

Synbiotic effects of lactitol and *Lactobacillus acidophilus* NCFM™ in a semi-continuous colon fermentation model

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Abstract

The effects of *Lactobacillus acidophilus* NCFM™, lactitol, and the combination of lactitol and *L. acidophilus* NCFM™ were studied with a semi-continuous colon fermentation simulation; consisting of compartments mimicking, ascending, transverse, descending and sigmoid colon and their conditions with faecal inoculation. *L. acidophilus* NCFM™ was detected throughout the colon simulator. Lactitol was utilised early on by the microbes in the proximal part of the simulator. Lactitol increased the total numbers of microbes and bifidobacteria, and decreased clostridia cluster IV, while *L. acidophilus* NCFM™ alone decreased the numbers of clostridia cluster XIV. Combination treatment increased the numbers of bifidobacteria. Furthermore, concentrations of acetic acid, butyric acid and the sum of total short-chain fatty acids were increased by both lactitol-including treatments. The treatment with *L. acidophilus* NCFM™ alone increased the concentration of propionic acid and butyric acid. *L. acidophilus* NCFM™ tended to increase the total concentrations of biogenic amines, while lactitol suppressed production of biogenic amines also in the presence of *L. acidophilus* NCFM™. True synergistic effects are suggested in stimulation of the production of butyrate, an important microbial metabolite for colon health. In conclusion, lactitol as well as the combination of lactitol and *L. acidophilus* NCFM™ were found to exhibit complementary beneficial effects on the colon microbial composition and activity.

Keywords: lactitol, *Lactobacillus acidophilus* NCFM™, colon fermentation, *in vitro* simulation

1. Introduction

In recent years, various combinations of prebiotic substances and probiotic strains have been studied in clinical trials and by using both animal and *in vitro* models. The current hypothesis states that prebiotic substances aid the survival and growth of the probiotic strains especially in the colon (Ouwehand *et al.*, 2007). This hypothesis leads to the assumption that a prebiotic substance is selectively utilised by a particular probiotic strain and it might be able to assist the probiotic strain's survival when digested simultaneously. These potential synergistic effects of combinations of particular probiotic strains and prebiotic substances in the human colonic microbiota have attracted increasing interest during the last decade. The development

of new synbiotic combinations typically requires a long-term screening process, during which combinations of selected prebiotic substances and probiotic strains are tested both *in vitro* and *in vivo* in order to find the most appropriate and synergistic pre- and probiotic pairs.

Lactitol and *Lactobacillus acidophilus* NCFM™ have previously been tested for their respective prebiotic and probiotic properties. Lactitol has been shown to have bifidogenic and bowel movement increasing properties (Finney *et al.*, 2007; Oku *et al.*, 2005), while *L. acidophilus* NCFM™ has been reported to improve metabolism of small intestinal microbiota (Dunn *et al.*, 1998) and to act as an immune modulatory agent in the gastrointestinal tract (Foligne *et al.*, 2007). The synbiotic properties of the

combination of lactitol and *L. acidophilus* NCFM™ have recently been reported in a clinical trial conducted with healthy elderly volunteers (Ouweland *et al.*, 2009).

In order to expand the knowledge on the synbiotic effects of lactitol and *L. acidophilus* NCFM™ on the human colonic microbiota, *in vitro* colon fermentation simulation was utilised. Previously, fermentation of different prebiotics and prebiotic candidates and *in vitro* digested cocoa mass and probiotic cheese have been subjected to the same simulation system (Mäkeläinen *et al.*, 2007, 2009; Mäkivuokko *et al.*, 2005, 2006, 2007) for studying the metabolic activity of the microbes in a simulated human colonic environment. In the present study, the model was used for the first time to investigate potential synergistic activity of a probiotic and prebiotic combination by testing the effects of lactitol and/or *L. acidophilus* NCFM™ on the human colonic microbiota.

2. Materials and methods

The EnteroMix® simulator

The EnteroMix® colon simulator has previously been described in (Mäkeläinen *et al.*, 2007, 2009; Mäkivuokko *et al.*, 2005, 2006, 2007). Briefly, a single unit of the model consisted of four sequentially connected glass vessels (V1, V2, V3 and V4), with conditions adjusted to represent ascending, transverse, descending and sigmoid compartments of the human colon. Three separate simulations, each with four parallel simulation units, were run.

The simulator units were inoculated with fresh faeces collected from a single healthy male donor as described previously in (Mäkeläinen *et al.*, 2007, 2009; Mäkivuokko *et al.*, 2005, 2006, 2007). A single faecal donor was used in all three simulation runs to increase the total number of simulated channels with similar starting material to 12 and, thus increasing the statistical power of channel-wise comparisons. Briefly, faecal material was preconditioned by mixing it with 3 parts (wt/wt) of anaerobic simulation medium (Macfarlane *et al.*, 1998) and incubated 24 hours at 37 °C in an anaerobic serum bottle. After the preconditioning, 10 ml of the inoculum was injected anaerobically into the first vessel of each simulator unit and subsequently transferred through the following three vessels. All the vessels were pre-filled with 0.9% pre-reduced NaCl, and thus the original inoculum was diluted during the transfers. The final fermentation volumes of V1, V2, V3, and V4 in all units were 6, 8, 10 and 12 ml, respectively.

The studied substances, 150 mg (2.3×10^9 cfu) freeze-dried *L. acidophilus* NCFM™ (ATCC 700396, supplied by Danisco Cultures, Paris, France) and 2 g lactitol monohydrate powder (supplied by Danisco Sweeteners, Redhill, United Kingdom) were anaerobically mixed into 50 ml of simulator

medium and stored at 4°C, to avoid bacterial growth, for the duration of the simulation. During the simulation runs, the simulator contents were transferred from vessel to vessel in 3 ml pulses once every three hours. Thus, on estimate 1.4×10^8 cfu *L. acidophilus* NCFM™ and 120 mg lactitol were pumped into the first vessel (V1) every three hours along with the basal simulator medium. The liquid transitions occurred in similar fashion in all four separate units. Half of the fermented liquid (3 ml) in the last vessel (V4) was first pumped to effluent, and then similar liquid transitions continued vessel by vessel in reverse sequence (pumping 3 ml from V3 to V4, from V2 to V3, from V1 to V2 and finally 3 ml from fresh medium to V1). Simulation was stopped after 48 hours and samples were collected from the vessels V1 through V4.

Chemical analysis

Analysis of short chain fatty acids (SCFAs) was performed essentially as described in (Fava *et al.*, 2007) using gas chromatography, analysing the concentration of acetic, propionic, butyric, isobutyric, valeric, isovaleric and 2-methylbutyric.

Concentrations of biogenic amines; methylamine, ethylamine, tryptamine, 2-methyl-butylamine, putrescine, cadaverine, histamine, tyramine, spermidine, and spermine; were determined as dansyl derivatives by reversed phase HPLC as previously reported by Saarinen (2002).

Lactitol concentrations were determined with an in-house validated HPLC-analysis method using anhydrous lactitol as reference material. Samples were injected (25 µl) to a cation-exchange column (Aminex HPX-87P, 30×7.8 mm in Pb⁺-form, BioRad, Hercules, CA, USA), running temperature 85 °C, pumping water as carrier liquid at 0.7 ml/min and the lactitol peaks were detected with a refractive index detector (BioRad).

Microbiological analysis

The number of bacteria was determined by using a flow cytometric FACSCalibur-system (BD Biosciences, San Jose, CA, USA) as previously described (Apajalahti *et al.*, 2002). For determination, the formaldehyde fixed samples were stained with a fluorescent, nucleic acid binding dye, SYTO 24 (Molecular Probes, Leiden, the Netherlands).

Bifidobacteria-genus specific (Mäkivuokko *et al.*, 2005) and *L. acidophilus* specific (Ouweland *et al.*, 2009) primers and probes labeled with TaqMan® methodology (Applied Biosystems, Foster City, CA, USA) and quantitative real-time polymerase chain-reaction (QPCR) were used to determine total bifidobacteria and *L. acidophilus* concentrations. The SYBR green methodology (Applied Biosystems) was used for quantification of clostridia cluster XIV (Song *et al.*, 2004),

clostridia cluster IV (Matsuki *et al.*, 2004) and *Roseburia intestinalis* and closely related strains. The *Roseburia* primer pair was designed to amplify a 16S rRNA gene sequence of 146 bp located at bp 264-410 in the DNA sequence of AJ312385. The sequence of the forward Roseint_F primer was 5'-GCATGACCTGGTGTGAA-3', and for the reverse gRose_R primer 5'-TTGGGCCGTGTCTCAG-3'. The specificity of this primer pair was tested by PCR, and identified *R. intestinalis* and *Ruminococcus productus*. The 25 µl PCR reaction mixture was composed of Power SYBR green PCR Master Mix (Applied Biosystems) containing 300 nM of each primer. The annealing temperature was 56 °C and *R. intestinalis* DSM 14610 was used as a standard.

Statistical analysis

To determine the statistical significances between different simulated treatments Student's two sample t-test was used. *P*-values were associated with the significances and differences were considered to be significant at $P < 0.05$. The relative changes (\pm standard error of mean; SE) of measurements compared with the corresponding baseline values are also reported.

3. Results

Effects on residual lactitol

Three independent colon fermentation simulations, each including four parallel treatments, control, lactitol, *L. acidophilus* NCFM™ and lactitol + *L. acidophilus* NCFM™, were performed. In the two treatments including lactitol, lactitol was rapidly consumed by the microbes in the proximal and ascending colon simulating vessels (1 and 2). On average 571 mg (\pm SE 265 mg) and 0.8 mg (\pm SE 0.8 mg) lactitol were measured respectively in vessels V1 and V2 in the lactitol treatment. In lactitol + *L. acidophilus* NCFM™ treatment on average 486 mg (\pm SE 249 mg) and 0 mg lactitol were measured respectively in vessels V1 and V2. No measurable concentrations of lactitol were detected in the descending and distal colon simulating vessels (V3 and V4).

Effects on microbes

Both treatments including lactitol increased the total number of microbes by supporting the whole colon fermentation, although only lactitol + *L. acidophilus* NCFM™ treatment reached statistical significance ($P = 0.02$). The *L. acidophilus* NCFM™ treatment had no effect on the total microbial cell numbers during the fermentation when compared with the control treatment (Table 1). However, supplementation with *L. acidophilus* NCFM™ increased the total numbers of *L. acidophilus* NCFM™-like counts significantly ($P = 0.04$) and decreased the counts of clostridia cluster IV ($P = 0.05$), while it had no effect on the numbers

of bifidobacteria, *R. intestinalis*-like or clostridia cluster XIV, when compared to the control. In contrast, lactitol treatment increased the total numbers of bifidobacteria ($P = 0.03$) and decreased numbers of clostridia cluster XIV ($P = 0.02$), while it had no effect on the *L. acidophilus* NCFM™-like, clostridia cluster IV, or *R. intestinalis*-like numbers. The combination treatment resulted only in a significant increase in bifidobacteria ($P = 0.006$), when compared to the control treatment (Table 1).

Effects on microbial metabolites

The *L. acidophilus* NCFM™ treatment significantly increased the total concentrations of propionic and butyric acid ($P = 0.01$, $P = 0.03$, respectively) in the whole simulator, as compared to the control simulations (Figure 1). When compared with the respective concentrations in the control simulations, the concentrations of acetic, butyric and total short-chain fatty acid concentrations were all increased approximately two to three-fold by the treatments supplemented by lactitol ($P = 0.008$, $P = 0.05$, $P = 0.006$, respectively). Lactitol also tended to increase the concentrations of lactic acid, although this increase did not reach statistical significance ($P < 0.09$). The combination treatment increased concentrations of acetic, butyric, and short-chain fatty acids similar to the lactitol treatment ($P = 0.01$, $P = 0.007$, $P = 0.008$, respectively). Similarly, there was a trend towards an increase in lactic acid concentrations ($P = 0.09$) (Figure 1).

Supplementation of the colon simulator by *L. acidophilus* NCFM™ tended to increase cadaverine ($P < 0.1$), putrescine ($P < 0.09$) concentrations and the total biogenic amine concentrations (Figure 2). The concentrations of biogenic amines were also significantly affected by the lactitol including treatments. Compared to the control treatment, concentrations of cadaverine in NCFM+lactitol channels and methylamine in lactitol-channels were significantly reduced ($P = 0.03$ and $P = 0.03$, respectively) and a strong reducing trend was observed in cadaverine in lactitol-channels and putrescine in NCFM+lactitol channels ($P = 0.06$ and $P = 0.06$, respectively), while tyramine concentrations were significantly increased in both lactitol including simulations ($P = 0.02$ and $P = 0.02$, respectively). The sum concentrations of the biogenic amines also tended to be lower than in the control treatment ($P = 0.06$). Combined supplementation with lactitol and *L. acidophilus* NCFM™ treatment resulted in slightly higher concentration of tyramine ($P = 0.03$), but lower concentration in total biogenic amines ($P = 0.03$) (Figure 2).

4. Discussion

Different *in vitro* models have recently been utilised to test the synbiotic effects of combinations of various probiotic strains and prebiotic substances. Simple *in vitro* pure culture

Table 1. The *L. acidophilus* NCFM™-like, *Bifidobacterium*-genus, clostridia cluster XIV, clostridia cluster IV, *R. intestinalis*-like and the total numbers of microbes in the simulator counts/ml after three 48-h fermentation simulations; ±SE indicate standard error of mean. Statistical significance ($P<0.05$) of all treatment vessels combined (V1-V4) compared to corresponding control vessels (V1-V4) is marked by asterisks following the treatment name.

		Control	NCFM	Lactitol	NCFM+Lactitol	Control	NCFM	Lactitol	NCFM+Lactitol
		<i>L. acidophilus</i>				<i>Bifidobacterium</i> spp.			
Vessels	V1	1.17E+02	1.09E+06*	5.72E+02	1.45E+04	6.80E+07	9.91E+07	1.65E+09*	1.81E+09*
Qty/ml	V2	2.40E+01	1.10E+06*	4.57E+03	6.56E+06	4.51E+07	4.92E+07	3.11E+09*	1.33E+09*
	V3	1.39E+01	1.44E+05*	5.57E+02	2.54E+05	5.51E+07	1.06E+08	3.52E+08*	1.45E+09*
	V4	5.44E+01	2.46E+06*	1.82E+02	4.28E+05	1.60E+08	9.27E+07	1.95E+09*	8.02E+08*
±SE	V1	7.66E+01	6.65E+05	4.37E+03	6.46E+06	6.21E+07	6.00E+07	1.46E+09	2.65E+09
	V2	1.71E+01	4.04E+05	5.00E+02	1.08E+05	2.36E+07	2.70E+07	1.99E+09	7.04E+08
	V3	2.15E+01	2.22E+06	1.25E+02	2.94E+05	9.69E+06	5.71E+07	1.58E+08	1.03E+09
	V4	2.47E+02	1.80E+05	7.37E+01	9.03E+04	7.82E+07	3.37E+07	8.78E+08	5.42E+08
		Clostridia cluster XIV				Clostridia cluster IV			
Vessels	V1	2.11E+09	4.46E+09	6.10E+08*	6.46E+07	1.52E+09	8.93E+08*	4.20E+08	3.06E+08
Qty/ml	V2	2.12E+09	5.68E+08	1.43E+09*	4.31E+08	1.04E+09	3.05E+08*	5.69E+08	2.13E+08
	V3	1.31E+09	1.82E+09	3.43E+08*	1.57E+09	4.40E+08	5.07E+08*	5.17E+07	5.82E+08
	V4	1.08E+09	8.92E+08	7.13E+08*	3.20E+08	6.73E+08	2.67E+08*	6.59E+08	1.25E+08
±SE	V1	1.83E+09	3.84E+09	3.07E+08	6.45E+07	1.32E+09	6.35E+08	3.69E+08	2.70E+08
	V2	1.20E+09	1.46E+08	1.16E+09	2.87E+08	6.92E+08	1.09E+08	2.88E+08	1.23E+08
	V3	8.75E+08	1.65E+09	2.02E+08	1.27E+09	2.07E+08	3.79E+08	2.91E+07	1.68E+08
	V4	6.71E+08	6.63E+08	4.53E+08	1.91E+08	2.65E+08	1.03E+08	4.91E+08	2.07E+07
		<i>Roseburia</i>				Total microbes			
Vessels	V1	2.55E+06	9.46E+06	2.62E+06	2.76E+06	2.62E+09	3.12E+09	2.35E+09	3.06E+09*
Qty/ml	V2	1.54E+07	4.57E+06	2.94E+06	1.61E+06	5.29E+09	5.91E+09	6.28E+09	8.64E+09*
	V3	3.29E+06	1.65E+06	1.78E+06	1.17E+07	7.62E+09	7.77E+09	9.78E+09	1.09E+10*
	V4	4.21E+06	3.20E+06	2.55E+06	1.03E+06	9.16E+09	7.36E+09	1.19E+10	1.13E+10*
±SE	V1	1.62E+06	4.61E+06	2.07E+06	2.56E+06	7.27E+08	5.93E+08	9.83E+08	1.60E+09
	V2	1.40E+07	3.39E+06	1.46E+06	3.10E+05	1.86E+09	1.39E+09	1.18E+09	1.05E+09
	V3	1.96E+06	3.14E+05	1.68E+06	5.92E+06	1.53E+09	9.27E+08	2.78E+09	2.11E+09
	V4	1.25E+06	8.83E+05	1.19E+06	6.89E+05	7.34E+08	8.49E+08	2.28E+09	2.33E+09

models (e.g. Crittenden *et al.*, 2001)) are essential tools in large-scale screening processes of several combinations of pro- and prebiotics, enabling the growth of the selected strain under optimal or near-optimal cultivation conditions, while utilising a single prebiotic substance as prime carbon and energy source. Evaluation of the prebiotic utilisation by the probiotic strains and the bacterial growth rates enables the selection of the most potent pairs for further analysis. More sophisticated *in vitro* models, however, allow a detailed study of the gastrointestinal effects of a smaller number of potential synbiotic pairs under controlled fermentation conditions in the presence of the complex human colon microbial community (Finney *et al.*, 2007). Importantly, these models also enable studies with potential synbiotic pairs in parallel with only the probiotic and only prebiotic treatments that are often too expensive to include in clinical trials assessing the health effects of diets supplemented with synbiotic products (Finney *et al.*, 2007).

In the current study, the synbiotic potential of a well-documented probiotic strain, i.e. *L. acidophilus* NCFM™ (Sanders and Klaenhammer, 2001), and a sugar alcohol with potential prebiotic effects, i.e. lactitol, were studied with *in vitro* colon simulation model mimicking closely colonic fermentation conditions (Mäkeläinen *et al.*, 2007, 2009; Mäkiyuokko *et al.*, 2005, 2006, 2007). Previously lactitol has been reported to exhibit acetic and lactic acid increasing effects in human faecal samples (Finney *et al.*, 2007) and, as reviewed in (Drakoularakou *et al.*, 2007), to lower pH, reduce concentrations of aromatic compounds and reduce the activity of carcinogenetic enzymes in faeces. Consistent selective increases in faecal lactobacilli, bifidobacteria or both by lactitol have been observed in human studies (Hotten *et al.*, 2003). The combination of lactitol and *L. acidophilus* NCFM™ has been previously reported to promote synbiotic effects in healthy elderly persons (Ouwehand *et al.*, 2009). When administered twice

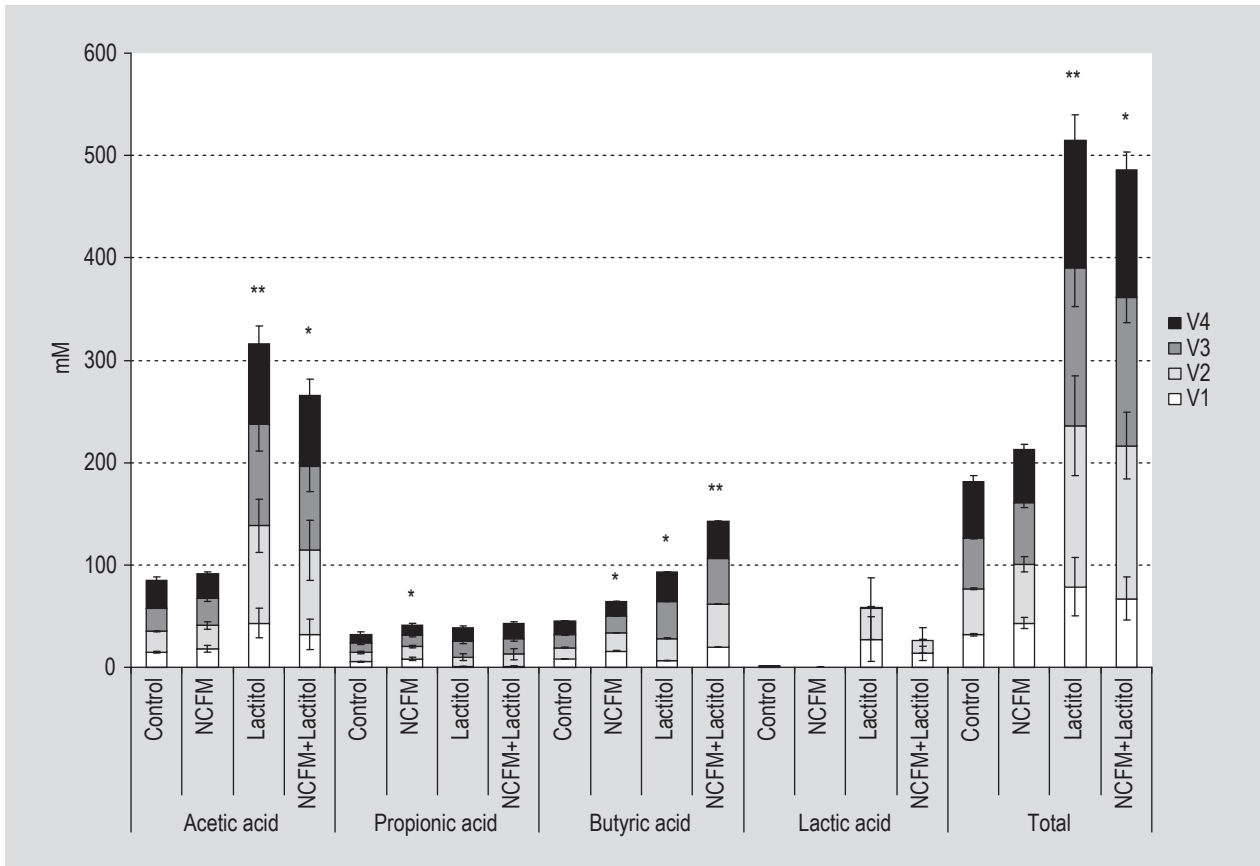


Figure 1. The sums of the most abundant short chain fatty acids in vessels 1 to 4 after three 48-h fermentation simulations. Statistical significances compared to baseline values are marked with asterisks (* = $P \leq 0.05$, ** = $P \leq 0.005$) and error bars indicate standard error of mean.

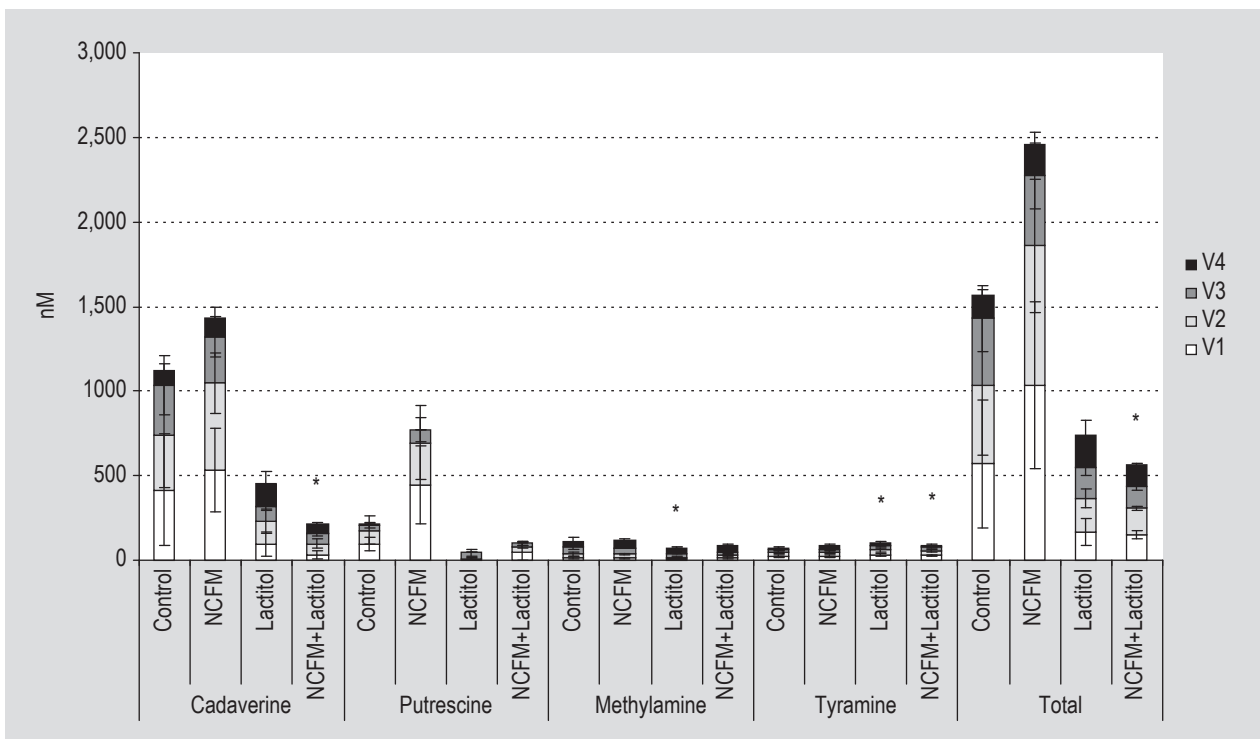


Figure 2. The sums of the most abundant biogenic amine concentrations in vessels 1 to 4 after three 48-h fermentation simulations. Statistical significances compared to baseline values are marked with asterisks (* = $P \leq 0.05$), error bars indicate standard error of mean.

daily, consumption of the combination was associated with an improvement in intestinal microbiota composition and mucosal functions: increased stool frequency, higher faecal numbers of *L. acidophilus* NCFM™ and bifidobacteria, as well as increased concentration of spermidine and PGE₂ when compared with the placebo group.

In this study, the treatments including *L. acidophilus* NCFM™ yielded consistently higher levels of *L. acidophilus* strains than with the feeding medium only; indicating the strain was able to multiply in human colonic conditions. Supporting the previous clinical findings, lactitol either in combination with *L. acidophilus* NCFM™ or alone had a general bifidobacteria increasing effect in the simulated colon, although no additional effect on the bifidobacteria by the combination product was observed. *L. acidophilus* NCFM™ alone did not affect the growth of bifidobacteria. The levels of clostridia cluster IV and XIV harbouring several known butyrate producers, such as *Roseburia* and ruminococci, were analysed for in-depth comparison between the numbers of butyric acid producers and butyric acid concentrations in different simulation vessels. Decreases in levels of clostridia cluster IV after *L. acidophilus* NCFM™ inclusion and in clostridia cluster XIV after lactitol treatment compared to control levels were observed. However, more targeted analysis of numbers of *R. intestinalis*-like species, i.e. members of clostridia cluster XIV, compared to control values did not reveal significant differences between treatments. When compared to baseline fermentations, and similar to previous *in vivo* animal trials (Islam *et al.*, 2004), lactitol treatment alone promoted acetic and butyric acid production, whereas *L. acidophilus* NCFM™ treatment alone increased propionic and butyric acid production. In this study, the increase in butyric acid production could not be explained by microbes belonging generally to clostridia clusters IV and XIV in lactitol and *L. acidophilus* NCFM™ treatments or by the targeted analysis of *R. intestinalis*-like species. This result indicates that other colonic microbiota residing butyrate acid producing species activated by lactitol and/or *L. acidophilus* NCFM™ are thus responsible for elevated butyric acid production during the simulations.

The addition of *L. acidophilus* NCFM™ along with lactitol specifically promoted a strong synergistic butyric acid promoting effect throughout the *in vitro* colon. Another strain of *L. acidophilus*, 74-2, has previously been reported to increase butyrate production *in vitro* in combination with a prebiotic, fructooligosaccharide (Gmeiner *et al.*, 2000). Butyrate is utilised as an energy source by mucosal epithelial cells, and thus the maximum potential of butyrate stimulating effect can be only measured from *in vitro* samples. Butyrate has also been reported to reduce the growth of colon carcinoma cells and reverse carcinogenic alterations (Scheppach and Weiler, 2004). In the present study, the measured biogenic amines are

fermentation metabolites produced from proteins. *In vivo*, it is more difficult to estimate the impact of diet-induced microbial fermentation on biogenic amines since both the host and the intestinal microbes produce biogenic amines in the faeces (Priebe *et al.*, 2002). Importantly in this study it was described that lactitol can suppress the biogenic amine concentrations overall when compared to control, even when in combination with *L. acidophilus* NCFM™. This is noteworthy since in general protein fermentation in the colon is considered an adverse effect, and can be associated with e.g. an increased risk of colon cancer (Smith and Macfarlane, 1997a,b). As an indication of the sustained synbiotic effect in the present study, the combination of lactitol and *L. acidophilus* NCFM™ increased SCFA concentrations and decreased biogenic amine concentrations throughout the simulated colon.

In conclusion, lactitol and the combination of lactitol and *L. acidophilus* NCFM™ were found to exhibit many beneficial effects in the colon microbial composition and function. True synergistic synbiotic effects are suggested in stimulation of production of butyrate, an important microbial metabolite for colon health.

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