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Review

Application of *In Vitro* Gut Fermentation Models to Food Components: A Review

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Abstract *In vitro* fermentation models have been developed for study of relationships between gut microbiota and food components. *In vitro* fermentation gut models involve use of pure cultures, mixed cultures, and human feces, and range from simple batch style fermentations performed in serum bottles to sophisticated pH-controlled multistage continuous culture systems. These models are increasingly used as an alternative to *in vivo* assays not only for disclosure of physiological activities of food components in the human intestine, but also for development of novel health functional foods. The purpose of this review is to introduce the present status and challenges of use of *in vitro* gut fermentation models in food studies.

Keywords: human large intestine, gut microbiota, *in vivo* test, *in vitro* fermentation model, food component

Introduction

Digestion is a complex process that is essential for health wherein ingested food is broken down into nutrients that can be used by the human body for growth, maintenance, and energy. During human digestion, the 2 main processes that occur simultaneously are mechanical transformations that reduce the size of food components and enzymatic transformations where macromolecules are hydrolyzed into smaller elements that are absorbed into the bloodstream (1). Food disintegration commonly occurs in the mouth and stomach, whereas enzymatic digestion and absorption of nutrients takes place in the small intestine. Non-absorbed materials travel to the large intestine for absorption of water and electrolytes, reabsorption of bile salts, formation, storage, and elimination of feces, and fermentation of polysaccharides and phytochemicals by gut microbiota (2).

Gut microbiota are improtant for human health primarily in absorption of nutrients and energy, and other roles (3). In particular, microbial infections and imbalances of gut microbiota are related to inflammatory bowel diseases, and to other immune related disorders (4,5). Although genetic and environmental factors influence the balance of gut microbiota, diet also affects microbial fermentation and the microbial compositional balance in the human intestine (6). *In vivo* testing involving use of humans and animals would be the best models for investigation of diet effects on intestinal microbiota. However, such testing is often expensive and time consuming, usually with limited study numbers. Animal testing is also becoming unpopular for ethical reasons. Furthermore, many of these types of investigations suffer from low compliance and high drop-out rates (7). These problems can be solved using *in vitro* methods. *In vitro* fermentation gut models involve use of pure cultures, mixed cultures, and human feces, and can range from simple batch style fermentations performed in serum bottles to sophisticated pH-controlled multistage continuous culture systems (7). Therefore, the aim of this review is to introduce potential and perspective advances based on *in vitro* gut fermentation models for development of novel functional food components.

Human large intestine The human large intestine contains 10^{10}-10^{11} cells/g in the proximal colon, 10^{10}-10^{12} cells/g in the transverse colon, and 10^{12} cells/g in the distal colon (Fig. 1). Using microflora, the colon is capable of complex hydrolytic digestive functions involving breakdown of dietary complex carbohydrates and some proteins that are not hydrolyzed or absorbed in the upper digestive tract. The level of carbohydrate consumption is subsequently lowered in the transverse and descending portions of the colon (8). The large intestine is the area most populated with microflora due to favorable conditions, including slow gut transit times, nutrient availability, and a favorable pH (8). Up to 10^{12} microbes per g inhabit the colon, accounting for a total of 1-2 kg of human body weight. Over 50 bacterial phyla have been identified, but human-associated microbiota are dominated by only the 4 main phyla of Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (9,10). Inclusion of

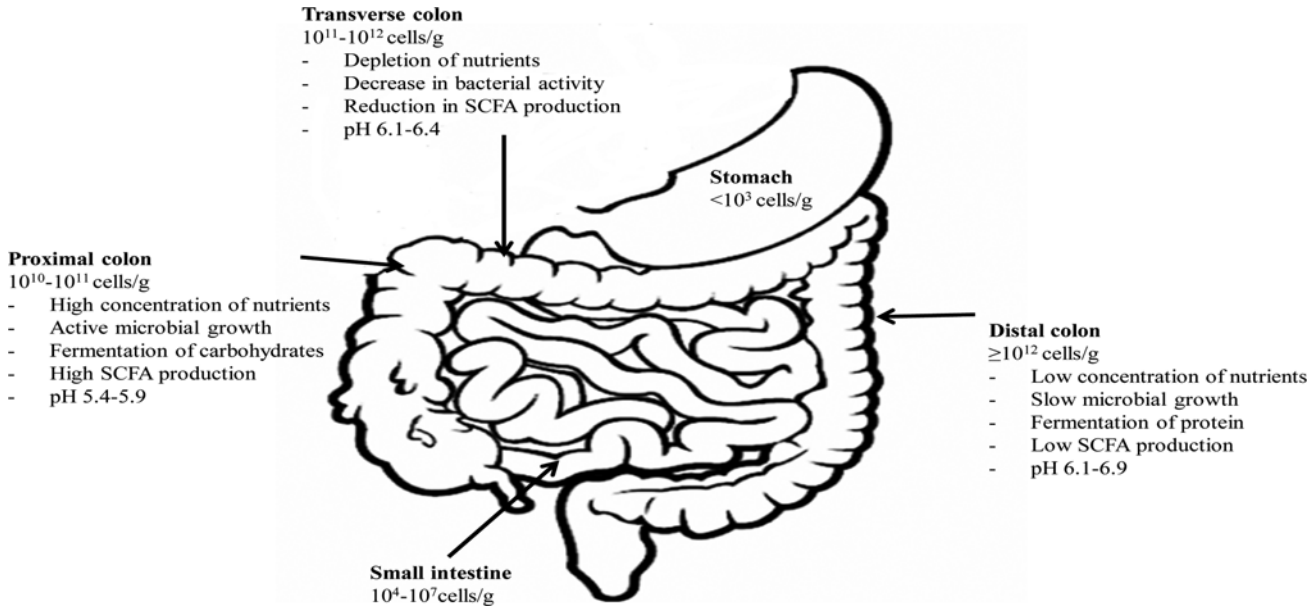

Fig. 1. Different segments of the human gastrointestinal tract. Approximate numbers of microbial cells per g of intestinal content, nutrient availability, and types of fermentation that are typical for each segment of a healthy gut are indicated.

Clostridia within Firmicutes makes it the phylum containing the most members represented in the microbiota, constituting 46-58% of total gut bacteria (11,12). Intestinal metabolism involves a variety of enzymes and biochemical pathways provided by specialized members of the microbial community that are distinct from host supplied resources. This metabolic capacity is estimated to be encoded in a total of 2-4 million microbial genes, which is 70-140x greater than the number of genes in the human genome (13,14).

Initial digestion processes in the human intestine require specific microbial glycosidases encoded in the DNA of hydrolytic gut microbiota. These microbes hydrolyze otherwise indigestible polysaccharides, such as resistant starch, both soluble and insoluble dietary fiber, proteins or host-innate mucins, such as glycosylated high Mw proteins, and phytochemicals reaching the large intestine. Subsequent microbial fermentation of hydrolyzed non-absorbed material in the large intestine results in production of short chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, branched chain fatty acids, as well as lactate, formate, ethanol, and mixed gases, such as CH_4, CO_2, and H_2 (15). Dissimilatory metabolic processing of proteinaceous material produces ammonia, amines, mercaptans, and H_2S, as well as some toxic indolic and phenolic compounds (16). SCFA production resulting from microbial fermentation of hydrolyzed dietary fibers, and sugars provides an estimated 10% of daily dietary energy resources to the host (17-19).

***In vitro* fermentation models** Design of *in vitro* fermentation systems includes simple batch cultures and single-stage or multistage continuous flow models that use different fecal inoculation (7,20-22). *In vitro* gut fermentation models allow stable cultivation of gut microbiota for a known period of time. Selection of a proper model requires cautious evaluation for research purposes considering both the advantages and limitations of different systems (Table 1). Each system type uses a different medium for microbial cell growth (Table 2).

***In vitro* gut models for digestion and/or fermentation** Batch fermentation models are closed systems in which microorganisms grow in a fermenter after cell inoculation (Fig. 2A). Batch cultures are inexpensive and easy to set up and are especially useful for determining metabolite profiles produced by fecal microflora that consume diet components (23) and are, therefore, used for fast testing of different substrates or fecal samples (7). However, substrate depletion and pH change during batch fermentations can result in distorted fermentation profiles based on a lack of steady state conditions and, thus, these methods are used for limited purposes of short-term testing (23). Uninterrupted medium flow through continuous culture fermentation models is regarded as a better system for mimicking *in vivo* contitions (7,23). Continuous fermentation chemostat models in which fresh medium is added and spent medium is removed allows long-term testing with high cell density cultures (7). Single-stage models consist of a single fermenter jar corresponding to a part of the gastrointestinal (GI) tract, whereas multi-stage models consist of several jars corresponding to multiple parts of the GI tract, such as the ascending, transverse, and descending colons (Fig. 2B). Operation of most *in vitro* systems requires a liquid fecal suspension as an inoculum, resulting in several limitations due to the free-cell state of bacterial populations (21). Systems with liquid fecal inocula generally experience a rapid washout of less competitive bacteria and are, consequently, limited in operational time to less than 4 weeks (24-26). These systems also fail to reproduce

Table 1. Advantages and limitations of *in vitro* fermentation models

Models	Advantages	Limitations	Refs
Batch	Easy to set up, convenient for fermentation studies and especially food component digestion assessment	Short-term studies and weakness in microbiological control	(48)
Continuous	Continuous liquid mimicking conditions found *in vivo*. Environmental parameters are well controlled	No host functionality and experiments are time limited to few days	(49)
Immobilized continuous	High-cell density and long-term studies of a continuous fermentation system with immobilized fecal microbiota	No host functionality	(27)
Multistage continuous	Continuous flow into several vessels mimicking conditions found in portions of the digestive tract	No host functionality and experiments are time limited to days or weeks	(50)
Digestion-fermentation-absorption	Continuous flow with metabolites and water exchange mimicking *in vivo* conditions	No immune response and experiments are limited to few days	(51)

Table 2. Different media for microbial cell growth

Models	Nutritional medium (g/L)	Refs
Batch	Peptone water (2), yeast extract (2), NaCl (0.1), K_2HPO_4 (0.04), KH_2PO_4 (0.04), $MgSO_4 \cdot 7H_2O$ (0.01), $CaCl_2 \cdot 6H_2O$ (0.01), $NaHCO_3$ (2), Tween 80 (2 mL), hemin (0.02), vitamin K1 (10 mL), cysteine HCl (0.5), and bile salts (0.5)	(52)
Immobilized continuous	Bile salts (0.05), porcine gastric mucin type (4.0), yeast extract (2.5), hemin (0.01), Tween 80 (1.0), peptone (0.5), tryptone (0.5), salts (NaCl, 4.5; KCl, 4.5; $MgSO_4 \cdot 7H_2O$, 1.25; $CaCl_2 \cdot 2H_2O$, 0.15; K_2HPO_4, 0.5; $NaHCO_3$, 1.5; $FeSO_4 \cdot 7H_2O$, 0.005), and cysteine (0.8)	(7)
Multistage continuous (Three-stage model)	Pectin (9.4), xylan (9.4), arabinogalactan (9.4), amylopectin (9.4), casein (47.0), starch (78.4), Tween 80 (34.0), bactopepton (47.0), and oxbile (0.8). $K_2HPO_4 \cdot 3H_2O$ (2.5), NaCl (4.5), $FeSO_4 \cdot 7H_2O$ (0.005), $MgSO_4 \cdot 7H_2O$ (0.5), $CaCl_2 \cdot 2H_2O$ (0.45), bile (0.05), cysteine (0.4), and 1 mL vitamin mixture containing (mg/L) menadione (1.0), D-biotin (2.0), vitamin B_{12} (0.5), pantothenate (10.0), nicotinamide (5.0), ρ-aminobenzoic acid (5.0), and thiamine (4.0)	(32)
Multistage continuous (SHIME)	Arabinogalactan (1.0), pectin (2.0), xylan (1.0), starch (4.0), glucose (0.4), yeast extract (3.0), peptone (1.0), mucin (4.0), and cystein (0.5). The pancreatic juice contained $NaHCO_3$ (12.5), bile salts (6.0), pancreatin (0.9)	(22)

both the planktonic free-cell and sessile biofilm-associated states of bacterial populations in the colon (7,27-29). To address problems associated with inoculum washout, a process for immobilization of fecal microbiota has been developed (21,28) wherein fecal microbiota are suspended within a porous polysaccharide matrix resulting in formation of fecal beads. The beads are transferred to the first part of a multistage continuous fermentation model (Fig. 2C). Limitations of substrate and toxic product diffusion within beads result in formation of a high-cell density peripheral layer in which cell release occurs spontaneously as a result of active cell growth (21,30-32). The operational system time for studies using immobilized cells varies and has been functionally demonstrated for time frames of 29, 54, and up to 71 days (21,33,34).

Digestion and fermentation systems include simulation of the human intestinal microbial ecosystem (SHIME), as a multi-stage sequential batch model of semi-continuous culture (Fig. 2D). The system has 5 fermenter jars connected in series corresponding to the stomach and the small intestine, and the ascending, transverse, and descending portions of the colon. Working volumes in these vessels are 300 mL for the stomach and small intestine, 1,000 mL for the ceacum and ascending colon, 1,600 mL for the transverse colon, and 1,200 mL for the desending colon. The system is inoculated using daily introduction of a 10 mL supernatant of a human diet suspension into the first vessels for 8 successive days. The remaining 3 vessels representing the 3 different compartments of the colon are inoculated with a 50 mL of a fecal suspension for 10 successive days. Contents of these vessels are pumped continuously from vessel to vessel and finally to a discard bottle. The transit time of the system is 84 h (35).

***In vitro* gut models for digestion-fermentation-absorption** Artificial digestion-fermentation-absorption systems have been developed based on modification of multi-stage gut fermentation models to mimic human physiological functions (23). *In vitro* gut models for digestion-fermentation-absorption differ from *in vitro* models for digestion and/or fermentation in that, during fluid transportation, water is absorbed apart from fermentation products using dialysis membranes.

There are 2 TNO (Dutch Organization for Applied Scientific Research) Intestinal Models (TIMs) known as the TIM-1 small intestine model, which is equipped for functions of the upper intestine, such as bile secretion, motility, pH control, and absorption (Fig. 3A) (36,37), and the TIM-2 proximal colon model, which is equipped for functions of the proximal colon like peristaltic mixing and metabolite absorption (Fig. 3B) (38). The TIM-1 and TIM-2 models have also been combined for investigation of the nutritional effects of novel food compounds, and for pharmaceutical studies (36,39-41). For TIM-1, a homogenized human meal is introduced into the gastric compartment at pre-set times and the homogenate is pumped through the system. During

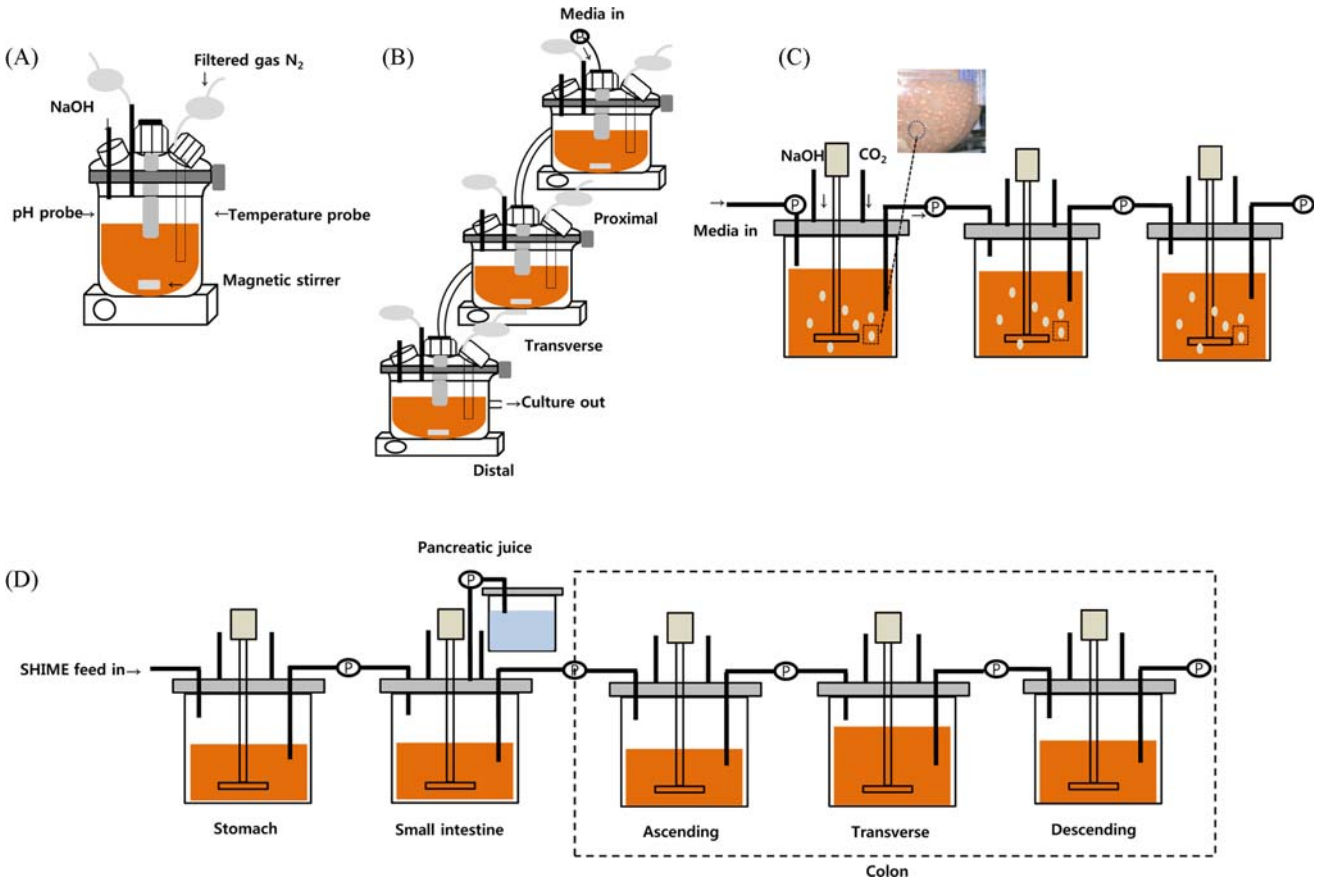

Fig. 2. Different *in vitro* gut models for digestion and/or fermentation. (A) Batch fermentation model used for culture of microorganisms without addition of additional nutrients. (B) Multi-stage chemostat used for simulation of multiple segments of the GI tract, such as the proximal, transverse, and distal colon. (C) Proximal colon reactor containing polysaccharide beads with immobilized fecal microbiota. (D) SHIME model simulation of numerous segments of the GI tract, including the stomach, small intestine, ascending colon, transverse colon, and descending colon.

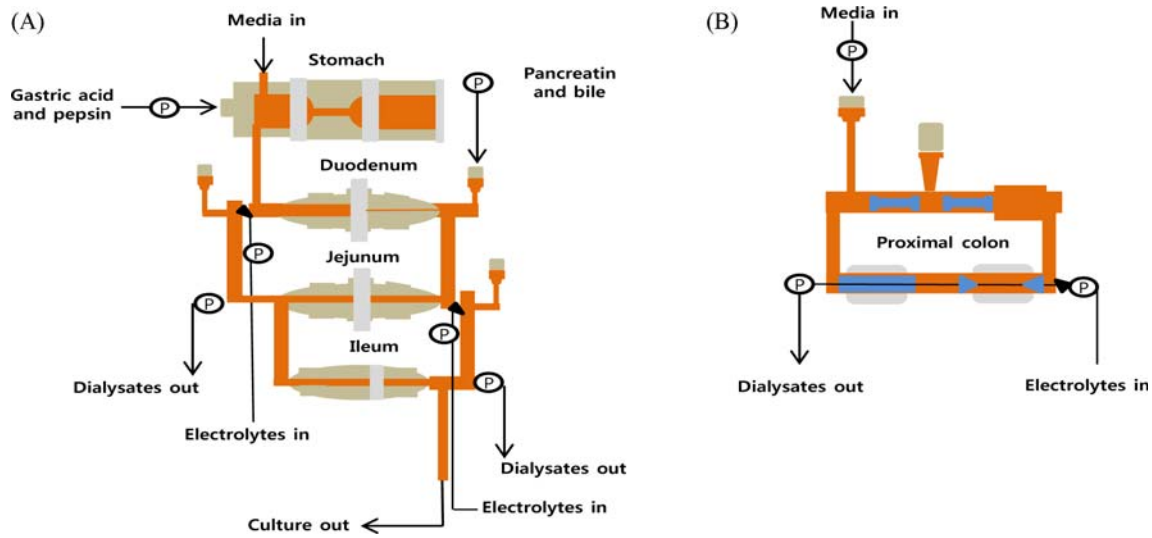

Fig. 3. Different *in vitro* gut models for digestion-fermentation-absorption. (A) TIM-1 model simulation of the upper intestine, including the stomach, duodenum, jejunum, and ileum. The TIM-1 model can also mimic host bile secretion, motility, pH, and absorption abilities of this region of the GI tract. (B) TIM-2 model simulation of the proximal colon. The TIM-2 model mimics host peristaltic mixing, water absorption, and metabolite absorption.

the operational period, secretion of enzymes, bile, and pancreatic juice and pH-control of the stomach in a gradient from 5.0 to 1.8 for 80 min from the beginning while maintaining the duodenum at a constant pH of 6.5, are regulated using a computer. For TIM-2, 200 mL of a fecal inoculum is first introduced and fecal microbiota are allowed to adapt to conditions for 16 h, after which the actual simulation is started based on addition of an ileal environment medium semi-continuously with or without the tested substate. The pH value is constantly maintained at 5.8 for representation of the pH level in the proximal colon.

Monitoring of the gut microbiotal composition Culture-based techniques used for bacterial identification are laborious and time-consuming, and provide a limited view of the diversity and dynamics of the GI microbiota with less than 30% of gut microbiota taxa having been cultured to date (9). Since the 1990s, introduction of novel molecular biological procedures has made it possible to overcome some of these limitations based on culture-independent methods (10) based on sequence divergence, including techniques such as denaturing gradient gel electrophoresis (DGGE) (42), terminal restriction fragment length polymorphism (TRFLP) (43), fluorescence *in situ* hybridization (FISH) (4), quantitative polymerase chain reaction (qPCR) (44), DNA microarray analysis (5), and next-generation sequencing (NGS) (43). FISH has been used for quantification of

Table 3. Studies using *in vitro* fermentation models[1)]

Food components	Type of study	Duration	Dose	Microbial techniques	Population increase	Population decrease	Refs
Oligosaccharides							
Fructo-oligosaccharides	Batch	48 h	0.3 g/100 mL	qPCR	*Bifidobacterium* spp.	*Clostridium* spp.	(13)
Galacto-oligosaccharides	Three-stage continuous	3 weeks	2x daily (4.0 g)	qPCR	*Lactobacillus* spp.		(8)
Xylo-oligosaccharides	Batch	24 h	0.5 g/50 mL	FISH	*Bifidobacterium* spp. *Lactobacillus* spp.	*Clostridium* spp.	(53)
Isomalto-oligosaccharides	Batch	24 h	0.5 g/50 mL	FISH	*Bifidobacterium* spp.	*Clostridium* spp.	(53)
Arabino-oligosaccharides	Batch	24 h	1.0 g/100 mL	qPCR	*Bifidobacterium* spp.		(44)
Fucosyllactose	Batch	48 h	5.0 g/L	qPCR	*Bifidobacterium* spp.	*Escherichia coli* *C. perfringens*	(6)
Sialyllactose	Batch	24 h	1.0 g/ 100 mL	qPCR	*Bifidobacterium* spp.		(52)
Polysaccharides							
Inulin	TIM-2	2 weeks	3.0 g/day	Intestinal-Chip Plate count PCR-DGGE	*B. adolescentis*		(5)
Dextran	Batch		1.0 g/100 mL	FISH	*Bacteroides-Prevotella*	*Faecalibacterium prausnitzii*	(4)
Phytochemicals or polyphenols							
Gallic acid	Batch	24h	1 mg/mL	FISH	*Lactobacillus* spp.	*Clostridium* spp.	(54)
Complex food extracts							
Soy germ powder	SHIME	2 weeks	2.5 g/day	Plate count	Enterobacteriaceae Coliforms *Lactobacillus* spp. *Staphylococcus* spp. *Clostridium* spp.		(46)
Black tea extract	Twin-SHIME	2 weeks	3x daily (1,000 mg)	Plate count qPCR PCR-DGGE pyrosequencing	*Klebsiella* spp. Enterococci *Akkermansia* spp.	*Bac. coccoides* *Anaeroglobus* spp.	(47)
Red wine-grape juice	Twin-SHIME	2 weeks	3x daily (1,000 mg)	Plate count qPCR PCR-DGGE pyrosequencing	*Klebsiella* spp. *Alistipes* spp. *Cloacibacillus* spp. *Victivallis* spp. *Akkermansia* spp.	*Bac. coccoides* *Anaeroglobus* spp.	(47)

[1)]Fermentation conditions for type of study, food components, duration, and dosage, and microbial techniques used, and main effects on bacteria groups, such as population increase and decrease, are included.

bacterial cells with 16S rRNA-targeted oligonucleotide probes combined with fluorescent light microscopy, confocal laser microscopy, or flow cytometry (11). FISH-based bacterial enumeration is based on cell counts and, consequently, provides accurate quantification.

Recently, a qPCR method has been used for quantification of different microfloral taxa within a large population. The main benefit of this technique is speed and the possibility for detection of minor populations of bacteria within microbial communities (12). For detection of a genus, a species, or a strain of microorganism, a primer set is designed to be genus, species, or strain-specific and PCR amplification with primers can be conducted. Comparison of cycles to threshold (Ct) values for each species in unknown cultures with reference cultures gives the cell number of the strain in the unknown mixed culture, determined based on colony forming units on agar plates (45).

Food studies using *in vitro* gut fermentation models Different modulation studies of gut microbiota using different food components with *in vitro* gut fermentation models are shown in Table 3. Details of fermentation conditions, including type of study, food components, duration, and dosage, microbial techniques used, and main effects on bacteria groups, such as population increase and decrease, have been included. Prebiotic effects of fructo-, galacto-, xylo-, isomalto-, and arabino-oligosaccharides and inulin, dextran, and arabinan polysaccharides have been examined using both batch and continuous models. Phytochemicals and polyphenolic compounds have been used for testing of prebiotic and microbiota modulatory effects in the large intestine. Among phytochemicals, gallic acid and derivatives showed potential prebiotic effects. Moreover, complex food substances, including soy germ powder, black tea extracts, red wine, and grape juice, have been used for investigation of effects on microbiota in the large intestine.

Prebiotic effects of arabino-oligosaccharides were investigated in our previous study using an *in vitro* batch fermenter and they were slowly degraded compared with fructo-oligosaccharides, possibly permitting SCFA production in the distal colon. This result demonstrated that arabino-oligosaccharides are promising dietary substrates for human health (44). Using the SHIME model, De Boever *et al.* (46) found that addition of soy germ powder during a 2 week treatment period resulted in a significant increase of 2 log10 units in the *Lactobacillus* spp. population. More recently, Kemperman *et al*. (47), using the twin-SHIME model, studied the influence of complex dietary polyphenols from black tea and red wine grape extracts on colonic microbiota, showing that these complex polyphenols, in the context of a model system, can modulate select members of the human gut microbiota.

In conclusion, *in vitro* fermentation models provide innovative technological platforms with a virtually limitless experimental capacity and no restrictions based on ethical concerns. To mimic the human GI tract, further improvement of *in vitro* fermentation models is needed. Use of a combined model system comprising *in vitro* gut compartments coated with the human intestinal cell lines Caco-2 and HT-29 may provide useful information on host responses to colonal fermentation, including competitive colonization and adhesion between beneficial and harmful or pathogenic bacteria, inhibitory activity of prebiotics on attachment of harmful bacteria, and immune modulatory activity of prebiotics and probiotics. Standardization of medium compositions used in an *in vitro* gut fermenation model is also necessary for different ethnic groups that follow dietary restrictions.

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