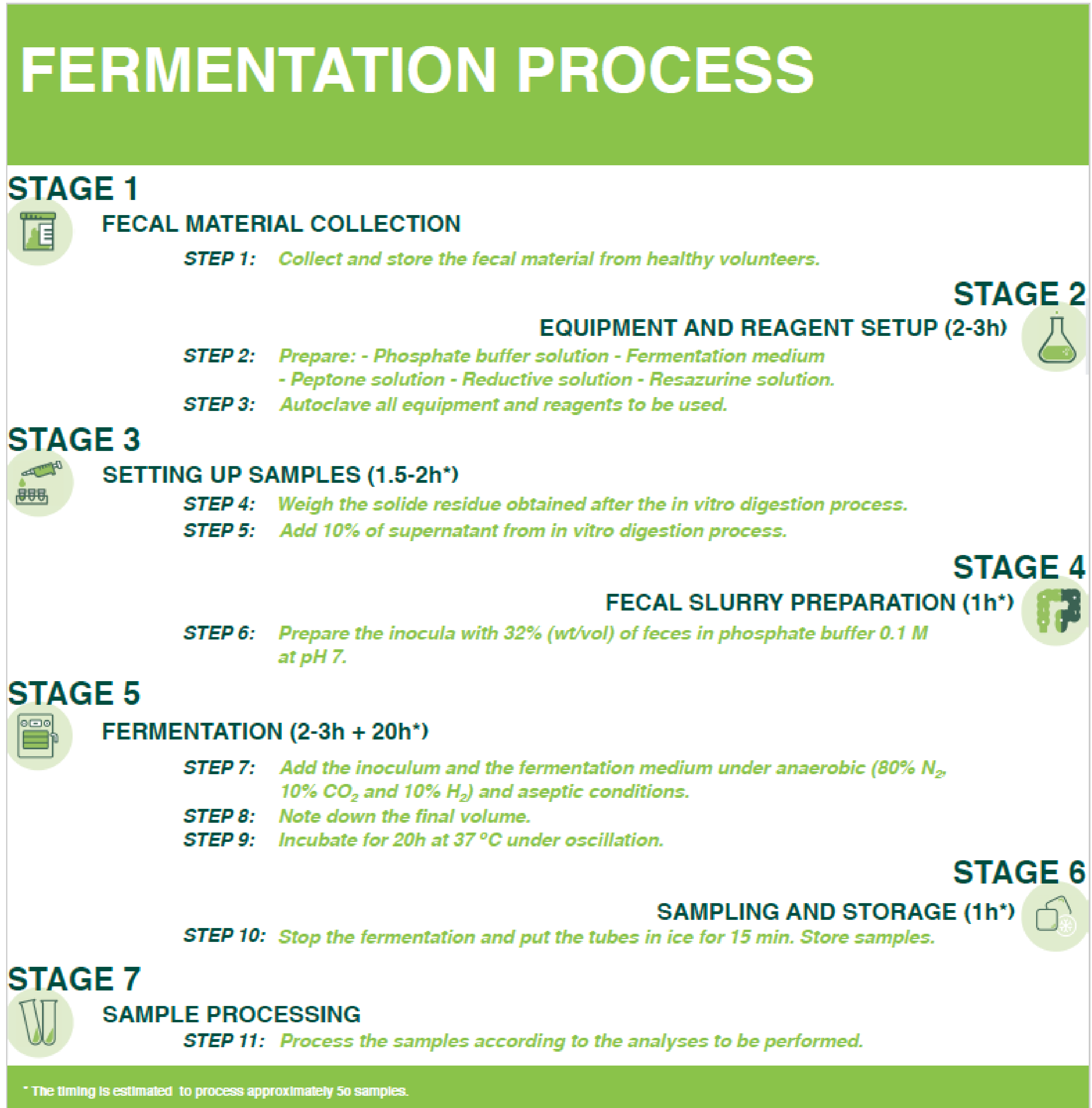

Figure 2.

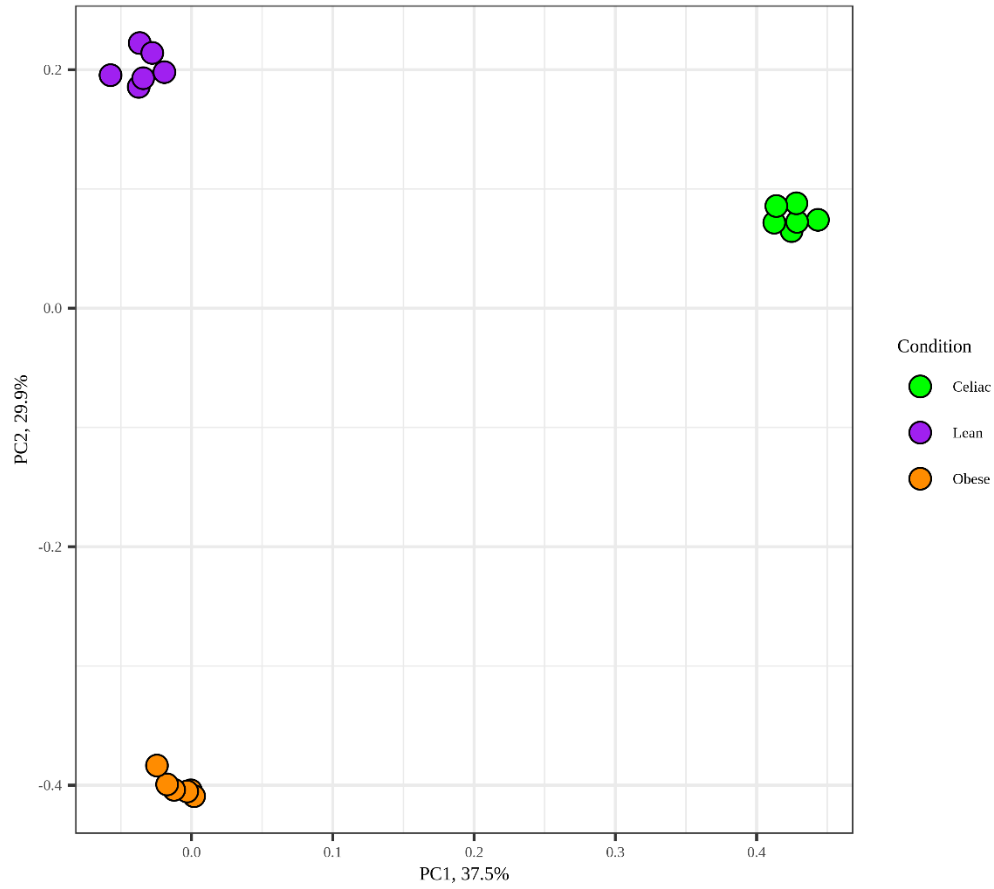

Figure 3.

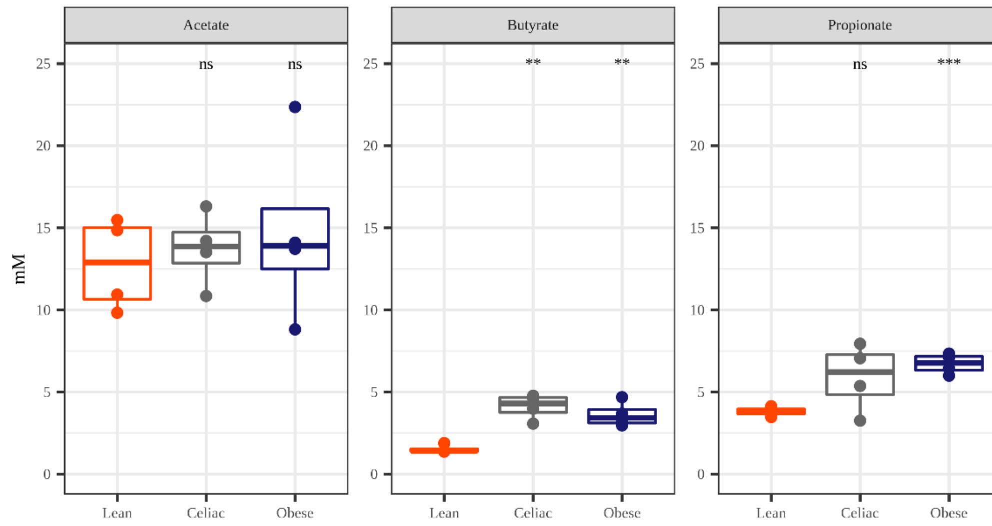

Figure 4.