

The Effect of Cocoa and Polydextrose on Bacterial Fermentation in Gastrointestinal Tract Simulations

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Received December 18, 2006; Accepted April 24, 2007; Online Publication, August 7, 2007

[doi:10.1271/bbb.60706]

Effects of cocoa mass and supplemented dietary fiber (polydextrose) on microbial fermentation were studied by combining digestion simulations of stomach and small intestine with multi-staged colon simulations. During the four phases of digestion, concentrations of available soluble proteins and reducing sugars reflected *in vivo* absorption of nutrients in small intestine. In colon simulation vessels, addition of polydextrose to digested cocoa mass significantly increased concentrations of total short-chain fatty acids and butyric acid, from 103 to 468 mM ($P < 0.01$) and from 12 to 22 mM ($P < 0.01$), respectively. Long-chain fatty acid concentrations (decreasing from 1,222 to 240 mM) were mainly affected by the presence of digested cocoa mass. Cocoa mass with or without polydextrose addition significantly decreased production of cadaverine ($P < 0.02$) and branched-chain fatty acids compared to control during colon simulations. Results indicate beneficial effects on metabolism of colonic microbiota after digestion of cocoa mass, and even more so with polydextrose addition.

Key words: intestinal bacteria; digestion simulation; colon fermentation simulation; polydextrose; cocoa mass

In recent years claims have been made by several confectionary companies regarding the potential health benefits of dark chocolate consumption. The claims suggest that consumption of dark chocolate may lead to benefits such as improvement in the endothelial and platelet functions, reduction in blood pressure, and increased HDL cholesterol concentration. In particular, the antioxidative effects of polyphenols from cocoa bean derivatives (cocoa mass, butter, or powder) on human health have been studied.^{1–4} Even though cocoa butter

consists mainly of saturated fatty acids, which are generally regarded as blood cholesterol elevating substances, the primary component (about 1/3 of the total fatty acids) of cocoa butter, stearic acid, does not elevate blood cholesterol.^{5,6} Palmitic acid, the secondary component of cocoa butter, is potentially a blood cholesterol elevator, although this appears to be the case only in people with hypercholesterolemia.⁷

Nutritional ingredients with prebiotic and fiber status, *e.g.*, fructo- and galacto-oligosaccharides and polydextrose have been proven to have a beneficial effect on colonic microbiota *in vitro* and *in vivo*.^{8–15} Hence these substances are postulated to play an essential role in the nutrition metabolism of the distal human intestine, and further, in reducing the risk of colon cancer.¹⁶

The impact of dark chocolate ingredients on gastrointestinal tract function and the fermentation of chocolate's fatty acid components by human colonic microbiota are not yet known. Digestion of long-chain fatty acids in the bovine rumen suggests that anaerobic bacteria are able to hydrolyze unsaturated fatty acids into more saturated ones.^{17–19} In humans, unsaturated fatty acids are absorbed more effectively than saturated fatty acids.^{20,21} Absorption of dietary fatty acids occurs mainly in the small intestine. Unabsorbed fatty acids entering the colon are subjected to a very reductive environment maintained by colonic anaerobic microbiota.²² Cocoa mass also contains 2–6% soluble fiber,^{23,24} mainly hemicellulose, which might have a positive effect on the colonic microflora.

It is generally accepted that the fiber intake level is too low in Western diets.²⁵ Novel food applications with fiber supplementation are constantly being developed, and fiber is added to several different types of matrices, including confectionary products such as chocolate. The present study examined the potential effects of the

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Abbreviations: V1–V4, vessels 1–4 in the simulator; SCFA, short-chain fatty acids; PDX, polydextrose

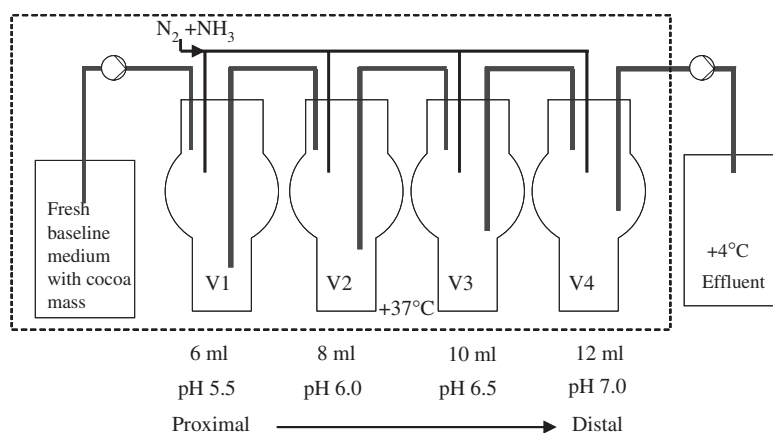


Fig. 1. A Single Unit of the EnteroMix™ Colon Simulator System.

A schematic presentation of a single unit of the EnteroMix™ colon simulator, consisting of four anaerobically sealed glass vessels with computer controlled semi-continuous fluid transfer and pH control. The target pH levels are representative of those found in the respective sections of the human colon.

addition of polydextrose to dark chocolate on the function of colonic microbiota. Due to the difficulties inherent in sample collection *in vivo*, the present study introduced a model that consists of two *in vitro* simulation techniques, mainly gastric and small intestinal digestion simulation combined with an automated four-stage colon simulator.

Materials and Methods

Digestion simulation. The digestion simulation method used in this study was an adaptation of a method, compiled previously by EnteroMix® Research from a book edited by Fuller²⁶⁾ as a reference, mimicking the upper gastric tract of pigs and chickens. The cocoa mass (Lotte, Tokyo, Japan) was weighed and cut into thin chips. The chips were mixed with a citrate-phosphate (50 mM Na-citrate, 100 mM Na-phosphate, pH 5.0) buffer solution at room temperature (1 g of chips/15 ml of buffer). The chocolate was dissolved into the buffer by warming and shaking the bottle in a water bath of approximately 70 °C (running hot tap water). A 1-ml starting point sample was drawn from the solution. For simulation of gastric digestion, the chocolate-buffer mixture was adjusted to pH 2 with 1 M HCl. Pepsin (P7000, Sigma-Aldrich, St. Louis, MO) was added at 1.7 ml/5 g of cocoa mass, and the sample was placed at 37 °C for 1 h with horizontal shaking (100 rpm). After the gastric digestion phase, a 1-ml sample was drawn from the mixture.

Pancreatin (7130, Merck, Whitehouse Station, NJ), a mixture of amylase (pH optimum 6.8), proteases (pH optimum 8), and lipases (pH optimum 9), and taurodeoxycholic acid (T 0875, Sigma-Aldrich) was added at 60 µl/ml and 2.5 mg/ml of cocoa slurry respectively, to simulate digestion process in the small intestine. This part of the digestion was divided into two pH phases: the first, 30 min, was at pH 6.5, followed by 30 min at pH 8,

both at 37 °C with horizontal shaking (100 rpm). After pancreatic digestion was completed, a 1-ml sample was taken.

In the dialysis phase of the simulation, a semi-permeable tube modelled the uptake of sugars, amino acids, triglycerides, and other nutrients by the small intestine. The digested solution was packed into a semi-permeable tube (CelluSept T1 micro, cut-off 4,000–6,000 Da, Membrane Filtration Products, Sequin, TX), which was sealed at both ends and submerged in a large volume of buffer (2,000 ml buffer/95 ml digested fluid). After 6 h of incubation in the buffer, the total volume of the fluid inside the tube was measured. A 1-ml sample of the fluid was taken, and the rest of the liquid was divided into 30-ml aliquots and frozen at –20 °C before it was used as a substrate in colonic simulations.

The 1-ml samples, taken before the digestion phases, and after the pepsin phase, pancreatic phase, and absorption phase, were analyzed to determine the concentrations of reducing sugars and total soluble proteins.

Four-stage colon simulator. The design and operation of the EnteroMix® four-stage colon simulator was evaluated earlier with polydextrose by Mäkiyuokko and co-workers.¹³⁾ The basic structure and flow are presented in Fig. 1. The run-parameters and the artificial basal simulation media were similar, with the exception that feeding of the digested cocoa mass to the system was performed at +37 °C instead of +4 °C to avoid clotting of the cocoa fats. The artificial medium contained the following constituents (g/l) in distilled water: starch, 5.0; peptone, 0.05; tryptone, 5.0; yeast extract, 5.0; NaCl, 4.5; KCl, 4.5; mucin (porcine gastric type III), 4.0; casein, 3.0; pectin (citrus), 2.0; xylan (oatspelt), 2.0; arabinogalactan (larch wood), 2.0; NaHCO₃, 1.5; MgSO₄, 1.25; guar gum, 1.0; inulin, 1.0; cysteine, 0.8; KH₂PO₄, 0.5; K₂HPO₄, 0.5; bile salts no. 3, 0.4; CaCl₂·6 H₂O, 0.15; FeSO₄·7 H₂O, 0.005;

hemin, 0.05; Tween 80, 1.0. The simulator system consisted of four independent parallel units, each constructed of four sequentially connected glass vessels, pH-controlling devices, and N₂-tubing to maintain of an anaerobic environment (Fig. 1). The digested cocoa mass obtained from the digestion simulation was mixed in an anaerobic cabinet to release its residual oxygen, and 1 part of the digestion slurry was diluted with 3 parts of artificial simulator media in order to simulate the effect of ileal fluids. Polydextrose (2% wt/vol) was added anaerobically to the diluted slurry, which was then sealed in a glass serum bottle. The simulator system was inoculated with preconditioned human feces (17 g of feces from a single donor was diluted anaerobically with 51 ml of artificial simulation fluid, and the slurry was incubated for 24 h in +37 °C in a sealed glass serum bottle). Each of the simulations was run for 48 hours, after which samples were collected from each vessel for further analysis. Two healthy male fecal donors were used in this study.

The proportions of the various bacteria were determined by flow cytometry by a procedure described earlier by Apajalahti and co-workers.²⁷⁾ Analysis of short-chain fatty acids (SCFAs) was performed by a protocol by Mäki vuokko *et al.*¹³⁾ The concentrations of biogenic amines (methylamine, ethylamine, tryptamine, 2-methyl-butylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine) were measured from the supernatant as dansyl derivatives by reversed phase HPLC by a method described by Saarinen.²⁸⁾

Analysis of free and esterified fatty acids from samples of both digestion and colon simulation was performed using a modification of the methods of El-Hamdy and Kaluzny.^{29,30)} Lipids were extracted from the simulator sample with a mixture of methanol and dichloromethane. The extract was evaporated, until dry and redissolved in chloroform and hexane. The free fatty acids and neutral esterified fatty acids were separated in an aminopropyl solid-phase extraction column. The free fatty acids and esterified fatty acids were derivatized to methyl esters with trimethyl sulphonium hydroxide and determined by gas chromatography with a flame ionization detector.

The total soluble proteins at different stages of digestion simulation were determined using a BCA-protein assay (Pierce, Boston, MA) according to the manufacturer's instructions. The reducing sugars were determined by heating samples in NaOH with dinitrosalicylic acid, which led to the formation of an intense color with absorption maxima in the range of 500–550 nm.³¹⁾

The concentration of polydextrose in the colon simulator vessels was measured by a HPLC-method presented by Mäki vuokko and co-workers.¹³⁾

To determine statistical significance between baseline and cocoa mass simulations, Student's t-test was used. *P*-values were associated with the corresponding values and the differences were considered to be significant at

P < 0.05. The relative increase or decrease (\pm SE) in measurements as compared to the baseline are reported with the values.

Results

Two parallel digestion simulations each consisting of four different stages were performed with the same batch of cocoa mass. In order to monitor the effects of digestion on the cocoa mass, the concentrations of the total released proteins and reducing sugars were determined during the different phases of digestion. These concentrations were nearly identical between the two digestion simulations (Fig. 2), showing the highest concentrations of both soluble proteins (270 mg/g of cocoa mass) and reducing sugars (97 mg/g of cocoa mass) in the ileal phase. The dialysis phase of digestion effectively simulated *in vivo* absorption of amino acids and sugars by lowering the concentration of soluble proteins by 24%, to 207 mg/g cocoa mass, and of reducing sugars by 48%, to 50 mg/g cocoa mass, as compared to the respective ileal phases. There are dark chocolate products on the Japanese market that are supplemented with polydextrose. One such product with polydextrose supplementation (2%) was subjected to the same gastric and ileal simulation as used for the cocoa mass in the present study. Analysis of the digested material revealed that the polydextrose concentration was not affected by the digestion of chocolate product (data not shown).

After each digestion simulation, a colon simulation was run using fecal inocula from a healthy donor. Both of these simulations included one baseline channel and two channels containing digested cocoa mass either with or without the addition of 2% polydextrose. The colon simulations were conducted over a 48-h period, after which samples were drawn from four subsequent vessels, V1 to V4, representing conditions in the different compartments of the colon for microbial and chemical analysis. The effects of polydextrose in the same colon model have been studied by Mäki vuokko *et al.*,¹³⁾ and hence polydextrose as such was not fermented in this study. Polydextrose was added to the diluted slurry after gastric simulation so that the concentration of polydextrose entering the colon (2 mg/100 ml) would be exactly the same as in the previous colon simulator study,¹³⁾ thus making possible accurate comparison of the results. Polydextrose was found to degrade in colon simulations in a fashion similar to previous colon simulations. The degradation began in V1 and proceeded to V4; 40% was left in V1 and 13% in V4 (data not shown).

The total bacterial densities measured by flow cytometry in vessels with cocoa mass digestion remained at the same levels as compared to the respective baseline channel vessels. The average densities varied from 5.5×10^9 (SE $\pm 1.6 \times 10^9$) to 1.4×10^{10} (SE $\pm 3.5 \times 10^9$), but no significant changes (*P* > 0.08) were measured among the different treatments.

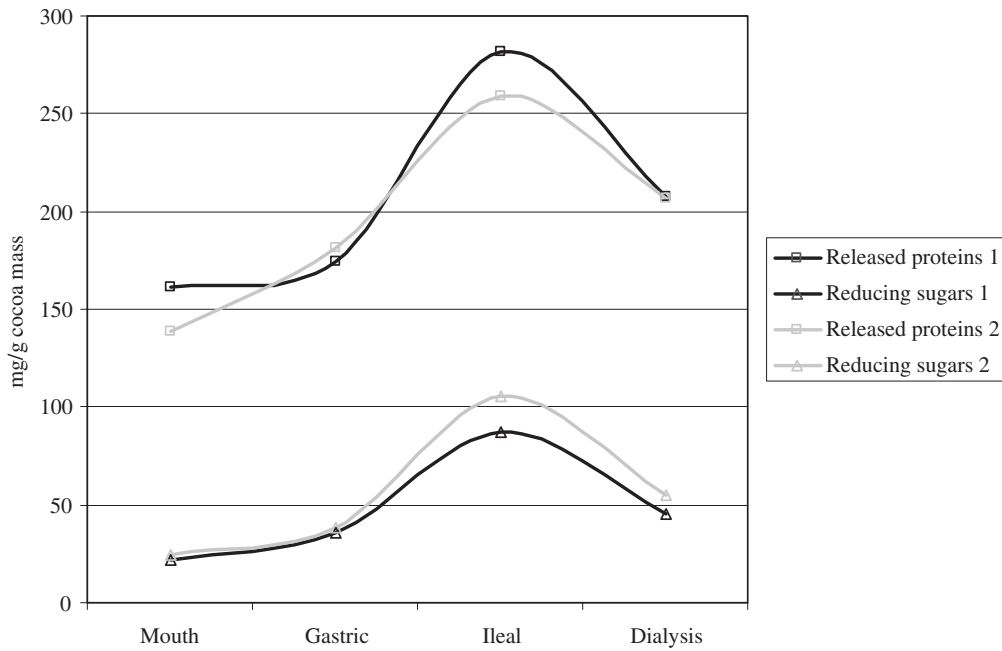


Fig. 2. Total Proteins and Reducing Sugars during Digestion.

Average concentrations of total proteins and reducing sugars during different stages of two independent small intestine digestion simulations of cocoa mass.

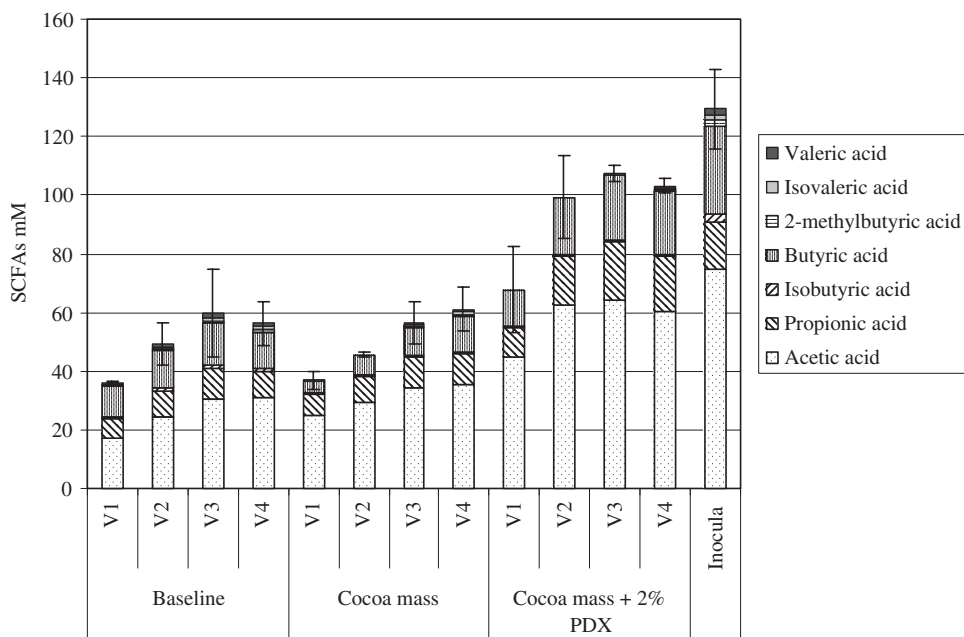


Fig. 3. Short-Chain Fatty Acids in the Inoculum and in Colon Simulator Vessels.

This figure shows average concentrations of the most abundant and the total (with \pm SE) short-chain fatty acids in fecal slurry before inoculation of the colon simulator and in the different cocoa mass fermenting vessels of the simulator (from V1 to V4) after six independent 48-h colon simulations.

The production of SCFAs was highest for the most abundant acids (acetic, butyric and propionic acid), and for the total SCFAs in each vessel of the cocoa mass + 2% polydextrose-channel as compared to the baseline- and plain cocoa mass-channels (Fig. 3). The difference between the treatments was greatest in terms

of butyrate production in the channels with cocoa mass digestion, where butyrate production was 2–3 times greater in the polydextrose-containing vessels, V1 and V2, at 12 and 22 mM respectively, as compared to the vessels without polydextrose ($P < 0.0001$). Total production of SCFAs in the channels with polydextrose

Table 1. Total Concentrations of Branched-Chain Fatty Acids.

Total concentrations (nmol/ml) of branched-chain fatty acids (isobutyric, isovaleric, and 2-methylbutyric acids) detected in different vessels (from V1 to V4) of the three colon simulator channels (cocoa mass, cocoa mass + 2% polydextrose, and baseline) after six independent 48-h fermentation simulations. The statistical difference between each cocoa mass vessel and the corresponding baseline vessel is presented as a *P*-value in brackets.

	Vessel 1	Vessel 2	Vessel 3	Vessel 4
Cocoa mass	0.459 (0.040)	0.499 (0.004)	1.495 (0.007)	2.650 (0.201)
Cocoa mass + 2% PDX	0.390 (0.030)	0.407 (0.005)	0.628 (0.002)	1.634 (0.010)
Baseline	1.296	2.119	3.129	2.889

increased steadily from vessel V1 (68 mm) to vessel V3 (107 mm), and declined slightly in V4 (103 mm). The SCFA concentrations in the pure cocoa mass channel increased steadily from V1 to V4, but concentrations were nearly identical with the baseline channel vessels. The concentrations of total branched-chain fatty acids (2-methylbutyric, isobutyric and isovaleric acid) increased from vessel V1 to vessel V4 in each channel, but were generally low in all channels. The lowest concentrations of total branched-chain fatty acids were measured in the vessels with added polydextrose, followed by those in the cocoa mass vessels and those in the baseline vessels (Table 1). Statistically, the total branched-chain fatty acid concentrations were significantly decreased in the cocoa mass vessels with or without additional polydextrose as compared to the corresponding concentrations in the control vessels, except for the V4 vessel of cocoa mass channel, where the decrease did not reach statistical significance ($P < 0.20$).

In contrast to the SCFA concentrations, cocoa mass digestion had a significant effect on long-chain fatty acid concentrations in all the vessels, as compared to the respective baseline vessels, while 2% polydextrose addition to cocoa mass digestion had a statistically insignificant increasing effect on the stearic acid concentration in the two first vessels as compared to the vessels with cocoa mass. The concentrations of the major long-chain fatty acids, palmitic (C16) and stearic (C18) acids, and long-chain poly/mono-unsaturated fatty acids, oleic (C18:1) and linoleic (C18:2) acids, were significantly higher in all of vessels of the channels with cocoa mass digestion ($P < 0.05$), as compared to the baseline channel, where the long-chain fatty acids were supplied only *via* a single dose of faecal inoculum (Fig. 4). The concentrations of long-chain fatty acids decreased steadily in the cocoa mass-fermenting channels from vessels V1 to V4, the respective average total concentrations being 1,222 mM and 240 mM. In the starting material for colon simulation, digested cocoa mass, the average total long-chain fatty acid concentration was 2,054 mM. Concentrations of saturated fatty acids appeared to be higher in vessels V1 and V2 of the cocoa mass + 2% polydextrose channel, while concen-

trations of the unsaturated fatty acids appeared constantly higher in all the vessels of the cocoa mass channel; no statistical significance was determined in either case. It is to be noted that the fatty acid profiles of the distal colon-simulating vessels (V4) of both channels with digested cocoa mass and with fecal samples obtained previously from a clinical study with chocolate³²⁾ were similar with proportions of 52–59% stearic acid, 29–34% palmitic acid, 6–9% oleic acid, and 2–4% linoleic acid. This strongly suggests that the combined gastric-small intestine digestion and colon fermentation simulations mimicked *in vivo* long-chain fatty acid digestion well (Fig. 5).

The average total concentrations of both biogenic amines were very low during all simulations, remaining below 300 μM, as compared to baseline fermentation in the respective vessels (Fig. 6). Digested cocoa mass with and without the addition of polydextrose decreased the concentration of biogenic amines as compared to the control concentrations, but the decrease was statistically significant only in cadaverine concentrations ($P < 0.04$).

Discussion

In the present study, a combination of different simulation techniques was used for the first time to study the potential effects of the addition of polydextrose to cocoa mass. Cocoa mass contains naturally soluble fiber hemicellulose,^{23,24)} but the effects of cocoa fibers on human colonic microbiota have not been studied. Fiber entering the colon can increase bacterial carbohydrate fermentation in the colon, thereby increasing the production of SCFAs. SCFAs in the feces are metabolites of polysaccharide fermentation by colonic microbiota that have not been absorbed by the host colon mucosa.

Effects of polydextrose

The addition of polydextrose to cocoa mass digestion significantly increased the SCFA concentration in colon simulations as compared to fermented cocoa mass digestion without the addition. The profiles, in other words, the relative proportions of the most abundant SCFAs (acetic, butyric and propionic acids), did not differ among the three treatments. The dose-dependent increase in SCFA concentrations due to polydextrose has been shown *in vitro* in both the EnteroMix[®] model and in a continuous flow colon model from the University of Reading,^{11,13)} and *in vivo* in a large-scale human trial on Chinese subjects.¹²⁾ The concentrations were slightly higher in the vessels of the channel cocoa mass digestion with 2% polydextrose as compared to those previously reported colon simulations with 2% polydextrose,¹³⁾ but the overall profiles were similar: increasing concentrations from V1 to V3, followed by a small decline to V4.

Polydextrose is a relatively slowly fermenting substance, and it has been reported to be gradually

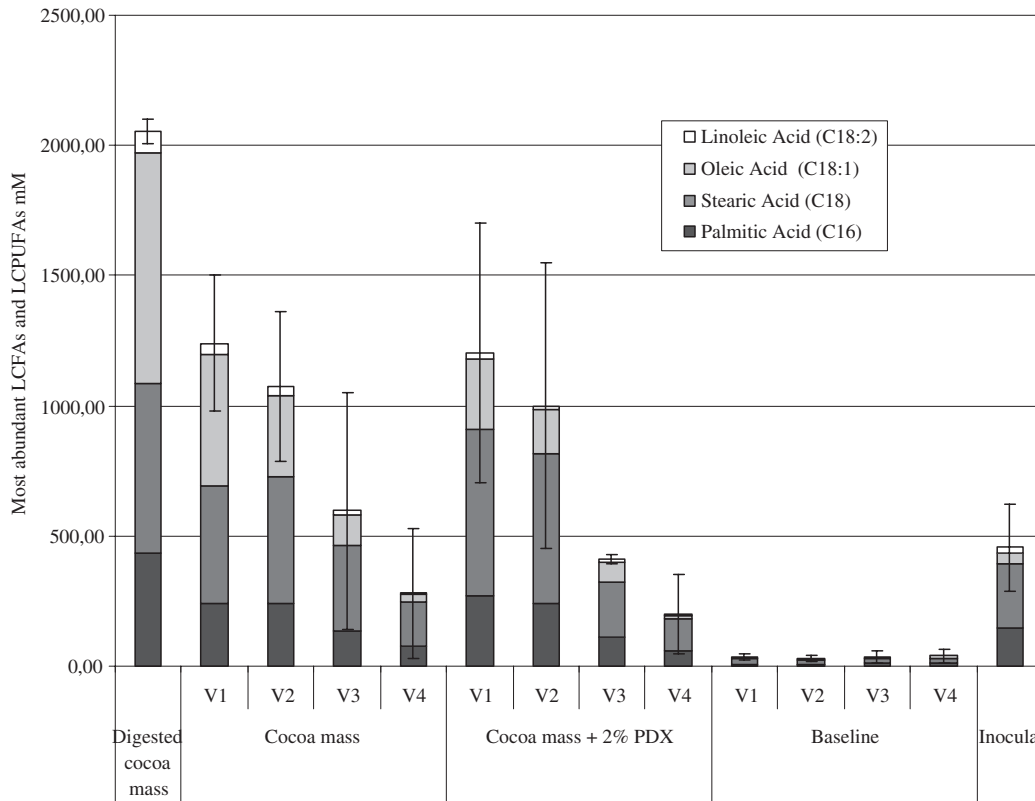


Fig. 4. Long-Chain Fatty Acids in Colon Simulator Vessels.

This shows average concentrations (mM) of free long-chain fatty acids (with \pm SE of total concentrations) in digested cocoa mass after the dialysis phase, in fecal slurry before inoculation of the colon simulator, and in the different parallel vessels (from V1 to V4) of the simulator after six independent 48-h semi-continuous fermentation simulations.

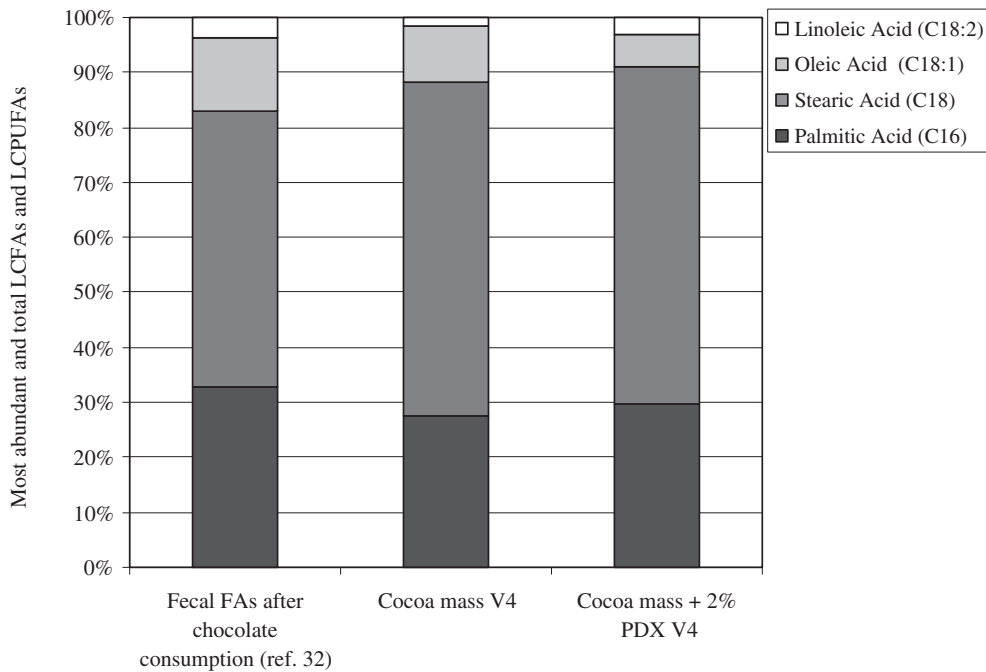


Fig. 5. Long-Chain Fatty Acid Concentrations Measured *in Vivo* and *in Vitro*.

This figure shows a comparison of average long-chain fatty acid concentrations measured in human fecal samples after dark chocolate consumption in a clinical trial³²⁾ and in the distal colon mimicking last vessels (V4) of the colon simulator after six independent 48-h fermentation simulations of cocoa mass digestion, both with and without 2% polydextrose.

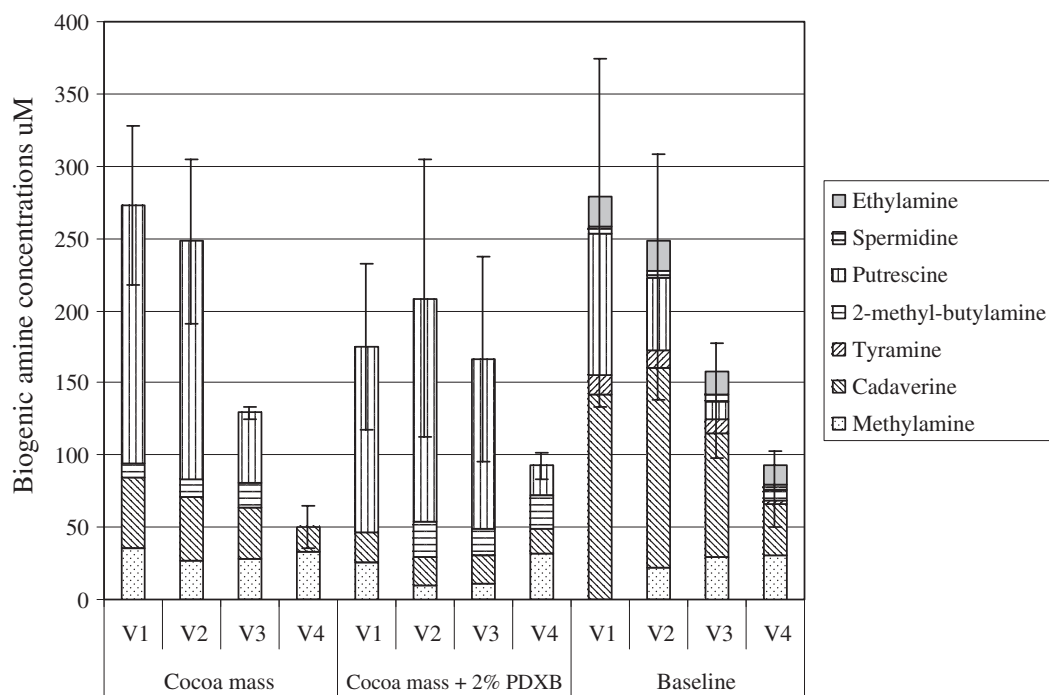


Fig. 6. Concentrations of Biogenic Amines in the Simulator Vessels.

This shows the concentrations of the most abundant and total (with \pm SE) biogenic amines detected in different vessels (from V1 to V4) of the three colon simulator channels (cocoa mass, cocoa mass + 2% polydextrose, and baseline) after six independent 48-h fermentation simulations.

fermented throughout an *in vitro* simulated human colon.¹³⁾ Gradual, saccharolytic fermentation of carbohydrates and fiber (*e.g.*, polydextrose) is essential in the prevention of the harmful effects of putrefaction. Polysaccharides are the preferred energy source for colonic microbiota. When they are not available, colonic microbes begin to ferment proteins and amino acids into biogenic amines, ammonia, and branched-chain fatty acids. These putrefactive components cause adverse effects on the colon epithelium.^{33–35)}

In the present study, both digested cocoa mass and polydextrose-supplemented cocoa mass decreased the total concentration of biogenic amines in all vessels as compared to baseline vessels, even though the decreases were not statistically significant. Furthermore, digested cocoa mass with and without additional polydextrose decreased the total concentrations of the branched-chain fatty acids in all six vessels (statistically significant reduction in five vessels), as compared to baseline channels. The presence of polydextrose did not increase microbial numbers in the vessels as compared to the baseline, thus promoting the saccharolytic fermentation of polydextrose. Similarly, increased concentrations of SCFA in the human colon indicate a healthy state of the colonic mucosa, since short-chain fatty acids are an energy-source for epithelial and immune cells.^{36–38)} Butyrate has been found to have functional properties in the prevention of colon cancer.^{13,39,40)}

Due to its highly branched structure, polydextrose might act as a lipid-carrier in the human ileum, thus increasing the amount of fatty acids entering the colon.

Cocoa mass digestion and fermentation

Cocoa mass contains carbohydrates and soluble fibers, some of which can be digested by ileal enzymes, and some that are fermented in the colon.²⁶⁾ In the present study, gastric digestion of cocoa mass did not increase the concentration of reducing sugars, while ileal digestion significantly increased the concentration. The dialysis, or absorptive, phase of the digestion simulation reduced the concentration of reducing sugars, reflecting the *in vivo* situation. The dialysis membranes have been validated as *in vivo* mimicking absorptive elements in an automated ileal simulator.⁴¹⁾ In the colonic simulation, cocoa mass treatment as such did not increase the production of SCFA. Hence, it can be concluded that the carbohydrate fraction of the digested cocoa mass had little effect on colonic fermentation.

The ileal digestion simulation released proteins from the cocoa mass. The dialysis phase of the digestion simulation decreased the concentration of proteins effectively, but some protein remained after the absorption phase. Similarly, digestion of the dietary proteins and amino acids in the *in vivo* gut have been found to be incomplete throughout the small intestine. *In vivo* concentrations are also increased by the release of endogenous proteins from the gut lumen, thus enabling the passage of proteins to the colon where they are subjected to microbial fermentation,⁴²⁾ which may lead to the above-mentioned state of putrefaction. In our model, digested cocoa mass mainly reduced the production of cadaverine in the colon simulation as compared to the baseline, and additional polydextrose

decreased biogenic amine production even further, as discussed above. Thus the proteins remaining after the digestion and absorption phases of cocoa mass did not have a significant impact on colonic fermentation.

Long-chain fatty acid concentrations of the simulated colonic fluid were greatly increased by the inclusion of cocoa mass. The effect of human colonic microbes on long-chain fatty acids is still poorly understood, even though it is clear that long-chain saturated fatty acids in the diet are an important energy source in human nutrition. In human intestine, unsaturated fatty acids are absorbed more effectively than saturated fatty acids,^{20,21)} and the absorption of dietary fatty acids occurs mainly in the small intestine. Unabsorbed fatty acids entering the colon are subjected to a very reductive environment maintained by colonic anaerobic microbiota.²²⁾ Mono-unsaturated fatty acids, *e.g.*, oleic acids (C18:1, n-9), are almost completely absorbed from the intestine and are either oxidized for energy, converted into other fatty acids, or incorporated into tissue lipids.⁴³⁾

In the present study, the concentrations of oleic acid, linoleic acid, and saturated fatty acids (palmitic and stearic acids) increased in a response to the inclusion of cocoa mass in the baseline fluid, and their concentrations decreased along the vessels. The long-chain fatty acid profiles from the fecal samples of the two different donors used to inoculate the colon model had a similar composition in terms of both saturated and unsaturated fatty acids, stearic acid and palmitic acid being the dominant acids over linoleic and oleic acids. These profiles were greatly affected by the introduction of digested cocoa mass into the simulator and 48-hour semi-continuous fermentation in the vessels. After fermentation, stearic acid was the most abundant acid in all the vessels, except for vessel V1, with cocoa mass, where oleic acids were dominant. However, the oleic and linoleic acid concentrations in the last vessels of both cocoa mass-fermenting channels were close to those monitored in the baseline channels. This indicates that microbial fermentation favored unsaturated acids over saturated ones, which is in accordance with the *in vivo* fatty acid metabolism discussed earlier. In the baseline vessels, the lack of long-chain fatty acids in the artificial simulation medium reduced the fatty acid concentrations dramatically as compared to the feces. Notably, the profiles of vessel V4 from both cocoa mass fermenting channels, and those measured previously in human feces after dark chocolate consumption,^{32,44)} all had a close resemblance (Fig. 5), even though the colon model was fed only with artificial ileal fluid, cocoa mass digestion, and polydextrose. This similarity between the distal-colon simulating vessels and the fecal samples supports the *in vivo* mimicking nature of the colon simulator used.

In vivo resemblance of the digestion model and the colon simulator

Replicable studies of the gastrointestinal functions of

a selected food matrix require the use of *in vitro* models due to difficulties in obtaining *in vivo* human samples. Presently only one model that is able to simulate the whole gastrointestinal tract has been published,⁴⁵⁾ while there are several multi-compartmental models capable of simulating either the human small intestine or the human colon.^{13,46–49)} These models differ structurally and functionally, but the basic solutions for simulating the gastrointestinal tract function as closely to *in vivo* situation as possible are very similar.

In the digestion model presented here, the digestion of a single food component or product is modelled by simulating the enzymatic, chemical, and pH environment along the gastrointestinal tract.²⁶⁾ Simulated digestion and absorption differ from the natural environment in many respects. For example, the absorptive phase of digestion using a dialysis membrane is not as effective as the gut *in vivo*, but it is comparable to the efficacy of other models, *e.g.*, TIM-1.⁴⁷⁾ This is noticeable in Fig. 2, where the concentrations of released proteins and reducing sugars did not decrease below the starting point (mouth) values after the dialysis (absorptive phase). Moreover, a large proportion of the long-chain fatty acids of cocoa mass survived through digestion and dialysis. The lack of indigenous microbes in the simulated small intestine further affects the utilization of nutrients. This is a general problem in all the small intestinal models which have used only chemical and enzymatic digestion, due to the difficulty of access to healthy ileal microflora in humans.^{45,47)}

Simulated ileal fluid entering the colon simulator is subjected to fermentation by fecal microbes. The fecal bacteria are not fully representative of those found in the proximal caecum and colon, but all current colon simulation models rely on fecal microbes of healthy donors.^{45,46,48)} The concentrations of long-chain fatty acids decreased from vessel V1 to vessel V4, which might be partly due to the possibility that the fatty acid-rich digesta mass was not totally transferred from vessel to vessel. Therefore, if any quantitative assessment of long-chain fatty acid concentrations is made, special attention should be paid to this. Nevertheless, the long-chain fatty acid profiles of the distal colon-simulating vessels were well in line with those measured in human fecal samples after consumption of dark chocolate.^{32,44)}

The short-chain fatty acid concentrations in different vessels of the colon simulator reflected well the previous results obtained with the same model, when simulating the effects of baseline medium supplemented with 0.5–2.0% of polydextrose.¹³⁾ In that study, polydextrose was found to have a dose-dependent SCFA-concentration increasing effect during transit through the simulated colon. In addition to polydextrose, lactose has recently been found to have an even more dramatic increasing effect on both SCFA production and bifidobacterial numbers in the same colon model.⁴⁹⁾ However, lactose also had a bactericidal effect on the total microbial numbers in the first vessel, probably due to rapid

fermentation of lactose by lactic acid bacteria, while polydextrose steadily increased total microbial numbers in both the present and the previously reported colon simulation study. This stable effect on microbes, increased production of SCFAs, and low concentrations of biogenic amines in all vessels are indicators of host-benefiting saccharolytic fermentation.

Conclusions

In this study, a model combining human gastric and small-intestine digestion with colonic fermentation simulations is presented.

The function of the small intestine digestion-model was evaluated by cocoa mass digestion. The digested cocoa slurry was supplemented with 2% polydextrose and subsequently introduced to a previously evaluated small-scale semi-continuous colon simulator model. This study suggests that the addition of polydextrose to cocoa mass increases carbohydrate fermentation of the local microbiota in a colon simulator environment. Also the cocoa mass components promoted a decrease in putrefactive markers in the simulated colon. Together with an intraluminal increase in the production of short-chain fatty acids, reduced putrefaction in the colon may induce positive health effects also in colonic mucosa.

Acknowledgments

We wish to acknowledge the skillful work of Brita Mäki in the digestion simulations, of Kirsi Stenström in various chemical analyses, and of Jaana Larsson-Leskelä in flow-cytometry analyses.

References

- Hermann, F., Spieker, L. E., Ruschitzka, F., Sudano, I., Hermann, M., Binggeli, C., Luscher, T. F., Riesen, W., Noll, G., and Corti, R., Dark chocolate improves endothelial and platelet function. *Heart*, **92**, 119–120 (2006).
- Grassi, D., Necozione, S., Lippi, C., Croce, G., Valeri, L., Pasqualetti, P., Desideri, G., Blumberg, J. B., and Ferri, C., Cocoa reduces blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives. *Hypertension*, **46**, 398–405 (2005).
- Vlachopoulos, C., Aznaouridis, K., Alexopoulos, N., Economou, E., Andreadou, I., and Stefanadis, C., Effect of dark chocolate on arterial function in healthy individuals. *Am. J. Hypertens.*, **18**, 785–791 (2005).
- Mursu, J., Voutilainen, S., Nurmi, T., Rissanen, T. H., Virtanen, J. K., Kaikkonen, J., Nyyssonen, K., and Salonen, J. T., Dark chocolate consumption increases HDL cholesterol concentration and chocolate fatty acids may inhibit lipid peroxidation in healthy humans. *Free Radic. Biol. Med.*, **37**, 1351–1359 (2004).
- Hegsted, D. M., McGandy, R. B., Myers, M. L., and Stare, F. J., Quantitative effects of dietary fat on serum cholesterol in man. *Am. J. Clin. Nutr.*, **17**, 281–295 (1965).
- Kris-Etherton, P. M., and Mustad, V. A., Chocolate feeding studies: a novel approach for evaluating the plasma lipid effects of stearic acid. *Am. J. Clin. Nutr.*, **60**, 1029S–1036S (1994).
- Wan, Y., Vinson, J. A., Etherton, T. D., Proch, J., Lazarus, S. A., and Kris-Etherton, P. M., Effects of cocoa powder and dark chocolate on LDL oxidative susceptibility and prostaglandin concentrations in humans. *Am. J. Clin. Nutr.*, **74**, 596–602 (2001).
- Fooks, L. J., Fuller, R., and Gibson, G. R., Prebiotics, probiotics and human gut microbiology. *Int. Dairy J.*, **9**, 53–61 (1999).
- Gibson, G. R., and Roberfroid, M. B., Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.*, **125**, 1401–1412 (1995).
- Gibson, G. R., Dietary modulation of the human gut microflora using the prebiotics oligofructose and inulin. *Am. Soc. Nutr. Sci., Suppl.*, 1438s–1441s (1999).
- Probert, H. M., Apajalahti, J. H., Rautonen, N., Stowell, J., and Gibson, G. R., Polydextrose, lactitol, and fructo-oligosaccharide fermentation by colonic bacteria in a three-stage continuous culture system. *Appl. Environ. Microb.*, **70**, 4505–4511 (2004).
- Jie, Z., Bang-Yao, L., Ming-Jie, X., Hai-Wei, L., Zu-Kang, Z., Ting-Song, W., and Craig, S. A., Studies on the effects of polydextrose intake on physiologic functions in Chinese people. *Am. J. Clin. Nutr.*, **72**, 1503–1509 (2000).
- Mäkivuokko, H., Nurmi, J., Nurminen, P., Stowell, J., and Rautonen, N., *In vitro* effects on polydextrose by colonic bacteria and Caco-2 cell cyclooxygenase gene expression. *Nutr. Cancer*, **52**, 94–104 (2005).
- Morelli, L., Zonenschain, D., Callegari, M. L., Grossi, E., Maisano, F., and Fusillo, M., Assessment of a new synbiotic preparation in healthy volunteers: survival, persistence of probiotic strains and its effect on the indigenous flora. *Nutr. J.*, **2**, 11 (2003).
- Bartosch, S., Woodmansey, E. J., Paterson, J. C., McMurdo, M. E., and Macfarlane, G. T., Microbiological effects of consuming a synbiotic containing *Bifidobacterium bifidum*, *Bifidobacterium lactis*, and oligofructose in elderly persons, determined by real-time polymerase chain reaction and counting of viable bacteria. *Clin. Infect. Dis.*, **40**, 28–37 (2005).
- Andoh, A., Tsujikawa, T., and Fujiyama, Y., Role of dietary fiber and short-chain fatty acids in the colon. *Curr. Pharm. Des.*, **9**, 347–358 (2003).
- Polan, C. E., McNeill, J. J., and Tove, S. B., Biohydrogenation of unsaturated fatty acids by rumen bacteria. *J. Bacteriol.*, **88**, 1056–1064 (1964).
- Wood, R. D., Bell, M. C., Grainger, R. B., and Teekel, R. A., Metabolism of labeled linoleic-1-C¹⁴ acid in the sheep rumen. *J. Nutr.*, **79**, 62–68 (1963).
- Fujimoto, K., Kimoto, H., Shishikura, M., Endo, Y., and Ogimoto, K., Biohydrogenation of linoleic acid by anaerobic bacteria isolated from rumen. *Biosci. Biotechnol. Biochem.*, **57**, 1026–1027 (1993).
- Hashim, S. A., and Babayan, V. K., Studies in man of partially absorbed dietary fats. *Am. J. Clin. Nutr.*, **31**, S273–S276 (1978).
- Nelson, S. E., Rogers, R. R., Frantz, J. A., and Ziegler,

- E. E., Palm olein in infant formula: absorption of fat and minerals by normal infants. *Am. J. Clin. Nutr.*, **64**, 291–296 (1996).
- 22) Howard, F. A., and Henderson, C., Hydrogenation of polyunsaturated fatty acids by human colonic bacteria. *Lett. Appl. Microbiol.*, **29**, 193–196 (1999).
 - 23) Valiente, C., Esteban, R. M., Martin, M. A., Molla, E., and Lopez-Andreu, F. J., Study of the dietary fibre content in cocoa. *Eur. J. Clin. Nutr.*, **49**(Suppl 3), S222–S223 (1995).
 - 24) Kamiwaki, T., Tsuji, K., and Nakagawa, Y., Effects of dietary fiber from cacao bean on blood pressure and lipid metabolism in spontaneously hypertensive rats. *Nippon Shokuhin Kagaku Kougaku Kaishi*, **46**, 581–586 (1999).
 - 25) Valsta, L. M., Food-based dietary guidelines for Finland: a staged approach. *Br. J. Nutr.*, **81**(Suppl 2), S49–S55 (1999).
 - 26) Fuller, M. F., “*In Vitro* Digestion for Pigs and Poultry,” C.A.B. International, Oxfordshire (1991).
 - 27) Apajalahti, J. H., Kettunen, H., Kettunen, A., Holben, W. E., Nurminen, P. H., Rautonen, N., and Mutanen, M., Culture-independent microbial community analysis reveals that inulin in the diet primarily affects previously unknown bacteria in the mouse cecum. *Appl. Environ. Microb.*, **68**, 4986–4995 (2002).
 - 28) Saarinen, M. T., Determination of biogenic amines as dansyl derivatives in intestinal digesta and feces by reversed phase HPLC. *Chromatographia*, **55**, 297–300 (2002).
 - 29) El-Hamdy, A. H., and Christie, W. W., Preparation of methyl esters of fatty acids with trimethylsulphonium hydroxide - an appraisal. *J. Chrom.*, **630**, 438–441 (1993).
 - 30) Kaluzny, M. A., Duncan, L. A., Merritt, M. V., and Epps, D. E., Rapid separation of lipid classes in high yield and purity using bonded phase columns. *J. Lipid Res.*, **26**, 135–140 (1985).
 - 31) Miller, G. L., Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.*, **31**, 426–428 (1959).
 - 32) Shahkhalili, Y., Duruz, E., and Acheson, K., Digestibility of cocoa butter from chocolate in humans: a comparison with corn-oil. *Eur. J. Clin. Nutr.*, **54**, 120–125 (2000).
 - 33) Macfarlane, G. T., Gibson, G. R., and Cummings, J. H., Comparison of fermentation reactions in different regions of the human colon. *J. Appl. Bacteriol.*, **72**, 57–64 (1992).
 - 34) Macfarlane, S., and Macfarlane, G. T., Regulation of short-chain fatty acid production. *Proc. Nutr. Soc.*, **62**, 67–72 (2003).
 - 35) Priebe, M. G., Vonk, R. J., Sun, X., He, T., Harmsen, H. J., and Welling, G. W., The physiology of colonic metabolism. Possibilities for interventions with pre- and probiotics. *Eur. J. Nutr.*, **41**(Suppl 1), I2–10 (2002).
 - 36) Mortensen, P. B., and Clausen, M. R., Short-chain fatty acids in the human colon: relation to gastrointestinal health and disease. *Scand. J. Gastroenterol. Suppl.*, **216**, 132–148 (1996).
 - 37) Sakata, T., Stimulatory effect of short-chain fatty acids on epithelial cell proliferation in the rat intestine: a possible explanation for trophic effects of fermentable fibre, gut microbes and luminal trophic factors. *Br. J. Nutr.*, **58**, 95–103 (1987).
 - 38) Roediger, W. E., Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut*, **21**, 793–798 (1980).
 - 39) Kamitani, H., Geller, M., and Eling, T., Expression of 15-lipoxygenase by human colorectal carcinoma Caco-2 cells during apoptosis and cell differentiation. *J. Biol. Chem.*, **273**, 21569–21577 (1998).
 - 40) Litvak, D. A., Evers, B. M., Hwang, K. O., Hellmich, M. R., Ko, T. C., and Townsend, C. M., Jr., Butyrate-induced differentiation of Caco-2 cells is associated with apoptosis and early induction of p21Waf1/Cip1 and p27Kip1. *Surgery*, **124**, 161–169 (1998).
 - 41) Marteau, P., Minekus, M., Havenaar, R., and Huis in’t Veld, J., Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the effects of bile. *J. Dairy Sci.*, **80**, 1031–1037 (1997).
 - 42) Fuller, M. F., and Tome, D., *In vivo* determination of amino acid bioavailability in humans and model animals. *J. AOAC Int.*, **88**, 923–934 (2005).
 - 43) EFSA, Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on the request from the Commission related to nutrition claims concerning omega-3 fatty acids, monounsaturated fat, polyunsaturated fat and unsaturated fat. *EFSA J.*, **253**, 1–29 (2005).
 - 44) Shahkhalili, Y., Murset, C., Meirim, I., Duruz, E., Guinchard, S., Cavadini, C., and Acheson, K., Calcium supplementation of chocolate: effect on cocoa butter digestibility and blood lipids in humans. *Am. J. Clin. Nutr.*, **73**, 246–252 (2001).
 - 45) Molly, K., Vande, W. M., and Verstraete, W., Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. *Appl. Microb. Biotechnol.*, **39**, 254–258 (1993).
 - 46) Gibson, G. R., Cummings, J. H., and Macfarlane, G. T., Use of a three-stage continuous culture system to study the effect of mucin on dissimilatory sulfate reduction and methanogenesis by mixed populations of human gut bacteria. *Appl. Environ. Microb.*, **54**, 2750–2755 (1988).
 - 47) Minekus, M., Marteau, P., Havenaar, R., and Huis in’t Veld, J. H. J., A multicompartamental dynamic computer-controlled model simulating the stomach and small intestine. *ATLA*, **23**, 197–209 (1995).
 - 48) Minekus, M., Smeets-Peeters, M., Bernalier, A., Marol-Bonin, S., Havenaar, R., Marteau, P., Alric, M., Fonty, G., and Huis in’t Veld, J. H. J., A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and absorption of fermentation products. *Appl. Microb. Biotechnol.*, **53**, 108–114 (1999).
 - 49) Mäkituokko, H., Saarinen, M., Ouwehand, A., and Rautonen, N., Effects of lactose on colon microbial community structure and function in a four-stage semi-continuous culture system. *Biosci. Biotechnol. Biochem.*, **70**, 2056–2063 (2006).