Appendix 1

Effect of lactose on oro-cecal transit in lactose digesters and maldigesters

Tao He, Marion G. Priebe, Gjalt W. Welling and Roel J. Vonk

Adapted from:
European Journal of Clinical Investigation. 2006;36:737-42
Appendix 1

Abstract

**Background:** The transit time of the small intestine, in addition to the lactase activity, may also influence lactose digestion and thus play a role in the occurrence of lactose intolerance. The objective of this study was to investigate the effect of lactose on the oro-cecal transit time (OCTT) in lactose digesters and maldigesters and the possible mechanisms underlying the effects.

**Subjects and methods:** Twenty-eight Chinese subjects and 16 Dutch subjects underwent one glucose and one lactose challenge in two single-blinded tests. Twenty of the Chinese subjects without complaints after glucose underwent another lactose challenge. A 6-h symptom score (SSC) was recorded, breath hydrogen concentration measured and OCTT after consumption of glucose and lactose determined with the lactose-$^{13}$C ureide breath test. The lactose digestion index (LDI) was determined in the Dutch and the 20 Chinese subjects with the $^{13}$C/$^2$H-glucose test.

**Results:** Lactose digesters (n=13) and maldigesters (n=20) were classified based on the results of LDI and breath hydrogen test. The OCTT after challenges of lactose and glucose did not differ in digesters, while in maldigesters OCTT after the lactose challenge was shorter than that after glucose. There was no difference in OCTT after the glucose challenge between maldigesters and digesters. However, OCTT after the lactose challenge in maldigesters was shorter than that in digesters. LDI of the digesters was significantly higher than that of the maldigesters. OCTT after the lactose challenge is not correlated to LDI in maldigesters or in digesters. Based on SSC after the one glucose and two lactose challenges, a tolerant (n=7) and an intolerant (n=5) group were classified in the Chinese subjects. The two groups did not differ in LDI or OCTT after the lactose challenge. OCTT after the lactose challenge was not correlated to SSC or LDI.

**Conclusions:** Lactose triggers faster oro-cecal transit in lactose maldigesters, but not in digesters. This, however, cannot be explained by intestinal distension resulting from the osmotic load posed by maldigested lactose and suggests a direct effect of lactose on intrinsic factors regulating intestinal motility.
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Introduction

Several factors are considered to be involved in the occurrence of lactose intolerance, including the amount of lactose ingested, small-intestinal lactase activity, small-intestinal transit, and colonic processing of lactose (1,2). In lactose maldigesters, ingestion of a certain amount of lactose may result in acceleration of small intestinal transit, which is suggested to be caused by the osmotic load of maldigested lactose (3,4). However, scant attention has been paid to insights into the effect of the osmotic load of maldigested lactose on small intestinal transit. The accelerated transit could also be caused by intrinsic factors, e.g. lactose maldigesters, per se, might have a faster transit than digesters. In the present study, the degree of lactose digestion in the small intestine (as indicated with the lactose digestion index, LDI) and the oro-cecal transit time (OCTT) after challenges of glucose and lactose were compared between lactose digesters and maldigesters and between lactose tolerant and intolerant subjects. By measuring the degree of lactose digestion, the osmotic load posed by maldigested lactose can be estimated and thus, the correlation between osmotic load and OCTT can be analysed. Glucose is readily absorbed in the small intestine. Comparison of OCTT after the glucose challenge between lactose digesters and maldigesters can help to clarify whether lactose maldigesters, when without the influence of osmotic load, still have a faster transit than digesters. The lactose digesters and maldigesters were classified from a population with genetically determined lactase persistence (Dutch) and that with genetically determined lactase non-persistence (Chinese), respectively.

The objective of this study was to investigate the effect of lactose on OCTT in lactose digesters and maldigesters and the possible mechanisms underlying the effects. The results of this study may help to understand the pathophysiology of maldigestion of lactose and possibly that of others sugars as well.
Subjects and Methods

Subjects

Twenty-eight healthy Chinese subjects (temporarily living in The Netherlands, 16 females and 12 males, age range 20-31 years) and 16 healthy Dutch subjects (12 females and 4 males, age range 18-60 years) were recruited for this study. None of the subjects had diabetes or gastrointestinal disorders, or had taken antibiotics or laxatives during the three months prior to the study. Every subject signed a declaration of informed consent. The study was approved by the Medical Ethical Committee of the Groningen University Hospital and Faculty of Medical Sciences.

One glucose and two lactose challenges for the Chinese subjects

In order to well classify lactose tolerant and intolerant subjects from the Chinese subjects, the 28 Chinese subjects underwent two lactose challenges and one glucose (placebo) challenge. They first underwent a challenge of 25 g glucose and a challenge of 25 g lactose (lactose challenge 1) in two single-blinded tests (test 1 and 2). During both challenges a 6-h symptom score (SSC) was recorded and breath samples were collected for measurement of breath hydrogen concentration and OCTT after the challenge of glucose or lactose. The glucose challenge served as a placebo control. Twenty of the 28 subjects who reported SSC ≤ 2 after glucose challenge underwent a second lactose challenge of 25 g $^{13}$C-enriched lactose (lactose challenge 2), during which SSC was recorded, breath samples and blood samples were collected for determination of OCTT and lactose digestion index (LDI), respectively.

One glucose and one lactose challenge for the Dutch subjects

The 16 Dutch subjects underwent a challenge of 25 g glucose and a challenge of 25 g $^{13}$C-enriched lactose in two single-blinded tests. Breath samples were collected during both challenges for measurement of OCTT after challenges of glucose and
lactose and breath hydrogen concentration, and blood samples were collected during the lactose challenge for measurement of LDI. SSC was recorded during both challenges.

**SSC**

SSC was recorded as described before (2). Briefly, during 6 h after the lactose consumption, the subjects scored hourly the occurrence and severity of flatulence and abdominal cramps using a ranked scale: 0 = none, 1 = mild, 2 = moderate and 3 = severe. The occurrence and consistency of bowel movements were also registered: 0 = normal, 1 = loose, 2 = watery. To weigh the different complaints according to their significance, the scores were multiplied by different factors: 1 = flatulence, 2 = abdominal cramps, 4 = stool consistency. SSC is the sum of all scores recorded in 6 h. For Chinese subjects, the SSC after the glucose and the two lactose challenges was combined together to define subjects who were ‘truly’ lactose tolerant or intolerant according to the following criteria: (1) SSC ≤ 2 after glucose challenge; (2) SSC of the two lactose challenges was consistent. Subjects with SSC ≤ 2 in both lactose challenges were classified as lactose tolerant, and those with SSC > 2 as lactose intolerant.

**Measurement of OCTT and LDI, determination of breath hydrogen**

OCTT was measured with the lactose-[\(^{13}\)C] ureide breath test as described previously (5). OCTT was defined as the time elapsed between ingestion of the test substrate and a sustained (> 3 time points) rise in the \(^{13}\)C abundance in breath CO\(_2\) of > 2·0 \(^{13}\)C above baseline. In the case of a substrate which is digested and absorbed in the small intestine, OCTT is actually the oral-cecal transit time of lactose-[\(^{13}\)C] ureide under the influence of the substrate. Breath hydrogen concentration was analyzed as described before (6). LDI was determined with the \(^{13}\)C\(^2\)H-glucose test as described earlier (7) with a slight modification in calculation: LDI was not calculated as the mean value of the \(^{13}\)C/\(^2\)H concentration ratio in plasma of the 3 time points (45, 60 and 75 min), but of three different time points,
i.e. the time-point of the peak $^2$H-glucose concentration, the point before and after this point. The time point of the peak $^2$H-glucose concentration, which is strongly influenced by the gastric emptying rate, has been shown to vary among individuals. With this new method of calculation, the individual differences in gastric emptying rate can be better compensated. The data from the authors’ previous study (7) were re-calculated with this method to establish a new cut-off value for classifying lactose digesters and maldigesters.

Data analysis

Data are expressed as mean ± standard deviation (SD). The Student $t$-test (unpaired, two-tailed) and the Mann-Whitney $U$ test (independent samples, two-tailed) were applied as appropriate to assess differences in LDI and in OCTT between the maldigesters and digesters and between the lactose tolerant and intolerant subjects. The Student $t$-test (paired, two-tailed) and the Wilcoxon signed ranks test were used as appropriate to assess differences in OCTT between lactose and glucose in digesters and maldigesters, and between lactose challenge 1 and 2 in the Chinese subjects, and to assess differences in SSC between glucose and lactose challenges. Correlation was assessed by calculating the Spearman correlation coefficients as appropriate. $P < 0.05$ was regarded as significant. All analyses were performed using SPSS 12.0 for Windows software (SPSS Inc., Chicago, IL, USA). Figures were made using SigmaPlot 901 for Windows software (Systat Software Inc., California, USA).

Results

Classification of lactose digesters and maldigesters with LDI and breath - hydrogen

After the data from our previous study (7) were re-calculated for LDI, a new cut-off value for classifying lactose digesters and maldigesters was set at 0.29 (mean +
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Subjects with LDI lower than or equal to 0.29 and with a positive breath hydrogen response after ingestion of 25 g of lactose were classified as lactose maldigesters, otherwise the subjects were classified as digesters. When LDI and breath hydrogen response were not in agreement, the subject would not be included in the data analysis. Twenty subjects (19 Chinese, one Dutch) were classified as lactose maldigesters, and 13 (Dutch) as digesters.

LDI of the digesters (0.59 ± 0.12, mean ± SD) was significantly higher than that of the maldigesters (0.17 ± 0.06, mean ± SD) ($P < 0.001$).

After the glucose challenge, all subjects (Chinese and Dutch) had a negative breath hydrogen response. After the lactose challenge, a positive breath hydrogen response was observed in 3 of the 16 Dutch and in 26 of the 28 Chinese subjects.

**OCTT**

An increase in breath-$^{13}$CO$_2$ was measured in 15 and 6 of the maldigesters and digesters, respectively, after intake of glucose, and in 17 and 7 of the maldigesters and digesters, respectively, after lactose (lactose challenge 1 for Chinese). An increase in breath-$^{13}$CO$_2$ was measured in 17 of the 20 Chinese maldigesters who underwent the second lactose challenge.

In the case of digesters, there was no difference in OCTT between lactose and glucose ($P = 0.249$) (Fig. 1). In maldigesters, OCTT after the lactose challenge (OCTT of challenge 1 for Chinese) was significantly shorter than that of glucose ($P < 0.001$). The digesters and maldigesters did not differ in OCTT after the glucose challenge ($P = 0.970$), but OCTT after the lactose challenge of the maldigesters was significantly shorter than that of the digesters ($P < 0.001$).

OCTT after the lactose challenge (17 Chinese and one Dutch, lactose challenge 2 for Chinese) is not correlated to LDI in maldigesters ($r = -0.090, P = 0.723$) (Fig. 2) or in digesters ($r = -0.595, P = 0.159$) (not shown).

There was no difference in OCTT between lactose challenge 1 and 2 of the 20 subjects who underwent both lactose challenges ($P = 0.372$).
Table 1. Comparison of the lactose digestion index (LDI) and oro-cecal transit time (OCTT) after the lactose challenge between lactose tolerant and intolerant subjects (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>LDI</th>
<th>OCTT</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tolerant</td>
<td>7</td>
<td>0.22 ± 0.08</td>
<td>144 ± 58</td>
<td></td>
</tr>
<tr>
<td>Intolerant</td>
<td>5</td>
<td>0.16 ± 0.05</td>
<td>113 ± 68</td>
<td></td>
</tr>
</tbody>
</table>

*: challenge 2

Figure 1. The oro-cecal transit time (OCTT) after challenges of lactose and glucose in lactose maldigesters and digesters. md-lac1: lactose challenge 1, maldigesters, n = 17; md-lac2: lactose challenge 2, maldigesters, n = 17; md-glu: glucose, maldigesters, n = 15; d-lac: lactose, digesters, n = 7; d-glu: glucose, digesters, n = 6; *: P < 0.001 compared with md-gluc; **: P < 0.001 compared with d-lac; •: values lying outside the 10th and 90th percentiles.
After the glucose challenge, 8 and 1 of the 28 Chinese and the 16 Dutch subjects, respectively, had SSC > 2 while all of them had a negative breath hydrogen response. Only one Dutch subject, a lactose digester, reported SSC > 2 after the lactose challenge. For the Chinese subjects, SSC of glucose (1.8 ± 2.8, mean ± SD) was lower than SSC of lactose (challenge 1 (5.4 ± 6.1, mean ± SD), \(P < 0.001\); challenge 2 (3.0 ± 3.2, mean ± SD), \(P =0.018\)). In lactose challenge 2 with 20 Chinese subjects, SSC was not correlated with either LDI (\(r = -0.184, P = 0.436\)) or OCTT (\(r = -0.112, P = 0.659\)).

**Tolerant versus intolerant**

Based on the SSC of the one glucose and two lactose challenges, a lactose intolerant (n=5) and a tolerant group (n=7) were classified in the Chinese subjects according to the criteria described above. The two groups did not differ in LDI (\(P = 0.073\)), although there was a trend towards a lower LDI for the intolerant subjects. OCTT after the lactose challenge of the intolerant group was shorter than that of the tolerant group, but the difference was not significant (\(P = 0.440\)) (Table 1). OCTT after the glucose challenge could be measured only in two intolerant (285 and 300 min, respectively) and three tolerant subjects (165, 315 and 345 min, respectively) and therefore, was not included in statistic analysis. For the 12 subjects, SSC was not correlated with LDI of lactose (\(r = -0.377, P = 0.226\)) or OCTT (\(r = -0.227, P = 0.502\)).

**Discussion**

In the present study, OCTT after challenges of lactose and glucose was investigated in lactose digesters and maldigesters. Lactose shortened OCTT in maldigesters but not in digesters, while OCTT after the glucose challenge was not different between digesters and maldigesters.
Figure 2. Correlation between the oro-cecal transit time (OCTT) after the lactose challenge and the lactose digestion index (LDI) in lactose maldigesters (n = 18)

The mechanisms behind the lactose-induced acceleration of the small bowel transit in lactose maldigesters are not clear. The osmotic load posed by maldigested lactose is suggested to be the cause of the accelerated transit. The osmotic load causes secretion of fluid which causes dilatation of the intestine. Dilatation of the intestine induces an acceleration of small intestinal transit (1). The same mechanism is also suggested for accelerated transit after intake of lactulose, a sugar that is composed of galactose and fructose and cannot be digested in the small intestine (8,9). It has been shown that a hyperosmolar solution increases duodenal motor activity in a dose-dependent fashion. This effect speeds up the transit of intestinal contents and may serve to provide a means whereby hyperosmolar material is removed from the duodenum (10). This increase in motility may be due to two mechanisms, i.e. the distension resulting from osmotic equilibration or the triggering of a local osmoreceptor control of duodenal motility (11). When we
estimate the amount of fluid maldigested lactose would attract to the intestine in the lactose digesters and maldigesters, it is unlikely that this amount could cause intestinal distension which would result in alteration in the intestinal transit. We measured the degree of lactose digestion (indicated with LDI). On average, the lactose digesters and maldigesters digested 59% and 17% of the 25 g lactose ingested, respectively. The maldigested lactose, i.e. 10.2 g and 20.8 g, respectively, represents osmotic loads of 35 and 70 mOsm, which would bring approximately 120 and 240 ml water to the small intestine, respectively (12). It is unlikely that a volume of 120 or 240 ml would cause distension and that a difference of 120 ml could cause the difference in OCTT, considering that the volume of the small intestine is about 6 litres and approximately 9 litres fluid is absorbed every day (13).

As alteration in the transit is probably not caused by distension resulting from osmotic equilibration, it is hypothesized that maldigested lactose alters the transit by affecting the intrinsic factors that regulate intestinal motility. A hyperosmolar solution can increase intestinal motility by triggering local osmoreceptor control (11). Osmoreceptors are reported to exist in the upper duodenum and can increase duodenal motility and slow down gastric emptying as a result of a local osmoreceptor control mechanism. The effect of osmoreceptors is dose-dependent and regardless of whether intestinal distension is preserved or removed (10,11,14). We did not observe a dose-dependent correlation between the magnitude of osmolarity and change in intestinal transit as OCTT is not correlated to the amount of maldigested lactose. This suggests the involvement of other mechanisms underlying the accelerated transit, even if osmoreceptors would contribute partly to the effect. Postprandial motility of the gastrointestinal tract is controlled by nerves, hormones and paracrine mediators. Food intake is succeeded by the release of many gastrointestinal regulatory peptides such as pancreatic polypeptide, peptide YY and neuropeptide Y (15). Chemosensitive receptors are found to be present in the intestine and intervene in gastrointestinal motor coordination, e.g. glucoreceptors in the duodenum and jejunum (16, 17). It might be possible that undigested lactose alters the intestinal motility by stimulating the secretion of certain gastrointestinal hormones, or by stimulating the neural activities of certain chemosensitive receptors. As the digesters consisted only of Dutch and the
maldigesters mainly of Chinese subjects, another explanation could be that Chinese and Dutch subjects differ in some intrinsic factors that are involved in the regulation of gastrointestinal motility, which may lead to a different response in gastrointestinal motility towards the same substrate. Yue et al. (18) observed an ethnic difference in OCTT between Chinese and Caucasian subjects in the response to codeine. The possible explanations given are the differences in dietary habit (19) and endogenous opioids (20,21) involved in the regulation of gastrointestinal motility. In the present study, we did not observe differences in OCTT after the glucose challenge between the tolerant and intolerant group. However, this cannot exclude the possibility of different response from the Chinese and Dutch towards other sugars, especially those that are maldigested.

It is speculated that this hypothesis can be extended to explain the accelerated intestinal transit in malabsorption of other sugars and some food components, for instance, fructose and sorbitol (22-27). Malabsorption of lactose, fructose and sorbitol can be related, as fructose and sorbitol malabsorption are common when lactose malabsorption is present (27).

These results indicate that OCTT is not a major factor in the occurrence of symptoms of lactose intolerance, as SSC was not correlated with OCTT and there was no significant difference in OCTT between lactose tolerant and intolerant subjects. Similar results were observed by Roggero et al (28). In other studies it was shown that a slow oro-cecal transit contributes to less symptoms in lactose maldigesters (2,3,29,30). When the degree of lactose digestion and transit time in the small intestine are the same, what could be the reason that some maldigesters develop symptoms while others do not? One possibility could be the difference in the colonic fermentation of maldigested lactose (31). Another possible cause could be the difference in symptom perception (32) and/or intestinal dismotility, as suggested for irritable bowel syndrome (IBS) (33-35). IBS is shown to be strongly related to subjective lactose intolerance (36).

In conclusion, we observed that lactose accelerates the oro-cecal transit in lactose maldigesters, which is not caused by intestinal distension resulting from the osmotic load posed by undigested lactose. We propose that the presence of
undigested lactose itself in the intestinal lumen plays a role in acceleration of transit by affecting intrinsic factors that regulate intestinal motility.

Acknowledgments

We gratefully acknowledge Janneke Heimweg and Marianne Schepers for their assistance in the glucose and lactose challenge tests. We would like to thank Dr. Koen Venema of the Wageningen Centre for Food Sciences, the Netherlands, for critically reading the manuscript and helpful discussions.
Appendix 1

References

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Appendix 2

Identification of bacteria with β-galactosidase activity in faeces from lactase non-persistent subjects

Tao He, Marion G. Priebe, Roel J. Vonk and Gjalt W. Welling

Adapted from:
Abstract

**Background:** Previous studies suggested that besides the maldigestion of lactose in the small intestine, the colonic processing of lactose might play a role in lactose intolerance. ß-galactosidase is the bacterial enzyme which catalyzes the first step of lactose fermentation in the colon.

**Subjects and methods:** We propose a practical method to differentiate and identify bacteria with ß-galactosidase activity in faeces which combines a colony-lift filter assay with X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside) as substrate for differentiation and the fluorescent *in situ* hybridization technique for identification. The method was applied to faeces from lactase non-persistent subjects.

**Results:** After 28 subjects had undergone one glucose and two lactose challenges, consistent intolerant (n=5) and tolerant (n=7) groups were defined according to their symptom scores. Of the 28 faecal samples, 80.6% (mean, SD: 12.1, range: 47.8%-100%) of the total cultured bacteria were found to possess ß-galactosidase activity. The tolerant and intolerant groups did not differ in the percentage or composition of the bacteria with ß-galactosidase activity or ß-galactosidase activity in faeces.

**Conclusions:** The bacterial ß-galactosidase is abundant in the colon. The percentage or composition of the bacteria with ß-galactosidase activity in faeces is unlikely to play a role in lactose intolerance.
Faecal bacteria with β-galactosidase activity

Introduction

Several studies have been carried out to clarify the mechanisms involved in lactose intolerance (1). We recently demonstrated that lactose intolerant subjects with mild symptoms or with diarrhoea did not differ in degree of lactose digestion in the small intestine or orocecal transit time (2), which indicates that besides the digestion of lactose in the small intestine, other factors might influence the development of lactose intolerance, for instance, colonic factors (3,4). Therefore, colonic microbiota which ferment lactose, have become a focus of lactose intolerance research. In our subsequent study in which faecal bacteria were quantified with fluorescent in situ hybridization (FISH), the total number of hybridizable bacteria was found to correlate negatively with symptom scores (SSC) of lactose intolerance, based on which it was suggested that a higher fermentation capacity of the colonic microbiota contributes to the reduction of lactose intolerance (5).

The colonic capacity to ferment lactose could be determined by the amount, composition and enzyme activity of the lactose-fermenting bacteria in the colon. β-galactosidase is the enzyme that catalyzes the first step of lactose fermentation in the colon: the hydrolysis of lactose into glucose and galactose which will subsequently be degraded. Among the lactose-fermenting bacteria, the β-galactosidase activity may vary up to 4-fold (6). Therefore, the amount and composition of the bacteria with β-galactosidase activity could be determinants of the fermentative capacity of lactose in the colon and studies on this topic might elucidate the possible role of colonic microbiota in lactose intolerance.

In the present study, a method to differentiate and identify bacteria with β-galactosidase activity (intra- and extra-cellular) was developed and applied to faecal samples from subjects with genetically determined low lactase activity to investigate whether the relative amount (percentage) and composition of bacteria with β-galactosidase is related to the development of lactose intolerance. For differentiation of bacteria with the enzyme activity, faecal bacteria were first cultured on a general medium agar instead of a medium containing X-gal and then transferred on a filter with X-gal (colony-lift filter assay) to avoid possible
influence of X-β-galactosidase activity gal on growth of the bacteria. The bacteria were identified with FISH.

Subjects and Methods

Subjects

Twenty-eight healthy Chinese subjects (temporarily living in The Netherlands, 16 female and 12 male, age range 20-31 years) were recruited for this study. All subjects did not have diabetes or gastrointestinal disorders, and had not taken antibiotics or laxatives during the three months prior to the study. Every subject signed a declaration of informed consent. The study was approved by the Medical Ethical Committee of the Groningen University Hospital and Faculty of Medical Sciences.

Culturing of anaerobic bacteria in faeces

Faeces from each subject was collected in a sterile bag and maintained anaerobically with AnaeroGen™ COMPACT (Oxoid Limited, Hampshire, England). After arrival in the laboratory, samples were kept under 4 °C. All faecal samples were processed within 2 h after defecation.

Samples were homogenized by mechanical kneading for 2 min. For each sample, 10 g was suspended in 40 ml of an anaerobic salt solution (pH: 7.5; ingredients per litre: MgSO$_4$·7H$_2$O, 0.06 g; NaCl, 0.23 g; K$_2$HPO$_4$, 8.45 g; KH$_2$PO$_4$, 1.52 g; NaHCO$_3$, 9.50 g; 0.4% CaCl$_2$, 0.47 ml; 0.02% resazurine, 0.93 ml; gelatine, 0.47 g; cysteine, 0.12 g) and vortexed for 3 min together with a few glass beads. The suspension was centrifuged at 170 × g for 1 min and the supernatant was taken into an anaerobic chamber (Anaerobic workstation, Concept 400, West Yorkshire, UK; 10%H$_2$, 10%CO$_2$, 80%N$_2$) and further diluted with the anaerobic salt solution to make a tenfold dilution series of $10^7$, $10^8$ and $10^9$. Of each dilution, 100 µl was
Faecal bacteria with β-galactosidase activity

inoculated on Brucella blood agar (BBA, diameter of the plate: 14 cm) and incubated for 72 h.

Differentiation of bacteria with β-galactosidase activity with X-gal

A β-galactosidase colony-lift filter assay was carried out to differentiate the bacteria with β-galactosidase activity according to the product protocol of the MATCHMAKER Two-Hybrid System (CLONTECH, USA). With this method, bacteria with intra- or extra-cellular β-galactosidase will be detected. After incubation for 72 h, for each faecal sample, the dilution with 20-150 colonies per plate was selected for enumeration of the total amount of colonies. The colonies on the plate were transferred to a Whatman filter (Qualitative, Whatman International Ltd Maidstone, England), during which both the filter and agar were marked to give an indication for orientation. The plate was kept in the anaerobic chamber for another 48 h to let the colonies re-grow for identification of bacteria with β-galactosidase activity with FISH. The filter with colonies was submerged in liquid nitrogen for 10 sec to disrupt the cells. After thawing, the filter was placed on top of another filter pre-soaked in Z buffer/X-gal (ingredients per100 ml: X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 0.134 g, Roche Diagnostics GmbH, Mannheim, Germany; β-mercaptoethanol, 0.27 ml; Na₂HPO₄·7H₂O, 1.61 g; NaH₂PO₄·7H₂O, 0.55g; KCl, 0.075g; MgSO₄·7H₂O, 0.0246g) in a Petri-dish. The Petri-dish was sealed with Parafilm and kept under 32 °C for 1 h to let the colour develop. Then the amount of blue dots on the filter was counted.

Identification of bacteria with β-galactosidase activity with fluorescent in situ hybridization (FISH)

For each faecal sample, 18 colonies which expressed β-galactosidase activity were chosen for identification of bacteria with FISH. Separate colonies of diverse morphology were picked up randomly. The β-galactosidase-producing colonies were identified by aligning the filter to the agar plate using the orientation marks. The cells were fixed with phosphate-buffered saline:ethanol (1:1 (v/v)) and stored
Appendix 2

under -20°C until measurement. The identification of bacteria was carried out by using 16S rRNA oligonucleotide probes targeting the major bacterial groups in faeces. The probes are listed in Table 1 and were described earlier (7) with the exception of the Pep197 probe which was newly developed and validated in our laboratory (data not shown). With this set of probes, more than 90% of the total bacterial cells in the faeces of adults could be detected. The hybridization and visualization of fluorescent cells were carried out according to the methods described previously (8).

**Symptom score (SSC)**

After donating faecal samples, the 28 subjects first underwent a challenge of 25 g glucose and a challenge of 25 g of lactose in a blinded manner. SSC was recorded during the 6 h after the challenge according to the method described earlier (2). The purpose of the glucose and lactose challenges was to select subjects who did not report complaints after glucose ingestion. A SSC less than 2 was considered as ‘no complaints’. Among the 28 subjects, 20 reported a SSC of less than 2 after glucose and they underwent another challenge of 25 g of lactose after which SSC was also recorded. The SSC after the glucose and the two lactose challenges was combined together to define subjects who were ‘truly’ lactose tolerant or intolerant according to the following criteria: (1) SSC≤2 after glucose challenge; (2) SSC after the 2 lactose challenges was consistent. Therefore, lactose tolerant subjects are defined as having SSC≤2 after glucose and the two lactose challenges; while intolerant subjects are defined as having SSC≤2 after glucose and SSC > 2 after the two lactose challenges. The SSC recorded during the second lactose challenge were used in the analysis of possible correlation between bacteria with ß-galactosidase activity and SSC.
*Faecal bacteria with β-galactosidase activity*

**Table 1.** The 16S rRNA oligonucleotide probes used to identify bacteria with β-galactosidase activity in faeces

<table>
<thead>
<tr>
<th>Probes</th>
<th>Targeting groups</th>
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<tr>
<td>Ato291</td>
<td><em>Atopobium</em> group</td>
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<tr>
<td>Bac303</td>
<td><em>Bacteroides/Prevotella</em></td>
</tr>
<tr>
<td>Bif164</td>
<td><em>Bifidobacterium</em></td>
</tr>
<tr>
<td>Chis150/Clit135</td>
<td><em>Clostridium histolyticum/lituseburense</em> group</td>
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<td>Ecoli1531</td>
<td><em>Enterobacteriaceae</em></td>
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<td>Ecyl387</td>
<td><em>Eubacterium cylindroides</em> group</td>
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<td>Elgc01</td>
<td><em>Eubacterium low G+C2</em></td>
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<td>Erec482</td>
<td><em>Eubacterium rectale/Clostridium coccoides</em> group</td>
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<td><em>Lactobacillus/Enterococcus</em></td>
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<tr>
<td>Pep197</td>
<td><em>Peptostreptococcus</em></td>
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<td><em>Phascolarctobacterium</em> group</td>
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<td><em>Ruminococcus</em> group</td>
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<td>Stre493</td>
<td><em>Streptococcus/Lactococcus</em></td>
</tr>
<tr>
<td>Veil223</td>
<td><em>Veillonella</em></td>
</tr>
</tbody>
</table>

* All probes have been described and validated elsewhere (7) except for Pep197 which was recently validated in our laboratory (unpublished).

**Quantification of β-galactosidase activity in faeces**

Of the 28 subjects, 12 were defined as lactose tolerant or intolerant according to the criteria mentioned above. β-galactosidase activity in faeces from these 12 subjects was quantified. Faeces was 1:19 (w/v) diluted with the anaerobic salt solution and 0.5 ml of this suspension was sonicated on ice (4 × 1 min with 15 s interval, at an amplitude setting of 24) with a Soniprep 150 (Beun de Ronde BV, Abcoude, the Netherlands) followed by centrifugation (16100 × g, 10 min). The β-galactosidase activity in the supernatant was measured by determining the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside (PNPG) at 37 °C (9).
Appendix 2

Data analysis

Data are expressed as mean ± standard deviation (SD). The Mann-Whitney U test (independent samples, two-tailed) was applied to assess differences between groups in percentages of β-galactosidase-producing cells (with total bacteria as 100%), percentages of Bacteroides/Prevotella, Bifidobacterium and the Eubacterium rectale/Clostridium coccoides group (with total β-galactosidase-producing cells as 100%) and β-galactosidase activity in faeces. Correlation was assessed by calculating the Spearman correlation coefficients. $P < 0.05$ was regarded as significant. All analyses were performed using SPSS 12.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Results

The percentage and composition of bacteria with β-galactosidase activity in faeces

For the 28 samples, the counts of total bacteria cultivated on the BBA agar were $0.9 \times 10^{10}$ (SD: $0.5 \times 10^{10}$) cells per g of wet faeces. The colonies that produced blue colour on the filter with X-gal were regarded as bacteria with β-galactosidase activity, which would include bacteria with either extra- or intra-cellular β-galactosidase. The percentages of colonies with β-galactosidase activity (with the total colonies as 100%) and the composition of the blue colonies (with the blue colonies as 100%) are presented in Table 2. On average, in 80.6% (SD: 12.1) of the colonies from the faecal samples, the enzyme was found to be present. But the percentages varied among different faecal samples, with a range of 47.8%-100%.

In total, 504 of the blue colonies were chosen randomly for identification with FISH, of which 461 were identified with the 14 group-specific probes. Bacteroides/Prevotella was the major β-galactosidase producer in most of the samples, comprising 70% (SD: 21.9) of the chosen blue colonies. Furthermore, of all the bacteria with β-galactosidase activity, Bacteroides/Prevotella was the only
Faecal bacteria with β-galactosidase activity

group which was detected in every faecal sample. 9.7% (SD: 13.2) and 7.4% (SD: 8.3) of the chosen blue colonies, respectively, were identified as Bifidobacterium and the Eubacterium rectale/Clostridium coccoides group, and they were found in 42.9% and 57.1% of the faecal samples, respectively. In some faecal samples, other bacterial groups, such as the Atopobium group, Streptococcus/Lactococcus and Lactobacillus/Enterococcus, were also found to possess this enzyme activity. No colonies responded positively to the probes for Enterobacteriaceae, Eubacterium cylindroides group, Phascolarctobacterium group or Veillonella, therefore these probes are not included in Table 2.

Association between the percentages and composition of bacteria with β-galactosidase activity in faeces and lactose intolerance

After one glucose and two lactose challenges, 12 subjects were defined as lactose intolerant (n=5) or tolerant (n=7) according to the criteria described above. The other 16 subjects could not be classified into either of the two groups according to our criteria. The SSC, percentages and composition of bacteria with β-galactosidase activity, and β-galactosidase activity in faeces of these two groups are summarized in Table 3.

The two groups were not significantly different in the percentages or composition of bacteria with β-galactosidase activity. There was no significant correlation between SSC and percentages of β-galactosidase-producing colonies (with the total colonies as 100%), or the percentages of Bacteroides/Prevotella, Bifidobacterium and Eubacterium rectale/Clostridium coccoides group (with the blue colonies as 100%) in these 12 subjects.
Table 2. The percentages of β-galactosidase-producing colonies (blue/total, with the total colonies as 100%) and the percentages of the blue colonies responding positively to different probes (with the blue colonies as 100%) in faeces from 28 subjects (subject 1-5: intolerant group; subject 6-12: tolerant group)

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<th>Bif164/ blue</th>
<th>Erec482/ blue</th>
<th>Ato291/135blue</th>
<th>Chis150lit135/blue</th>
<th>Elgo01/ blue</th>
<th>Lab158/ blue</th>
<th>Pep197/ blue</th>
<th>Rbro729/ Rfla730/blue</th>
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<td>70.0</td>
<td>9.7</td>
<td>7.4</td>
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</table>

-, not detected

*, the average and SD of the percentages of blue colonies responding to ato291, chis150lit135, elgo01c, lab158, pep197, rbro729rfla730 and strc493 probes were not calculated because they were only detected in a few samples
Faecal bacteria with β-galactosidase activity

β-galactosidase activity in faeces and its relation to X-gal results

β-galactosidase activity was quantified in faeces from the 12 subjects mentioned above. The average enzyme activity (U/g faeces) was 22.3 ± 9.3 (mean ± SD) with a range of 7.4-39.2. β-galactosidase activity in faeces was not correlated to the percentages of bacteria with β-galactosidase activity, nor was it correlated to the percentages of Bacteroides/Prevotella, Bifidobacterium or Eubacterium rectale/Clostridium coccoides group. The tolerant and intolerant groups were not significantly different in β-galactosidase activity (20.4 ± 5.0 vs. 25.1 ± 13.6). There was no significant correlation between SSC and β-galactosidase activity.

Table 3. The SSC, percentages and composition of colonies with β-galactosidase activity, and β-galactosidase activity in faeces of the lactose tolerant and intolerant groups (mean ± SD)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>SSCβ</th>
<th>Blue/total (%)</th>
<th>Bac303/total (%)</th>
<th>Bif164/total (%)</th>
<th>Erec482/total (%)</th>
<th>β-galactosidase (U/g faeces)</th>
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<td>80.3±7.4</td>
<td>76.2±20.7</td>
<td>13.5±14.3</td>
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<td>intolerant</td>
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<td>8.9±16.9</td>
<td>5.6±9.6</td>
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</table>

β, SSC of the second lactose challenge
*, significantly different from the tolerant group (P = 0.002)

Discussion

A number of substrates and assays are available for detection of β-galactosidase activity in vivo and in vitro in microorganisms. Substrates available include ortho-nitrophenyl-β-D-galactoside (ONPG), X-gal, chemiluminescent and fluorescent substrates. β-galactosidase activity can be assayed by the colony-lift filter assay and overlay assay, or on plates with incorporated X-gal (10). Favier et al. (11,12)
Appendix 2

reported a method to detect bacteria with β-galactosidase activity with X-gal. Faecal samples were cultured on an enriched Columbia agar medium modified by addition of X-gal. Colonies growing on this medium with blue halos were regarded as bacteria which produced extra-cellular β-galactosidase. Some of these colonies were sub-cultured for identification by classical biochemical and morphological characteristics.

In a pilot experiments, X-gal was added to Columbia agar and Wilkins-Chalgren agar by spreading 80 µg of X-gal solution (20 mg/ml, in dimethylformamide) evenly on the agar surface. When three faecal samples were cultured on these two modified agars, however, more than 90% of the blue colonies (with or without halos) were identified with FISH to be *Bifidobacterium* (data not shown). We assumed that the modified media in some way favoured the growth of *Bifidobacterium*, which did not reflect the true conditions in the faeces. Therefore, in our subsequent experiment, the faecal samples were first cultured on BBA agar medium without X-gal added and subsequently the β-galactosidase activity was assayed by transferring the colonies to a filter followed by identification by FISH. This colony-lift filter method has been used to assay β-galactosidase activity in yeasts (10). Its principle should also be applicable to bacteria and when combined with FISH, it would be an easy and efficient method to differentiate and identify bacteria with β-galactosidase activity.

When the method was applied to the faecal samples from 28 subjects with genetically determined low lactase activity, on average 80.6% (SD: 12.1; range: 47.8 %-100%) of the total cultured bacteria were found to possess β-galactosidase activity. This is higher than what was reported by Favier *et al.* (57% ± 29) (11), which might be explained by the fact that the colonies without halos which are supposed to produce intra-cellular β-galactosidase were included in our but not in their counting. However, it is not known yet whether the percentage of bacteria with β-galactosidase activity would be influenced by maldigested lactose entering the colon. Our results indicate that a major part of the colonic microbiota is capable of hydrolyzing lactose, which is in accordance with what was described earlier (6). However, individuals varied considerably in the percentages of bacteria with β-galactosidase activity.
Strains from each of the bacterial groups identified in our experiment to be β-galactosidase producers were reported previously to possess this enzyme activity (6,13). In the study by Favier et al. (11), the blue colonies identified by culturing belonged to 3 groups: Bacteroides, Prevotella and Bifidobacterium. In our experiment, 7 other groups besides 3 three groups, i.e. Atopobium group, Streptococcus/Lactococcus and Lactobacillus/Enterococcus, Clostridium histolyticum/lituseburense group, Eubacterium low G+C2, Peptostreptococcus and Ruminococcus group, were identified with FISH to possess β-galactosidase activity. For the last few years, the FISH technique has been employed to identify and quantify colonic microbiota (7,14). In our study, FISH was shown to be an easy and efficient way to identify a large number of β-galactosidase-producing colonies.

During method development, 60 white colonies were also picked up randomly from cultures of three faecal samples on Columbia, Wilkins-Chalgren or BBA agar with X-gal. When hybridized with group-specific probes targeting Bacteroides/Prevotella, Bifidobacterium and Eubacterium rectale/Clostridium coccoides group, eight of them were identified as belonging to Eubacterium rectale/Clostridium coccoides group, three as Bacteroides/Prevotella, but none as Bifidobacterium (data not shown).

There has been discussions of the advantages and disadvantages of using faeces to study the composition and activities of the colonic microbiota. Some studies found that the bacteria in faeces reflect the microbiota in the colon (15) while other studies did not (16). The metabolic activities measured in faeces can be quite different from in the proximal colon where there is active fermentation of carbohydrates by bacteria. For comparing lactose-fermenting capacities and composition of the colonic microbiota among lactose maldigesters and also considering difficulties in sampling in the colon, we consider faecal samples as a reasonable alternative. Some studies found that carbohydrate fermentation properties of faecal bacteria are individual-dependent and rather stable through time (17,18).

The results of measurement of bacteria with β-galactosidase activity were analyzed in relation to lactose intolerant symptoms. Considering the fact that lactose maldigestion correlates poorly with the symptoms of intolerance and the
bias in scoring of symptoms possibly caused by familiarization with the test procedures or placebo effect (19), a placebo control (glucose) was included besides two lactose challenges in an attempt to reliably define groups of lactose tolerance and intolerance. A tolerant (n=7) and an intolerant (n=5) group were defined. The 2 groups did not differ, in either the percentages or in the composition of the bacteria with β-galactosidase activity in faeces. For these 12 subjects, there is no significant correlation between SSC and percentages of β-galactosidase-producing colonies, or the percentages of *Bacteroides/Prevotella, Bifidobacterium* and *Eubacterium rectale/Clostridium coccoides* group in the blue colonies. Therefore, our results suggest that the percentages and composition of bacteria with β-galactosidase activity do not play a role in lactose intolerance.

Based on the observation that a major part of the faecal microbiota is capable of hydrolyzing lactose, we can hardly expect that lactose itself will present a large osmotic threat in the colon as it should be quickly degraded by the majority of colonic microbiota. It is not yet known whether the subsequent processes of bacterial fermentation after the hydrolysis of lactose might play a role in lactose intolerance. Furthermore, besides the fermentation process of lactose, other colonic factors, such as the colonic capacity to remove fermentation products, might also be related to lactose intolerance.

The large individual variations in the percentages of each bacterial group with β-galactosidase activity, however, might make it difficult to clarify the differences between the 2 groups. Large individual differences were also observed in the numbers of total bacteria and main groups of bacteria in faeces in one of our previous studies (5).

β-galactosidase activity in the 12 faecal samples from both lactose tolerant and intolerant subjects was quantified. No correlation was observed between β-galactosidase activity and the percentages and composition of bacteria with β-galactosidase activity. The possible explanation for this could be that the enzyme activity may vary considerably among the lactose-fermenting bacteria. The total enzyme activity in faeces is determined not only by the amount but also by enzyme activity of the bacteria with the enzyme. No correlation was found between SSC and β-galactosidase activity. In several intervention studies (3,19), faecal β-
Faecal bacteria with β-galactosidase activity increased after lactose ingestion for a period of time while intolerance symptoms decreased. However, whether increased β-galactosidase activity played a role in the alleviation of symptoms needs further investigation.

In conclusion, we propose a method to differentiate and identify bacteria with β-galactosidase activity in a complex microbiota. The method was applied to investigate faecal microbiota of subjects with genetically determined low lactase activity. The percentages and composition of bacteria with β-galactosidase activity in the distal colon do not seem to be related to lactose intolerance.

Acknowledgments

We thank Gerwin C. Raangs, Sandra P. van Tongeren and Hermie J.M. Harmsen for inspiring discussions. We also thank the subjects who kindly participated in our study.
Appendix 2

References


Colonic fermentation may play a role in lactose intolerance in humans

Tao He, Marion G. Priebe, Hermie J.M. Harmsen, Frans Stellaard, Xiaohong Sun, Gjalt W. Welling and Roel J. Vonk

Abstract

Background: The results of our previous study suggest that besides the small intestinal lactase activity and transit time, colonic processing of lactose may play a role in lactose intolerance. We investigated whether colonic fermentation of lactose is correlated with lactose intolerance.

Subjects and methods: After 28 Chinese subjects had undergone one glucose (placebo) and two lactose challenges, consistent lactose tolerant (n= 7) and intolerant (n= 5) subjects with no complaints after glucose administration were defined based on the 6-h symptom scores. Prior to the challenges, fecal samples were collected for in vitro incubation with lactose. The incubation was carried out in a static system under anaerobic conditions for 5 h during which samples were taken for measurement of short-chain fatty acids, lactate, lactose, glucose and galactose. Fecal bacterial composition was determined by fluorescent in situ hybridization.

Results: The tolerant and intolerant groups did not differ in the rate or degree of hydrolysis of lactose or production of glucose and galactose. The intolerant group produced D- and L-lactate, acetate, propionate and butyrate significantly faster than the tolerant group. In the intolerant group, the amount of acetate, propionate, butyrate and L- lactate produced was higher than that in the tolerant group. Fecal bacterial composition did not differ between the two groups.

Conclusions: The results indicate that the degree and rate of lactose hydrolysis in the colon does not play a role in lactose intolerance. However, after lactose is hydrolyzed, a faster and higher production of microbial intermediate and end metabolites, may be related to the occurrence of symptoms.
Introduction

Lactose intolerance refers to the gastrointestinal symptoms related to incomplete digestion of lactose. The adult-type lactase non-persistence occurring in a large part of the world population leads to lactose maldigestion, which in turn can, though not in all cases, lead to lactose intolerance. The origin of the symptoms of lactose intolerance is not well understood. The osmotic load caused by the undigested lactose cannot be regarded as the only mechanism behind the symptoms as lactose intolerance is poorly related to lactose maldigestion (1). This is supported by our recent study in which we observed that lactose intolerant subjects with mild symptoms or with diarrhoea did not differ in degree of lactose digestion in the small intestine or orocecal transit time (2). Based on these observations, we suggest that besides the lactose digestion capacity in the small intestine, the colonic processing of maldigested lactose may play a role in lactose intolerance.

The colonic microbiota, which ferments lactose, is an important factor in the colonic processing of lactose. The colonic microbiota can be studied on the levels of composition and the metabolic activity. In our recent study in which fecal bacteria were quantified with fluorescent in situ hybridization (FISH), we did not observe significant differences in the composition of the fecal microbiota between lactose intolerant subjects with mild symptoms or with diarrhoea, possibly because of large inter-individual differences. However, there was a negative correlation between the total number of bacteria and the 6-h symptom score (SSC), suggesting that the fermentation capacity of the colonic microbiota may be correlated to lactose intolerance (3).

During colonic fermentation, lactose is first hydrolyzed to glucose and galactose, which are subsequently fermented, leading to the production of a series of intermediate (e.g. lactate, formate and succinate) and end-product metabolites (i.e. acetate, propionate and butyrate, gas (H₂, CO₂ and CH₄) and biomass) (4, 5). In the present study, we investigated whether the colonic fermentation of lactose was correlated to lactose intolerance by comparing the in vitro lactose-fermenting indices of fecal bacteria from lactose tolerant and intolerant subjects. The degree
and rate of hydrolysis of lactose and production of lactate and short-chain fatty acids (SCFA) were compared between the two groups.

Subjects and Methods

Subjects

Twenty-eight healthy Chinese subjects (temporarily living in The Netherlands, 16 females and 12 males, age range 20-31 years) were recruited for this study. All subjects did not have diabetes or gastrointestinal disorders, and had not taken antibiotics or laxatives during the three months prior to the study. Every subject signed a declaration of informed consent. The study was approved by the Medical Ethical Committee of the Groningen University Hospital and Faculty of Medical Sciences.

Collection of fecal samples

The 28 subjects donated fecal samples for the in vitro fermentation experiment. Feces were collected in a sterile bag and maintained anaerobically with AnaeroGen™ COMPACT (Oxoid Limited, Hampshire, England). After arrival in the laboratory, samples were kept under 4 °C. All samples were processed within 2 h after defecation. The processing procedure took about 20 min.

In vitro fermentation of lactose by fecal bacteria

Fecal samples were diluted five times as described previously (6), brought into an anaerobic chamber (Anaerobic workstation, Concept 400, West Yorkshire, UK; 10%H₂, 10%CO₂, 80%N₂) and further diluted two times. For each fecal sample, 20 mL of this suspension was added to 20 mL of the anaerobic salt solution either with lactose (final concentration approximately: 55.6 mmol/L, or 0.4 g/ g stool homogenate) or without lactose (control culture). The purpose of the control
culture was to estimate the endogenous production of SCFA and lactate from carbohydrates, mucin or other substrates in the original fecal samples. Both the control culture and the culture with lactose were done in duplicate. In the incubation medium the feces were diluted 20 times. The cultures were incubated at 37 °C under anaerobic conditions for 5 h. During the incubation, samples were taken from the cultures at 0, 0.5, 1, 2, 3 and 5 h for quantification of SCFA, lactate and sugars (lactose, glucose and galactose). For measurement of lactate, the samples were immediately stored at -20 °C until measurement. For measurement of SCFA and sugars, 1 mL of the samples was first mixed with 1.5 mL of 96% ethanol, centrifuged at 1500 x g for 5 min and the supernatant was stored at -20 °C until analysis.

Classification of lactose tolerant and intolerant subjects

One to three weeks after donating fecal samples, the 28 subjects first underwent a challenge of 25 g glucose and a challenge of 25 g lactose in two single-blinded tests. SSC was recorded during the 6 h after the challenge according to the method described earlier (2). The purpose of the glucose challenge was to select subjects who did not report complaints after glucose ingestion. A SSC less than 2 was considered as ‘no complaints’. Among the 28 subjects, 20 reported a SSC of less than 2 after glucose and they underwent a second challenge of 25 g of lactose after which SSC was also recorded. The SSC after the glucose and the two lactose challenges was combined together to define subjects who were ‘truly’ lactose tolerant or intolerant according to the following criteria: (1) SSC ≤ 2 after glucose challenge; (2) consistent SSC after the two lactose challenges. Therefore, lactose tolerant subjects are defined as having SSC ≤ 2 after glucose and the two lactose challenges; while intolerant subjects are defined as having SSC ≤ 2 after glucose and SSC > 2 after the two lactose challenges. The SSC recorded during the second lactose challenge was used in the analysis of possible correlation between SSC and the composition of fecal bacteria.
Quantification of lactate, SCFA (acetate, propionate and butyrate) and sugars (lactose, glucose and galactose) in the in vitro fermentation samples

Of the 28 subjects, 12 were defined as lactose tolerant or intolerant according to the criteria mentioned above. Sugars, SCFA and lactate were quantified in the in vitro fermentation samples of these 12 subjects. Sugars were determined by gas chromatography by the method of Jansen et al. (7) with a few modifications, i.e. before derivatization, after methanol was added to the samples and the internal standard, the solution was directly evaporated to dryness without other processing steps; after derivatization, heptane was used for extraction instead of hexane; for gas chromatography analysis, GC-MS was used instead of GC, 1 µL was injected instead of 2 µL. The L- and D-lactate were quantified by an enzymatic method using Enzytec™ L- and D-lactate kits (Scil Diagnostics GmbH, Viernheim, Germany). For measurement of SCFA, 50 µL 15 mmol/L iso-butyrate in water (internal standard), 100 µL of the sample or SCFA standards (0 – 2 µmol)/ 96% ethanol (1:1.5, v/v) and 900 µL 96% ethanol / water (3:2, v/v) were added to a headspace vial. 100 µL 96% H₂SO₄ was added for acidification. The vials were stored at room temperature until analysis. The analysis is based on headspace gas chromatography (GC). The SCFA were separated on a WCOT fused silica 25m x 0.32 mm ID 0.2 mm Poraplot Q column (Varian, Middelburg, The Netherlands) using an Agilent 6890 GC (Agilent technologies, Amstelveen, The Netherlands). The flow rate was kept constant at 1.1 mL/min. The temperature programme was as follows: initial temperature 100°C; 10°C/min to 150°C; 7.5°C/min to 250°C. The gas phase sample was injected in the splitless mode using a 1 mL loop injection system. The injection temperature and flame ionization detector temperature were 250°C. The headspace device was an Agilent 7694 headspace sampler. The headspace conditions were as follows: headspace temperature 90°C, loop temperature 95°C, tray line 100°C, vial equilibration time 15 min, pressurizing time 3.0 min, loop fill time 1.0 min, loop equilibration time 0.25 min, injection time 0.50 min.
Quantification of bacteria in feces with fluorescent in situ hybridization (FISH)

16S rRNA oligonucleotide probes were used to detect the numbers of total bacteria and major bacterial groups in the fecal samples from the 12 lactose tolerant and intolerant subjects (Table 1). With this set of probes, more than 90% of the total bacterial cells in the feces of adults could be detected (8,9). The hybridization and visualization of fluorescent cells were carried out according to the methods described previously (8,10). 4’, 6-diamidino-2-phenylindole (DAPI)-staining was used to enumerate the total amount of cells in feces (10).

Normalization of the data with the number of total bacteria

As the amount of fecal sample used for incubation was determined by wet weight, it was possible that for different samples, different numbers of bacteria were added to the incubations. Therefore, the in vitro fermentation data were normalized to the number of total bacteria (wet weight).

Data analysis

Data are expressed as mean ± SD. Logarithmic transformation of the data was performed when necessary to obtain normally distributed data and when the data stayed skewed after logarithmic transformation, nonparametric tests were applied. The hydrolysis rates of lactose and the production rates of SCFA, lactate, glucose and galactose were calculated for periods within 0-0.5, 0.5-1, 1-2, 2-3 and 3-5 h of incubation (Table 2). The slope of the curve between two sampling points was calculated and taken as the rate of the time period. The slope of the curve between 0-5 h was calculated and taken as the overall rate. As the rate of fermentation during different periods of incubation can differ among subjects, which might be correlated with the occurrence of symptoms, comparison between the tolerant and intolerant groups took all the rates or concentrations of metabolites of different periods into consideration. The Univariate test was applied to assess the overall differences between the lactose tolerant and intolerant groups in the rates of
hydrolysis and production, and the overall differences in the concentrations of sugars, SCFA and lactate at 0, 0.5, 1, 2, 3 and 5h. The Student t-test (unpaired, two-tailed), the Mann-Whitney U-test and the Multivariate test were applied to assess the difference between the two groups in numbers of total cells (DAPI), total bacteria (Eub338) and major bacterial groups in feces (Bac303, Erec482, Fprau645, Bif164y, Ato291 and Rbro729/Rfla730), respectively. Correlations were assessed by calculating the Spearman correlation coefficients. $P < 0.05$ was regarded as significant. All analyses were performed using SPSS 12.0 for Windows software (SPSS Inc., Chicago, IL, USA).

Results

Classification of lactose tolerant and intolerant subjects
After the 28 subjects had undergone one glucose and two lactose challenges, 12 subjects were defined as lactose intolerant (n=5) or tolerant (n=7) according to the following criteria: (1) SSC $\leq 2$ after glucose challenge; (2) consistent SSC after the two lactose challenges; (3) tolerant when SSC $\leq 2$ and intolerant when SSC $> 2$.

Hydrolysis of lactose
During the 5 h incubation, the tolerant and intolerant groups did not differ in the hydrolysis rates of lactose ($P > 0.1$) or the production rates of glucose ($P > 0.1$) and galactose ($P = 0.09$) (Table 2), or in the concentrations of lactose (Fig. 1), glucose and galactose (data not shown).

Figure 1. In vitro hydrolysis of lactose by fecal bacteria from lactose tolerant (n=7) and intolerant (n=5) subjects. Values are means +/- SD.
Production of SCFA

The amounts of SCFA produced in the control cultures were subtracted from those in the cultures with lactose. During the 5 h incubation, the production rates of acetate ($P = 0.05$), propionate ($P < 0.01$) and butyrate ($P = 0.03$) were higher in the intolerant group than in the tolerant group (Table 2). The intolerant group produced more acetate ($P = 0.03$), propionate ($P < 0.01$) and butyrate ($P < 0.01$) than the tolerant group (Fig. 2). At 5 h, the ratios of acetate, propionate and butyrate were 6:1:1 and 4:1:1 for the tolerant and intolerant group, respectively. The concentrations of the three SCFA at 5 h was not different between the two groups ($P > 0.1$).

Table 2. *In vitro* hydrolysis rates of lactose and production rates of glucose, galactose, SCFA and lactate during incubation of fecal bacteria from lactose tolerant and intolerant subjects.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Time after inoculation, h</th>
<th>Tolerant (mmol L$^{-1}$ h$^{-1}$)</th>
<th>Intolerant (mmol L$^{-1}$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose</td>
<td>0-0.5</td>
<td>5.1 ± 5.9</td>
<td>2.4 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>0.5-1</td>
<td>8.1 ± 7.7</td>
<td>5.2 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>3.1 ± 5.2</td>
<td>8.2 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td>7.0 ± 2.9</td>
<td>5.4 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>3-5</td>
<td>4.6 ± 1.8</td>
<td>4.9 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>0-5</td>
<td>5.2 ± 2.1</td>
<td>5.7 ± 3.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0-0.5</td>
<td>0.2 ± 0.2</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>0.5-1</td>
<td>0.4 ± 0.5</td>
<td>0.4 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>0.6 ± 0.9</td>
<td>1.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td>0.4 ± 0.7</td>
<td>-0.1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>3-5</td>
<td>0.8 ± 0.9</td>
<td>0.4 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>0-5</td>
<td>0.5 ± 0.6</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Galactose</td>
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<td>0.1 ± 0.1</td>
</tr>
<tr>
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<td>0.5-1</td>
<td>0.4 ± 0.5</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>0.9 ± 0.9</td>
<td>0.7 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td>0.9 ± 0.8</td>
<td>0.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>3-5</td>
<td>2.4 ± 0.8</td>
<td>1.6 ± 0.8</td>
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<tr>
<td></td>
<td>0-5</td>
<td>1.0 ± 0.7</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td>Acetate</td>
<td>0-0.5</td>
<td>0.9 ± 0.9</td>
<td>2.9 ± 2.5</td>
</tr>
<tr>
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<td>0.5-1</td>
<td>2.8 ± 2.5</td>
<td>5.0 ± 2.9’</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>4.1 ± 3.6</td>
<td>6.4 ± 3.4’</td>
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<td>4.1 ± 2.2</td>
<td>4.3 ± 2.0’</td>
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<tr>
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<td>3.3 ± 2.0</td>
<td>4.5 ± 2.1</td>
</tr>
<tr>
<td>Propionate</td>
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</tr>
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<td>0.7 ± 0.8</td>
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<tr>
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<td>0.8 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>2-3</td>
<td>0.5 ± 1.1</td>
<td>1.4 ± 1.6</td>
</tr>
<tr>
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<td>3-5</td>
<td>0.5 ± 1.1</td>
<td>1.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>0-5</td>
<td>0.5 ± 0.6</td>
<td>1.2 ± 1.0</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0-0.5</td>
<td>0.0 ± 0.1</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.5-1</td>
<td>0.2 ± 0.2</td>
<td>0.4 ± 0.3’</td>
</tr>
<tr>
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<td>1-2</td>
<td>0.5 ± 0.6</td>
<td>1.4 ± 0.9’</td>
</tr>
<tr>
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<td>2-3</td>
<td>0.6 ± 0.7</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>3-5</td>
<td>1.1 ± 1.5</td>
<td>2.3 ± 1.4’</td>
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<tr>
<td></td>
<td>0-5</td>
<td>0.5 ± 0.6</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>L-lactate</td>
<td>0-0.5</td>
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<td>0.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>0.5-1</td>
<td>0.3 ± 0.5</td>
<td>0.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>1-2</td>
<td>0.8 ± 1.0</td>
<td>1.5 ± 2.9</td>
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<tr>
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<td>1.3 ± 0.5</td>
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<td>1.3 ± 0.5</td>
<td>0.9 ± 1.1</td>
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<tr>
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<td>0-5</td>
<td>0.9 ± 1.1</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>D-lactate</td>
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<td>0.0 ± 0.1</td>
<td>0.2 ± 0.4</td>
</tr>
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<td>0.5-1</td>
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<td>0.7 ± 1.0</td>
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<td>0.7 ± 0.4</td>
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<td>0.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>0-5</td>
<td>0.9 ± 0.6</td>
<td>1.2 ± 1.3</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n=5 (intolerant) or 7 (tolerant). Different overall (taking all 5 time periods together) form tolerant, $P < 0.05$. 

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Colonic fermentation and lactose intolerance
Figure 2. *In vitro* production of acetate (A), propionate (B) and butyrate (C) by fecal bacteria from lactose tolerant (n = 7) and intolerant (n = 5) subjects. Values are means +/- SD. The intolerant group differed overall (taking all 6 time points together) from the tolerant group (*P* < 0.05).
Production of L- and D-lactate

As the concentrations of L- and D-lactate after 5 h of incubation were relatively low in the control culture (0.4 ± 0.3, 0.5 ± 0.5 mmol/L, respectively), the amounts of L- and D-lactate produced in the control cultures were not subtracted from those in the cultures with lactose. During the 5 h incubation, the production rates of L- ($P < 0.01$) and D-lactate ($P = 0.03$) were higher in the intolerant group than in the tolerant group (Table 2). The intolerant group produced more L-lactate than the tolerant group ($P < 0.01$; Fig. 3). At 5 h, the ratio between D- and L-lactate was 1:1.5 and 1:1.4 for the tolerant and intolerant group, respectively. The concentrations of D- and L-lactate at 5 h was not different between the two groups ($P > 0.1$).

**Figure 3.** *In vitro* production of D-lactate (A) and L-lactate (B) by fecal bacteria from lactose tolerant (n = 7) and intolerant (n = 5) subjects. Values are means +/- SD. (B) The intolerant group differed overall (taking all 6 time points together) from the tolerant group ($P < 0.05$).
Appendix 3

Composition of bacteria in feces
The tolerant and intolerant group did not differ in the numbers of total cells, total bacteria or the major bacterial groups (Table 1). The numbers of total bacteria or each bacterial group were not correlated with SSC (data not shown).

Normalization of the data to the number of total bacteria
After normalization, the results of the comparison between the two groups stayed the same except that the tolerant group produced more galactose ($P = 0.01$) more rapidly ($P = 0.05$) than the intolerant group (data not shown).

Table 1. Bacteria in feces from lactose tolerant and intolerant subjects quantified by FISH\(^1\).

<table>
<thead>
<tr>
<th>Stain or probes</th>
<th>Targeted groups</th>
<th>Intolerant</th>
<th>Tolerant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cells ($10^{10}$)$^2$</td>
<td>% total bacteria$^3$</td>
<td>Cells ($10^{10}$)$^2$</td>
</tr>
<tr>
<td>DAPI</td>
<td>Total cells</td>
<td>21.0±10</td>
<td>129.2</td>
</tr>
<tr>
<td>Eub338</td>
<td>Bacteria</td>
<td>16.0±6.4</td>
<td>100</td>
</tr>
<tr>
<td>Bac303</td>
<td>Bacteroides/Prevotella</td>
<td>4.8±2.2</td>
<td>34.6</td>
</tr>
<tr>
<td>Eroc482</td>
<td>Eubacterium rectale/Clostridium cocoides group</td>
<td>4.3±2.7</td>
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<td>Rmo720/Rfla730</td>
<td>Ruminococcus group</td>
<td>2.0±1.2</td>
<td>10.9</td>
</tr>
<tr>
<td>Fpra645</td>
<td>Fecalibacterium</td>
<td>1.5±1.0</td>
<td>9.2</td>
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<td>Bfl164y</td>
<td>Bifidobacterium</td>
<td>1.0±1.1</td>
<td>5.2</td>
</tr>
<tr>
<td>Ecy1387</td>
<td>Eubacterium cylindroides group</td>
<td>0.2±0.4</td>
<td>1.0</td>
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<tr>
<td>Ato291</td>
<td>Atopobium group</td>
<td>0.09±0.07</td>
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</tr>
<tr>
<td>Strc493</td>
<td>Streptococcus/Lactococcus</td>
<td>0.09±0.11</td>
<td>0.5</td>
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<td>Ec1531</td>
<td>Enterobacteriaceae</td>
<td>0.02±0.04</td>
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<tr>
<td>Lab158</td>
<td>Lactobacillus/Enterococcus</td>
<td>0.003±0.006</td>
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<td>Chis/Clit</td>
<td>Clostridium histolyticum / lituseburense group</td>
<td>0.002±0.002</td>
<td>0.01</td>
</tr>
<tr>
<td>Phasco741</td>
<td>Phascolarctobacterium group</td>
<td>nd$^4$</td>
<td>nd</td>
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<tr>
<td>Veil223</td>
<td>Veillonella</td>
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</table>

\(^1\) Values are means ± SD or %, n=5 (intolerant) or 7 (tolerant).
\(^2\) per g feces, dry weight
\(^3\) Percentage of Bacteria (Eub338).
\(^4\) nd, not detected (<0.001×10^{10} cells/g dry feces).
Colonic fermentation and lactose intolerance

Discussion

In the present study, in vitro incubation with lactose showed that the fecal microbiota from lactose intolerant subjects had faster production rates of D- and L-lactate, acetate, propionate and butyrate than that from the tolerant subjects. The amounts of L-lactate and SCFA (acetate, propionate and butyrate) produced were higher in the intolerant subjects than the tolerant subjects. The rate or degree of the hydrolysis of lactose was not different between the two groups.

Whether colonic fermentation of lactose would influence the occurrence of lactose intolerance, either aggravate or alleviate it, depends on the balance between the ability of the colonic microbiota to ferment lactose and the ability of the colon to remove the fermentation metabolites. A low lactose-fermenting capacity of the colonic microbiota, which leads to inefficient removal of the maldigested lactose (and/or its intermediate fermentation metabolites), or a low absorption capacity of the colon which leads to inefficient removal of the fermentation metabolites, may contribute to the occurrence of symptoms. When lactose is converted to SCFA by fermentation, the osmotic load is increased by about eight fold, which makes the efficiency of the colon to absorb these fermentation metabolites an important determinant for the outcome of the osmotic load caused by malabsorbed lactose (11). It is generally believed that the colon has a high capacity to absorb SCFA (12,13), the absorption rate is 6.1-12.6 µmmol/(cm².d) (14,15). However, there are differences among segments in colonic permeability for the three major SCFA. Acetate is absorbed at the highest rate in the cecum and proximal colon, and butyrate in the distal colon; propionate is absorbed at a similar rate in the proximal and distal colon (16). Lactate is an intermediary organic acid in the bacterial fermentation of carbohydrates and is further converted to SCFA and as a result, it is rarely present in large amounts in feces (15,17,18). If the colon can absorb SCFA at a sufficient rate, a higher lactose-fermenting capacity of the colonic microbiota may help to alleviate lactose intolerance.

However, our results do not support this assumption. A possible explanation for our observations could be that although the colon is thought to possess a high capacity to absorb SCFA, the absorption rate might not be sufficient to remove in a
short period in situ all the SCFA produced from rapid fermentation of lactose. Several studies reported increased cecal SCFA pools or lactate concentration in rats fed oligosaccharides or fructo-oligosaccharides, indicating colonic accumulation of organic acids produced from rapid fermentation of those carbohydrates (19-21). Accordingly, the rapid fermentation of undigested lactose may result in temporary accumulation of SCFA in the lumen, which causes symptoms. Lactate and other intermediate metabolites can also accumulate temporarily if their further conversion and absorption by the colon cannot counteract their production. Segmental differences existing in colonic absorption rates of SCFA might play a role in accumulation of SCFA in certain parts of the colon. For instance, the absorption rate of butyrate is lower in the cecum and proximal colon (16) which is a major site of carbohydrate fermentation. Therefore, fermentation of lactose might lead to accumulation of butyrate in this part of the colon. In the present study, we observed that the production rate of butyrate in vitro was faster in lactose intolerant subjects than tolerant subjects.

Several hypotheses may explain why the temporarily accumulated fermentation metabolites could cause symptoms. Firstly, the osmotic load posed by those temporarily accumulated metabolites will draw fluid to the colonic lumen. The 12 lactose tolerant and intolerant subjects digested 38±12% of the 25 g lactose in the small intestine (data not shown) as estimated by the lactose digestion index (22). In principle, colonic fermentation converts 1 mol of lactose to about 3.7 mol of organic acids (23), but in real situation the production will be less as a considerable part of hexose will be incorporated into bacterial mass (24). The malabsorbed lactose (25 g × 60% = 15 g), which we assume is completely converted to organic acids, represents an osmotic load of ~ 190 mOsm, which will result in 633 mL of water in the colon (11). But this amount of fluid is unlikely to cause symptoms considering the high capacity of the colon to absorb fluid (25). Secondly, symptoms could appear because of the altered intestinal motility. The temporarily accumulated fermentation metabolites could trigger motor events of the intestine. Colonic fermentation of undigestible carbohydrates or/and its products are regarded to affect the motility of the proximal part of the gastrointestinal tract (26-28). SCFA affect motility in rat colon in vitro (29,30) and in vivo (31,32). SCFA
Colonic fermentation and lactose intolerance

also affect motility of the upper gut. The motor effects of SCFA are dose-dependent, suggesting that excessive amounts of rapidly fermented sugars might induce undesirable motor and sensitive effects. However, those effects of SCFA are not observed yet in humans (33). Furthermore, chemosensitive intestinal receptors, e.g. glucoreceptors, acid and alkali receptors, are present in the stomach and the small intestine (34). It is not known whether chemosensitive receptors are also present in the colon and if so, whether they will respond by altering the motility of the intestine upon chemical stimulation of fermentation metabolites. Alterations in motility of gastrointestinal tract are believed to play an important role in the origin of symptoms in the functional gastrointestinal disorders (35,36), the symptoms of which resemble those of lactose intolerance. It is not clear whether changes in intestinal motility are correlated to symptoms of lactose intolerance. Jouet et al. found that only 37% of the symptoms after intake of 40 g lactulose coincided in time with colonic motor events (37). Moreover, the temporarily accumulated fermentation metabolites can cause colonic hypersensitivity. In two recent studies, butyrate enemas elicited colonic hypersensitivity in rats (38) and was used to develop a model of chronic colonic hypersensitivity as a tool for studying irritable bowel syndrome (39).

The hydrolysis of lactose to glucose and galactose is the first step of colonic fermentation of lactose, catalyzed by the enzyme β-galactosidase. β-galactosidase is often measured as an indicator of the colonic capacity to ferment lactose (40-42). However, we have recently observed that the majority (80.6%) of the fecal microbiota from lactase non-persistent subjects possesses β-galactosidase activity (6). It is unlikely that lactose itself will present a large osmotic threat in the colon as it should be quickly hydrolyzed by the colonic microbiota. Results from the present study are in agreement with this assumption. There were no differences in the hydrolysis of lactose between the lactose tolerant and intolerant subjects in the in vitro incubation. Based on the above observation, we conclude that the hydrolysis of lactose does not play a role in lactose intolerance. Instead, the fermentation steps following the hydrolysis of lactose are related to the development of symptoms.
Appendix 3

Similar to what we found in a previous study (3), the composition of fecal microbiota was not different between the lactose tolerant and intolerant subjects. The possible reasons why differences in the fecal microbiota between the two groups were found in metabolic activities but not in composition could be that the detection of bacteria with FISH is not based on a strain-specific but a genus- or group-specific level. Bacterial strains of the same genus or group may have different metabolic capacities. Furthermore, the detection limit of bacteria in feces with FISH is about $10^6$–$10^7$ cells/g feces (0.001-0.01% of the total fecal bacteria). Bacterial groups with amounts below this level cannot be detected with FISH. In addition, large variations in bacterial numbers among individuals are often reported (3,8), which makes it difficult to clarify the differences in bacterial composition.

Feces are often used in studies on fermentation properties of the colon, considering the difficulties to sample directly in the colon and the observations that the indices of *in vitro* incubation with feces can be used to predict or interpret *in vivo* conditions and are rather stable through time and individual-dependent (43-46). However, there might be differences between metabolic activities determined with *in vitro* fermentation of fecal bacteria and that present in the colon, especially the cecum and proximal colon which is the major site of carbohydrate fermentation. *In vivo* studies, e.g. those in which stable isotope techniques are applied (47), may help to shed more light on colonic fermentation of carbohydrates.

In summary, by comparing the *in vitro* lactose-fermenting indices of fecal bacteria from lactose tolerant and intolerant subjects, we suggest that the colonic fermentation of lactose by the microbiota plays a role in lactose intolerance. The fermentative processes after lactose is hydrolyzed are related to the development of symptoms, while the hydrolysis of lactose is not. Studies are needed to further investigate the mechanisms by which those fermentative processes following hydrolysis of lactose and the intermediate and end metabolites of those processes influence the development of symptoms. Furthermore, the reaction of the colon towards those metabolites, *i.e.* absorption rate and motility alterations, ought to be an interesting issue for studies on lactose intolerance.
Acknowledgments

We gratefully acknowledge Klaas Bijsterveld and Albert Gerding, Renze Boverhof, and Toos Dalenoort for their assistance in measurement of sugars, SCFA and lactate measurement, respectively. The help from Janneke Heimweg and Marianne Schepers in the glucose and lactose challenge tests and the help from Gerwin Raangs and Marga Wester with the *in vitro* fermentation experiment is greatly appreciated. We’d like to thank Koen Venema of the Wageningen Centre for Food Sciences, the Netherlands, for critically reading the manuscript and helpful discussions.
Appendix 3

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Abstract

**Background:** Colonic metabolism of lactose, in addition to the small-intestinal lactase activity and transit time, may play a role in lactose intolerance. We investigated whether supplementation of yogurt and bifidobacteria could modify the colonic microbiota and influence the symptom response in lactose intolerant subjects.

**Subjects and methods:** Eleven Chinese lactose intolerant subjects consumed a yogurt enriched with *Bifidobacterium animalis* and capsules with *Bifidobacterium longum* for two weeks. The total number of bacteria and major bacterial groups in faeces were quantified with fluorescent *in situ* hybridization. PCR-denaturing gradient gel electrophoresis was used to study dynamics of the faecal bifidobacterial population. The subjects received oral challenges of 25 g of lactose one day before and one day after the supplementation period, during which blood samples were collected for measurement of the degree of lactose digestion in the small intestine (indicated by lactose digestion index, LDI) and a 6-h symptom score (SSC) was recorded.

**Results:** The numbers of total cells, total bacteria and the *Eubacterium rectale/Clostridium coccoides* group, and faecal β-galactosidase activity increased significantly during the supplementation period. The number of *Bifdobacterium* showed a tendency to increase during and after supplementation. The percentages of the major bacterial groups remained similar throughout the study. LDI was not different before and after supplementation, whereas SSC decreased after supplementation. The bifidobacterial strain in the yogurt was present in faeces during supplementation, but disappeared after consumption had stopped.

**Conclusions:** The results suggest that supplementation of yogurt and bifidobacteria modifies the amount and metabolic activities of the colonic microbiota, but does not increase the endogenous lactase activity in the small intestine. The changes in the colonic microbiota might be among the factors modified by the supplementation which lead to alleviation of lactose intolerance.
**Introduction**

Besides the amount of lactose ingested, and the small-intestinal lactase activity and transit time, colonic processing of lactose (1,2), especially the fermentation of lactose by the colonic microbiota (3), may affect the occurrence of symptoms of lactose intolerance. Therefore, it can be hypothesized that modulating the composition and/or metabolism of the colonic microbiota may influence lactose intolerance. Modulation of the colonic microbiota may be achieved through the targeted use of dietary supplementation, *i.e.* probiotics, prebiotics and synbiotics (4).

In several reviews (5-8), some probiotics are regarded to be able to improve lactose digestion and eliminate symptoms of intolerance. The mechanisms by which these probiotics exert their effects are not fully understood yet, but may involve modifying gut pH, providing ß-galactosidase, exerting positive effects on intestinal functions and colonic microbiota. However, in a systematic review by Levri *et al* (9), it was concluded that probiotic supplementation in general did not alleviate the symptoms of lactose intolerance in adults. *Bifidobacterium* spp., together with *Lactobacillus* spp., are the bacteria most applied as probiotics because of their potential health benefits (10,11).

Yogurt is defined by the *Codex Alimentarius* of 1992 as a coagulated milk product that results from the fermentation of lactose in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (12). Some lactic acid bacteria can be combined with yogurt starters for their probiotic properties. In addition to the effects of yogurt-derived microbial ß-galactosidase, yogurts improve lactose digestion and tolerance by delaying gastric emptying, oro-cecal transit time, or both (6). The lactose contained in yogurts can be considered as a prebiotic for people with lactose maldigestion (13,14). Regular consumption of lactose influences their colonic microbiota (15,16) and reduces lactose intolerance (17).

Supplementation of yogurt and/or probiotics modified the composition and metabolism of the colonic microbiota in healthy adults (18,19), healthy infants (20) and patients with functional bowel disorders (21). In those studies, enumeration of faecal bacteria was often achieved with cultural methods which cannot provide a
very accurate picture of the composition of the colonic microbiota as not all bacteria can be cultured and media are not always specific (22).

The subjects for this study were selected from a group of Chinese lactose maldigesters based on their high symptom scores in lactose challenge tests. Those Chinese maldigestors harbored considerably less *Bifidobacterium* (23) in their colon than European subjects (24). The objective of this study was to investigate the effects of supplementation of a yogurt enriched with *Bifidobacterium animalis* and capsules with *Bifidobacterium longum* on the colonic microbiota and on symptom response in lactose intolerant subjects. Molecular techniques were applied to detect and quantify bacteria in faeces. We also investigated whether the supplementation would increase the endogenous lactase activity in the brush border of the small intestine. The design of the study does not include the instant effect of yogurt on hydrolysis of lactose in the small intestine.

**Subjects and Methods**

**Subjects**

Eleven healthy Chinese subjects (5 males and 6 females, age range 23-54 years) were recruited for this study. The subjects were selected from a group of lactose maldigesters based on their high 6-h symptom scores (>10) in oral lactose challenge (25 g lactose in water) tests in the year previous to the study. The subjects had not taken antibiotics or laxatives during the three months prior to the study. The subjects were asked to keep their habitual diet throughout the study period. All subjects gave a verbal informed consent. The study was approved by the Medical Ethical Committee of the Groningen University Hospital and Faculty of Medical Sciences, Groningen, The Netherlands, and of the West China University of Medical Sciences, Chengdu, China.
Yogurt and bifidobacteria supplementation

The yogurt used in this study was a fermented milk with the traditional yogurt strains (Lactobacillus bulgaricus and Streptococcus thermophilus) and a specific starter: Bifidobacterium animalis DN-173010 (approximately 10^8 colony forming units (CFU) per g of product) (Danone, Shanghai, China). The probiotic administered was encapsulated Bifidobacterium longum (Bifina®, Morishita Jintan Co. Ltd., Osaka, Japan), each capsule contained 2 × 10^8 CFU freeze-dried Bifidobacterium longum.

Study design

The study was divided into three periods (Figure 1): a 1-w baseline period, a 2-w supplementation period, and a 1-w follow-up period. During the supplementation period, each subject consumed per day three times three Bifina® capsules and three times 125g of yogurt.

Faecal samples were collected for analysis of composition of the faecal microbiota and β-galactosidase activity. In total five faecal samples were collected: two in the baseline period, two in the supplement period, and one in the follow-up period. Faeces was collected in a sterile bag, kept at 4 °C after arrival in the laboratory, and processed within 12 h after collection.

Two oral lactose challenge tests (25 g of lactose in water) were carried out: one day before and one day after the supplement period. A 6-h symptom score (SSC) was recorded as described earlier (2) and blood samples were collected for measurement of the lactose digestion index (LDI). LDI was determined with the ^13C/^2H-glucose test as described previously (25) with a slight modification: blood samples were collected before ingestion of lactose, 45 and 60 min after ingestion of lactose. LDI was calculated as the mean value of the two samples collected at 45 and 60 min.

SSC was also recorded in these 10 subjects after a lactose challenge in the year previous to the study.
Quantification of bacteria in faeces with fluorescent in situ hybridization (FISH)

16S rRNA oligonucleotide probes were used to detect the numbers of total bacteria and predominant bacterial groups in the faecal samples (Table 1). The hybridization and visualization of fluorescent cells were carried out according to the methods described previously (24,24,26,26). The detection limit of bacteria in faeces with our FISH technique is about $10^6$–$10^7$ cells/g faeces (0.001-0.01% of the total faecal bacteria). In addition to FISH, 4’, 6-diamidino-2-phenylindole (DAPI)-staining was used to enumerate the total amount of cells in faeces (26).

Analysis of the bifidobacterial population in faeces with PCR-denaturing gradient gel electrophoresis (PCR-DGGE)

DNA extraction and PCR.

0.5 g of each stool was suspended in 4.5 ml filtered PBS and homogenized on a vortex for 3 min together with a few glass beads (diameter, 4 mm). The suspension was centrifuged at 700 × g for 1 min, then 1 ml of the supernatant was centrifuged again at 14000 × g for 5 min. The pellet was stored at -20 °C until DNA extraction. Total DNA was extracted as described previously (27).

The DNA was diluted 100-fold for PCR amplification. The forward primer U515 (5'-GTGCCAGCAGCCGCGGT-3') and Bifidobacterium genus-specific reverse primer 1412 (lm3, 5'-CGGGTGCTICCCACTTTCATG-3') (28) were used for amplification of the 16S rRNA gene of Bifidobacterium. The reaction mixture (50μl) consisted of reaction buffer (final concentrations, 15 mmol/L Tris-HCl (pH 8.0), 50 mmol/L KCl, 0.01% (v/v) Tween 20), 2.5 mmol/L MgCl$_2$, 1.6 mmol/L of each deoxynucleoside triphosphate, 200 nmol/L of each primer, 2.5 U Hot Goldstar Taq polymerase (Eurogentec, Seraing, Belgium), and 1 μl 100-fold diluted template DNA. The PCR was performed in a T-gradient thermocycler (Biometra, Göttingen, Germany) using the following conditions: 95°C for 10 min; 34 cycles of 95°C for 45s, 50°C for 2 min and 72°C for 2min; finally 72°C for 5min. The
Figure 1. The study design. 1~5 in rectangles indicate the schedule of collection of the five faecal samples.
resulting amplicons were diluted 1000-fold and used as template for a second PCR. Forward U968-GC(5’-GCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGAACGCGAAGAACCTTAC-3’) and reverse U1406 (5’-ACGGGCGGTGTGTRC-3’) primers (29) were used to amplify the V6 – V8 regions of bacterial 16S rRNA gene. The PCR conditions were as follows: 95°C for 10 min; 25 cycles of 95°C for 30s, 56°C for 1 min and 72°C for 1 min; finally 72°C for 5 min. The PCR amplicons were checked for their size by electrophoresis on agarose (10 g/L) gel containing ethidium bromide.

**Bifidobacterium markers**

DNA fragments of **bifidobacterial strains**, in the yogurt and Bifina® capsules, and mixed DNA fragments of 6 bifidobacterial strains were prepared as markers for DGGE. The 6 bifidobacterial strains included: *B. adolescentis*, *B. bifidum*, *B. breve*, *B. dentum*, *B. longum* and *B. pseudo longum*. Each bacterial strain was cultured on Brucella blood agar (BBA) under anaerobic conditions at 37°C. The *B. animalis* in the yogurt and *B. longum* in Bifina® capsules were obtained by culturing the yogurt and capsules on BBA, followed by re-culturing a colony which hybridized with the 16S rRNA-based probe Bif164y (30). Sequence analysis confirmed that the strain obtained from the yogurt was a *B. animalis* subspecies *lactis*. DNA extraction of the bacterial colonies and PCR amplification were performed as mentioned above. PCR amplicons of the 6 strains were mixed together to make a combined marker.

**DGGE analysis**

Polyacrylamide gels (9% [w/v] acrylamide-bisacrylamide [37.5:1]) in 20 mmol/L Tris-acetate-EDTA buffer (pH7.4) with a denaturing gradient were prepared with a gradient mixer. The gel contained a 45 to 70% gradient of urea and formamide increasing in the direction of electrophoresis. A 100% denaturing solution contained 40% (v/v) formamide and 7.0 mol/L urea. The PCR amplicons were loaded on the gel and separated by electrophoresis in a PhorU system apparatus (Ingeny, The Netherlands) at a constant voltage of 140 V and temperature of 60°C.
Yogurt and bifidobacteria supplementation in lactose intolerant subjects

for 16h. After electrophoresis, the gel was silver-stained as described previously (31).

Quantification of ß-galactosidase activity in faeces

An aliquot of 0.5 g of each stool was kept at -20°C until the measurement of ß-galactosidase activity. The 0.5 g of stool was diluted with 4.5ml buffer (0.02 mol/L Na₂HPO₄, 0.01 mol/L MgSO₄, 0.001 mol/L dithiothreitol, pH 7.0) and 0.5 ml of this suspension was sonicated on ice (4 × 1 min with 15 s intervals) with a Soniprep 150 (Beun de Ronde BV, Abcoude, the Netherlands) followed by centrifugation (16100 × g, 10 min). The ß-galactosidase activity in the supernatant was measured by determining the hydrolysis of p-nitrophenyl-ß-D-galactopyranoside (PNPG) at 37 °C (32).

Data analysis

Data are expressed as mean ± SD. For statistical evaluation of FISH and ß-galactosidase results, the results for baseline and supplementation periods were the average of the two samples taken in each period. Logarithmic or square root transformation of the data was performed when necessary to obtain normally distributed data and when the data stayed skewed after transformation, nonparametric tests were applied. The Repeated measures followed by the Bonferroni method for pair-wise comparison was applied to assess differences among baseline, supplementation and follow-up periods in total number of cells and bacteria, numbers and percentages of Bacteroides/Prevotella, Eubacterium rectale/Clostridium coccoides group, Eubacterium low G+C2 and Ruminococcus group, and in ß-galactosidase activity and SSC. The Friedman test followed by a Wilcoxon test for pair-wise comparison was applied to assess differences among baseline, supplementation and follow-up periods in numbers and percentages of Bifidobacterium. The Student t-test (paired, two-tailed) was applied to assess differences in LDI before and after supplementation. Correlations were assessed by calculating the Pearson or Spearman correlation coefficients as appropriate. P <
0.05 was regarded as significant. All analyses were performed using SPSS 12.0 for Windows software (SPSS Inc., Chicago, IL, USA).

**Results**

**Effects of yogurt and bifidobacteria supplementation on the amount and composition of the colonic microbiota (Table 1)**

The numbers of total cells ($P = 0.05$), total bacteria ($P = 0.03$) and the *Eubacterium rectale/Clostridium coccoides* group ($P = 0.04$) increased significantly during supplementation, but returned to the level of that in the baseline period after supplementation had stopped ($P > 0.1$). The number of *Bifidobacterium* showed a tendency of increase in supplementation and follow-up periods, but this was not significant ($P = 0.07$). The percentages of *Bacteroides/Prevotella, Eubacterium rectale/ Clostridium coccoides* group, *Eubacterium* low G+C, *Ruminococcus* group or *Bifidobacterium* (with total bacteria as 100%) were not different among baseline, supplementation or follow-up periods ($P > 0.1$).

**Effects of yogurt and bifidobacteria supplementation on bifidobacterial population in faeces**

Dynamics in faecal bifidobacterial population was monitored with PCR-DGGE. As an example, Figure 2 shows the DGGE profile of four subjects. In the baseline period, no bands were present at the same level as the band from *B. animalis* in the yogurt in all faecal samples. In 10 of the 11 subjects, a band at the same level as the band from *B. animalis* appeared during supplementation, but disappeared again after supplementation had stopped. In six of the 11 subjects, a band at the same level as the band from *B. longum* in Bifina® capsules was already present in the baseline period and this did not change during or after supplementation. In others which did not have the *B. longum* band in the baseline period, this band was not
present after supplementation, although in three of these samples the band appeared during supplementation.

The DGGE profiles of bifidobacteria showed host-specific patterns. Differences were found among subjects in the positions of specific bands and the number of bands. The profile of each subject in the follow-up period was similar to that in the baseline period except for some slight changes, e.g., in three subjects, new bands appeared during and after supplementation.

In two of the 11 subjects, no band at all was present in the baseline period. In one of them, two bands at the same levels as bands from the consumed B. animalis and B. longum appeared during supplementation but disappeared after supplementation. In another subject, besides two bands as B. animalis and B. longum, another band appeared during supplementation and while these three bands disappeared after supplementation, a new band appeared.

Results of bifidobacteria measured with DGGE and FISH showed similar trends. Samples in which the numbers of bifidobacteria were below the detection limit of FISH had no band or just a few bands on DGGE profiles, while samples with bifidobacteria above the detection limit of FISH showed more bands on DGGE profiles. In some samples in which no bifidobacterium were detected with FISH, there were bands present on DGGE profiles (e.g. Figure 2, subject 1, sample 3).

**Effects of yogurt and bifidobacteria supplementation on faecal β-galactosidase activity**

β-galactosidase activity in faeces increased significantly during supplementation ($P = 0.01$; Table 2). In the follow-up period, β-galactosidase activity remained higher than that of baseline period, but the difference was not significant ($P = 0.37$). β-galactosidase activity was not correlated with the total number of cells or bacteria, numbers of *Bacteroides/Prevotella, Eubacterium rectale/ Clostridium coccoides* group, *Eubacterium* low G+C2, *Bifidobacterium* ($P > 0.1$) or *Ruminococcus* group ($P = 0.09$). β-galactosidase activity was not correlated with SSC ($P > 0.1$).
Table 1. Numbers of total cells, total bacteria, and predominant bacterial groups in faeces of lactose intolerant subjects detected with FISH and DAPI staining before, during and after supplementation of the yogurt and *Bifidobacterium longum*\(^1\).

<table>
<thead>
<tr>
<th>Stain or probes</th>
<th>Targeted groups</th>
<th>Baseline period</th>
<th>Supplementation period</th>
<th>Follow-up period</th>
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<tr>
<td></td>
<td></td>
<td>Cells (10(^10))(^2)</td>
<td>%total bacteria(^3)</td>
<td>Cells (10(^10))(^2)</td>
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<tr>
<td>DAPI</td>
<td>Total cells</td>
<td>14.5 ± 6.3</td>
<td>20.6 ± 4.9(^7)</td>
<td>17.4 ± 8.5</td>
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<tr>
<td>Eub338</td>
<td>Bacteria</td>
<td>12.9 ± 4.9</td>
<td>19.5 ± 5.5(^7)</td>
<td>17.6 ± 7.9</td>
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<td>Bac303</td>
<td><em>Bacteroides/Prevotella</em></td>
<td>3.4 ± 1.7</td>
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<td>Erec482</td>
<td><em>Eubacterium rectal Clostridium coccoides group</em></td>
<td>2.5 ± 1.4</td>
<td>19.6 ± 7.1</td>
<td>4.2 ± 1.3(^7)</td>
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<td>Elgc01</td>
<td><em>Eubacterium low G+C2</em></td>
<td>0.8 ± 0.5</td>
<td>6.4 ± 3.1</td>
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<tr>
<td>Rbro729/Rfla730</td>
<td><em>Ruminococcus group</em></td>
<td>1.1 ± 1.1</td>
<td>7.7 ± 6.5</td>
<td>0.6 ± 0.5</td>
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<tr>
<td>Bif164y</td>
<td><em>Bifidobacterium</em></td>
<td>0.1 ± 0.1</td>
<td>0.8 ± 1.0</td>
<td>0.2 ± 0.3</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SD or %, n=11 (baseline period and during) or 10 (after).
\(^2\) per g faeces, dry weight
\(^3\) Percentage of Bacteria (Eub338).
\(^7\) P=0.052 compared to baseline period
\(^7\) P=0.035 compared to baseline period
Figure 2. PCR-DGGE analysis of bifidobacterial population in faeces of four lactose intolerant subjects before, during and after supplementation of the yogurt and *Bifidobacterium longum*. (A) *Bifidobacterium animalis* obtained from the yogurt, (L) *Bifidobacterium longum* obtained from Bifina\textsuperscript{\textregistered} capsules, and (M) a mixture of 6 *Bifidobacterium* strains: (from top to bottom) *B. adolescentis, B. dentum, B. breve, B. longum & B. pseudolongum* and *B. bifidum*. For each subject, sample 1-2, 3-4 and 5 were from the baseline, supplementation and follow-up periods, respectively. Arrows indicate the presence of a band at the same level as the band from *B. longum* in the yogurt during the supplementation period. Numbers of *Bifidobacterium* \((10^9\) cells/g dry faeces) quantified with FISH are shown under the DGGE profile.

<table>
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</table>
Appendix 4

Effects of yogurt and bifidobacteria supplementation on digestion of lactose in the small intestine
LDI was measured one day before and one day after the supplementation as an indication of the degree of lactose digestion in the small intestine. Supplementation of yogurt and bifidobacteria did not change LDI ($P = 0.74$; Table 2), which indicates that the endogenous (brush-border) small-intestinal lactase activity was not stimulated by the supplementation.

Effects of yogurt and bifidobacteria supplementation on SSC
SSC recorded in the follow-up period was significantly lower than that in the baseline period ($P = 0.02$; Table 2). Compared to the SSC obtained in the year previous to the study (18.3 ± 10.3, means ± SD, n=10), SSC in the baseline period was not different ($P = 1.00$). However, SSC in the follow-up period was significantly lower than that of the previous year ($P = 0.01$; Figure 3).

![Figure 3](image_url)

**Figure 3.** 6-h symptom scores (SSC) of individual lactose intolerant subjects recorded in the year previous to the study (n=10), and in the baseline and follow-up periods of the study (n=11). SSC in the baseline period was not different from that of the previous year ($P = 1.00$). SSC in the follow-up period was significantly lower than those of the previous year ($P = 0.01$) and baseline period ($P = 0.02$).
Table 2. Faecal β-galactosidase activity, LDI and SSC of lactose intolerant subjects before, during and after supplementation of the yogurt and *Bifidobacterium longum*.

<table>
<thead>
<tr>
<th></th>
<th>Baseline period</th>
<th>Supplementation period</th>
<th>Follow-up period</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>β-galactosidase</strong></td>
<td>6.4 ± 6.3</td>
<td>10.6 ± 8.3*</td>
<td>8.9 ± 9.0</td>
</tr>
<tr>
<td>(U/mg feces)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LDI</strong></td>
<td>0.39 ± 0.14</td>
<td>nm²</td>
<td>0.38 ± 0.20</td>
</tr>
<tr>
<td><strong>6 h SSC</strong></td>
<td>16.1 ± 10.0</td>
<td>nm²</td>
<td>7.1 ± 5.5†</td>
</tr>
</tbody>
</table>

1 Values are means ± SD, n=11
2 Not measured
* P=0.01 compared to baseline period
† P=0.02 compared to baseline period

Discussion

This study shows that a 2-w supplementation of probiotic bacteria *Bifidobacterium longum* and a yogurt enriched with *Bifidobacterium animalis* modifies the amount of bacteria and increases β-galactosidase activity in faeces from lactose intolerant subjects. However, *Bifidobacterium animalis* does not colonize in the colon and composition of the faecal microbiota remains unchanged. The supplementation does not increase the brush-border lactase activity in the small intestine, however, SSC after the lactose challenge decreases after the supplementation.

Yogurt and probiotic supplementation increased numbers of total cells, total bacteria and *Eubacterium rectale/Clostridium coccoides* group in faeces in this study. The increase in bacterial numbers could be attributed to, at least partly, the lactose present in the yogurt. During the production of yogurts, only 20–30% of the lactose is hydrolyzed in the fermentation process (12). The supplemented yogurt per day contained about 11.5 g lactose. Only ~40% of the ingested lactose (in water) could be digested in the small intestine of the subjects (as indicated by LDI).
Lactose in yogurt is better ingested, but not all lactose in yogurt can be digested in lactase-deficient subjects (33,34). Thus, during the supplementation period, up to 7 g of lactose would enter the colon per day and serve as a substrate for fermentation by the colonic microbiota. Presence of lactose is expected to stimulate the bacterial β-galactosidase activity. The *Eubacterium rectale/Clostridium coccoides* group has been shown to possess β-galactosidase activity (35). In two other studies, yogurt supplementation did not result in an increase in the number of total anaerobes in healthy infants (20) or healthy German adults (18), which was probably caused by the fact that healthy infants and most German adults can digest lactose well. Supplementation of yogurt would thus not bring a considerable amount of lactose to their colon. In contrast to this, a study carried out in Chinese subjects in Taiwan (19) showed increased counts of anaerobes after yogurt ingestion. Most Chinese adults have genetically-determined low lactase activity (36). The above studies lend support to our assumption that maldigested lactose may serve as a substrate for the colonic microbiota and thus, will stimulate the growth of bacteria. Furthermore, the supplemented bifidobacteria, *esp. B. animalis* which was found to be present in the colon during the supplementation, may influence the colonic microbiota, considering their positive effects on intestinal ecology (18,37).

Despite the change in the numbers of bacteria, the composition of the faecal microbiota was not changed by the supplementation. The stability of the colonic microbiota in healthy subjects (38) and during similar dietary supplementation as the present study (27,39) were reported earlier.

*Bifidobacterium* in faeces from the 11 subjects was enumerated with FISH in the year previous to the study. The number (7.3 (8.7)×10^8 cells/ g dry faeces, mean (SD)) and the percentage of *Bifidobacterium* (0.7% of total bacteria) in these subjects were considerably lower than those in European subjects (6.0 (4.0)×10^9 cells/ g dry faeces, 4.8% of total bacteria) (24). *Bifidobacterium* is generally believed to possess health-beneficial properties (40). Unfermented milks containing *B. longum* might be effective in reducing breath hydrogen response and symptoms from lactose malabsorption (41). *Bifidobacterium animalis* DN-173 010 is shown to exhibit probiotic properties in the colon (37). Effects of supplementation of *B. longum* in capsules and a yogurt containing *B. animalis* on
Yogurt and bifidobacteria supplementation in lactose intolerant subjects

The faecal bifidobacteial population were investigated with FISH and PCR-DGGE in this study. FISH analysis shows a trend of increase in numbers of bifidobacteria. PCR-DGGE analysis shows host-specific patterns of *Bifidobacterium* that were relatively stable. A strain(s) similar to the *B. longum* in Bifina® capsules was already present in the colon of some of the subjects before the supplementation. In other subjects that did not have this *B. longum* strain before the supplementation, the strain did not colonize the colon after the supplementation had ceased. A strain(s) similar to the *B. animalis* strain in the supplemented yogurt was not present in any subject before the supplementation. It was transiently present in the colon during the supplementation, but disappeared after consumption had stopped. It has been reported that once the adult gut microbiota is established, the colonization with new strains is usually difficult and transient and sustained oral doses are required for their middle- and long-term maintenance (42). In most studies, supplemented probiotic strains did not colonize the intestine, as observed in the present study (43-45). Duez et al. detected *Bifidobacterium animalis* DN-173 010 one week after its ingestion had stopped with a colony immunoblotting method (46). It is good to bear in mind the possibility that the study of faecal samples alone might not be sufficient in evaluating colonization by a probiotic strain. In one study, the administrated probiotic strain was detected in colonic biopsies after its disappearance from faeces (47).

For detection of *Bifidobacterium* in faeces, PCR-DGGE is more sensitive than FISH in the present study. The detection limit of PCR-DGGE is lower than that of FISH.

Faecal β-galactosidase activity was increased during the supplementation period. One of our recent studies indicate that a major part of the colonic microbiota possess β-galactosidase activity (35) and the abundance of β-galactosidase is not involved in lactose intolerance (3). Therefore, the increase in faecal β-galactosidase activity may not necessarily be related to the reduced SSC, but rather, could be an indication of altered metabolic activities of the colonic microbiota or presence of the administered probiotic bacteria in the colon. Probiotics are found to be able to modify colonic fermentation (48,49). Modification of colonic fermentation of lactose might affect the occurrence of lactose intolerance. Increase in faecal β-
galactosidase activity could not be correlated with the changes in the amount of the colonic microbiota. The reasons for this can be that firstly, changes in metabolic activities occur without changes in composition of bacteria and secondly, our FISH method is not sensitive enough to detect the changes in bacterial composition responsible for the enzymatic changes. The large variation in bacterial numbers among individuals might make it difficult to clarify the relationship.

SSC was reduced after the supplementation. Hertzler et al. observed colonic adaptation to regular lactose ingestion and suggested that this adaptation reduces lactose intolerance symptoms (17). Changes in the amount and metabolic pattern of the colonic microbiota as observed in this study can be among those adaptive changes. Furthermore, yogurt and probiotics are generally regarded to be able to improve lactose digestion and alleviate symptoms of intolerance. In this study, SSC of the baseline period was not different from the SSC of the previous year, whereas SSC decreased significantly after the supplementation compared to the baseline values. This indicates that the supplementation of yogurt and bifidobacteria alleviates the symptoms of lactose intolerance.

Yogurt can efficiently improve lactose digestion through providing active microbial β-galactosidase and slowing gastrointestinal transit (6). However, consumption of yogurt or yogurt combined with probiotics cannot stimulate the endogenous (brush-border) lactase activity in the small intestine, as indicated by the study from Lerebours et al. (50) and the present study. The alleviation of intolerance symptoms observed in the follow-up period of this study is not caused by improvement in digestion of lactose in the small intestine.

Colonic metabolism of lactose has been suggested to play a role in lactose intolerance (1-3). In this study, alleviation of lactose intolerance is not caused by improved digestion of lactose in the small intestine. The changes in the colonic microbiota might be among the factors modified by the supplementation which lead to reduction of symptoms. Modulation of colonic metabolism through dietary supplementations could be a helpful approach for management of lactose intolerance.
Effects of yogurt and bifidobacteria supplementation on the colonic microbiota and lactose-induced symptoms in lactose intolerant subjects

Tao He, Marion G. Priebe, Yan Zhong, Chengyu Huang, Hermie J.M. Harmsen, Gerwin C. Raangs, Jean - Michel Antoine, Gjalt W. Welling and Roel J. Vonk

Submitted
Acknowledgments

We gratefully thank Danone, Shanghai, for kindly providing the yogurt. This work was also partly financially supported by the Commission of the European Communities, and specifically the RTD programme 'Quality of Life and Management of Living Resources', QLK 1-2001-00431 'Stable isotope applications to monitor starch digestion and fermentation for the development of functional foods' (EUROSTARCH).


Yogurt and bifidobacteria supplementation in lactose intolerant subjects


References

Appendix 5

Differential analysis of protein expression of Bifidobacterium grown on different carbohydrates

Tao He, Han Roelofsen, Gloria Alvarez-Llamas, Marcel de Vries, Koen Venema, Gjalt W. Welling and Roel. J. Vonk

Submitted
Abstract

Background: We observed recently that colonic fermentation of lactose might be a major factor in the pathophysiology of lactose intolerance. Proteomic techniques could be helpful in interpreting the metabolic pathways of lactose in the colon. The objective of this study was to explore proteomic methodologies for studying bacterial metabolism of lactose that can be used for identification of proteins involved in the colonic lactose metabolism which are associated with the onset of intolerance symptoms.

Materials and methods: Differential expression of cytoplasmic proteins of *Bifidobacterium animalis, breve and longum* grown on different carbohydrates (lactose, glucose, galactose) was analyzed with surface-enhanced laser desorption ionization - time of flight (SELDI-TOF) MS and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After fractionation by SDS-PAGE, differentially-expressed proteins among different carbohydrates were identified with LC-MS/MS.

Results: The three strains grown on the same carbohydrate or the same strain grown on glucose or lactose showed differences in SELDI-TOF MS protein profiles. Differences in protein expression were observed among *B. breve* grown on glucose, galactose or lactose as analyzed with SDS-PAGE. With LC-MS/MS, proteins related to *Bifidobacterium* were identified, which included enzymes for metabolism of lactose, glucose and galactose.

Conclusions: The applied techniques can discern differences in protein expression of bacteria metabolizing different carbohydrates. These under-developing techniques can be promising in studying metabolism of lactose and other substrates in a complex bacterial ecosystem such as the colonic microbiota.
**Introduction**

Lactose intolerance refers to the gastrointestinal symptoms related to incomplete digestion of lactose by the small intestinal enzyme lactase. The adult-type lactase non-persistence occurring in a large part of the world population leads to lactose maldigestion, which in turn can, though not in all cases, lead to lactose intolerance. The pathophysiology of lactose intolerance is not well understood. We recently described that besides the small intestinal lactase activity and transit, fermentation of lactose by the colonic microbiota may play a role in lactose intolerance (1). Dietary supplementations, esp. probiotics, have been used to alleviate symptoms of lactose intolerance (2-4). These supplementations are expected to modify metabolism of lactose in the small and large intestine in such a way that symptoms can be prevented or alleviated. We observed that supplementation of a yogurt and probiotic bifidobacteria could alter the amount and metabolic activity of the colonic microbiota and alleviate intolerant symptoms in lactose intolerant subjects (unpublished data).

The role of colonic microbiota in lactose intolerance can be studied on the levels of bacterial composition and metabolic activities. In our recent studies in which fecal bacteria were quantified with fluorescent *in situ* hybridization, we did not observe significant differences in the composition of the fecal microbiota between lactose tolerant and intolerant subjects (1), or between lactose intolerant subjects with mild symptoms or with diarrhea (5), possibly because of large inter-individual differences. Studies on colonic metabolism of lactose by the colonic microbiota may reveal useful information on the pathophysiology of lactose intolerance. Proteomics technology can be an appropriate approach to study bacterial metabolism of lactose. Study of differential protein expression of colonic bacteria grown on lactose and other carbohydrates as energy source will facilitate detection of proteins specifically involved in lactose degradation. Differential proteomic analysis of the colonic microbiota from lactose tolerant and intolerant subjects may help to unravel the complex network of the proteins involved in bacterial metabolism of lactose which are correlated to the onset of intolerant symptoms. Identification of those proteins will help to understand the pathophysiology of...
lactose intolerance and can be used to evaluate the effects of dietary intervention designed to alleviate lactose intolerance.

In this study, we aimed to explore proteomic methodologies for differential proteome analysis of the bacterial lactose metabolism. The profiles of cytoplasmic proteins of bifidobacteria grown on different carbohydrates were studied with the SELDI-TOF MS Proteinchip technology and SDS-PAGE. LC-MS/MS was applied to identify the differences in protein expression among bifidobacteria grown on different carbohydrates as analyzed with SDS-PAGE. With high throughput capacity and limited material requirements, SELDI-TOF MS has been shown to be useful for studying differential protein expression in bacteria (6-8). SDS-PAGE is used to separate proteins based on differences in mass. It is less powerful in resolution than two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), but is faster and easier to use. The colonic microbiota is very complex. Bifidobacteria are a predominant group of the colonic microbiota, accounting for ~5% of the total bacteria (9), and are generally regarded as health-promoting (10). As a first approach, Bifidobacterium was chosen as a model bacterium for studying fermentation of lactose by the colonic microbiota.

**Materials and Methods**

**Bacterial strains and growth condition**

Three *Bifidobacterium* strains were used in this study: *Bifidobacterium animalis* (DSM 20104) and *Bifidobacterium breve* (DSM 20213) were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *Bifidobacterium longum* (NCC 2705) was kindly provided by the Nestlé Research Center (Lausanne, Switzerland). The bacteria were grown at 37 °C under anaerobic conditions. For defining a method for bacteria culturing and protein extraction with SELDI-TOF Proteinchip arrays, *B. animalis*, *B. breve* and *B. longum* were grown in mLAPT medium (11) with lactose or glucose as energy source (20 g L\(^{-1}\)). Four cultures were prepared for each strain grown on each
Protein expression of Bifidobacterium grown on different carbohydrates

carbohydrate. For identification of proteins with LC-MS/MS, B. breve was cultured in mLAPT medium with lactose, or glucose or galactose as energy source (20 g L⁻¹). Growth of bacteria was monitored spectrophotometrically at 492 nm. When the optical density (492 nm) of the cultures reached ~0.7 (~1.5 × 10⁸ cells/ml) which is corresponding to the mid-exponential growth phase, cells were harvested by centrifugation at 16100 × g for 5 min. The pellet was suspended in 1 ml 100 mmol/L Tris-HCl (pH 7.4) and the suspension was centrifuged at 16100 × g for 5 min. This step was then repeated twice. The pellet of cells was stored at -20 °C until protein extraction.

Protein extraction

The cells were suspended in 0.5 ml 100 mmol/L Tris-HCl (pH 7.4). The suspension was sonicated on ice (4 × 1 min with 15 s interval, at an amplitude setting of 24) with a Soniprep 150 (Beun de Ronde BV, Abcoude, the Netherlands) followed by centrifugation (16100 × g, 10 min). The supernatant was stored at -80 °C until analysis. The protein concentration in the extracts was determined with the Bradford assay (Sigma, Saint Louis, USA).

SELDI-TOF MS analysis of cytoplasmic proteins

The protein extracts of bifidobacteria were analyzed using strong-anion-exchange (SAX2) ProteinChip® arrays which were imbedded in a 96-well bioprocessor (Ciphergen Biosystems, Inc., Fremont, USA). The SAX chip was chosen because it showed the best results in terms of peak number and reproducibility in pilot experiments. Each of the quadruple cultures for each bifidobacterial strain grown on each carbohydrate was analyzed on one spot on the arrays. The arrays were equilibrated in a binding buffer (100 mmol/L Tris HCl (pH 10)-0.05% (v/v) Triton X 100) on a shaker for 5 min. Subsequently, the protein extracts (~20 µg of protein) in the binding buffer (final volume: 200 µl) were applied on the arrays. The arrays were shaken for 45 min to allow binding. After the samples were removed, the arrays were washed three times in the binding buffer on a shaker. The arrays were
Appendix 5

air-dried and 0.5 µl of a saturated solution of sinapinic acid (Ciphergen Biosystems, Inc.) in 50% acetonitrile (v/v)-0.5% trifluoroacetic acid (v/v) was applied twice as matrix. The arrays were then analyzed with a SELDI-TOF mass spectrometer (Ciphergen Biosystems, Inc.). Before the analysis, the mass spectrometer was calibrated using the All-in-One protein mix (mass range: 12 kDa-147kDa) (Ciphergen Biosystems, Inc.).

Differences in protein spectra were analyzed using proteinchip software 3.1 with the integrated Biomarker Wizard™ cluster analysis software (Ciphergen Biosystems, Inc.) as described earlier (12).

**SDS-PAGE analysis of cytoplasmic proteins**

SELDI does not allow direct identification of the observed differences in protein expression. To obtain more information on the nature of the observed differences, we separated cytoplasmic samples of *B. breve* grown on glucose, galactose and lactose with SDS-PAGE and excised bands that showed clear differences in intensity for protein identification with LC-MS/MS. As *B. breve* showed the most differences in growth when grown on different carbohydrates, it was chosen for analysis with SDS-PAGE and LC-MS/MS. Proteins extracted as described above were precipitated in acetone before SDS-PAGE fractionation followed by in-gel digestion. The acetone precipitate was directly dissolved in sample buffer (NuPAGE®-Novex, Invitrogen, Carlsbad, CA, USA) according to the protocol of the manufacturer and applied to a 12% Bis-Tris gel with a MOPS buffer system. Protein separation occurred for 50 min at 200V and visualization of bands was performed overnight by Coomassie Brilliant Blue G-250 based staining (PageBlue Staining Solution, Fermentas). Bands were excised, cut into small pieces and stored at -20 ºC until further analysis.

**Identification of cytoplasmic proteins with LC-MS/MS**

Gel pieces were washed in ultra pure water and dehydrated in acetonitrile. In-gel reduction with 10 mM dithiothreitol (one h at 60 ºC) and carbamidomethylation
with 55 mM iodoacetamide (45 min at room temperature in the dark) were performed. Gel pieces were subsequently washed with ultra-pure water, 50% acetonitrile and 100% acetonitrile. 0.1 µg trypsin in 50 mM ammonium bicarbonate was added and gel pieces were allowed to rehydrate on ice for 20 minutes. Digestion was carried out overnight at 37 ºC and peptides were further extracted by shaking the gel pieces in 0.1% formic acid for 30 min.

Separation of the resulting tryptic peptide mixtures was performed by nanoscale reversed-phase liquid chromatography tandem mass spectrometry (LC-MS/MS). The Agilent 1100 nanoflow/capillary LC system (Agilent, Palo Alto, CA, USA) was equipped with a trapping column (5 x 0.3 mm C₁₈RP) (Dionex/LC Packings, Amsterdam, The Netherlands) and a nanocolumn (150 x 0.075 mm, C₁₈Pepmap) (Dionex/LC Packings). Peptides mixtures were injected into the trapping column at a flow rate of 10 µl/min (5% acetonitrile/0.1% formic acid). After 10 minutes the trapping column was switched into the nanoflow system and the trapped peptides were separated using the nanocolumn at a flow rate of 0.25 µl/min in a linear gradient elution from 95% A (5% acetonitrile/0.1% formic acid) to 50% B (95% acetonitrile/0.1% formic acid) in 60 min, followed by an increase up to 80% B in 5 min. The eluted peptides were on-line electrosprayed into the QStar XL Hybrid ESI Quadrupole time-of-flight tandem mass spectrometer, ESI-qQTOF-MS/MS (Applied Biosystems, Framingham, MA; MDSciex, Concord, Ontario, Canada) provided with a nanospray source equipped with a New Objective ESI needle (10 µm tip diameter). Typical values for needle voltage were 2 kV in positive ion mode. The mass spectrometer was set to perform data acquisition in the positive ion mode, typically with a selected mass range of 300-1400 m/z. Peptides with +2 to +4 charge states were selected for tandem mass spectrometry, and the time of summation of MS/MS events was set to be 2 seconds. The three most abundant charged peptides above a 30 count threshold were selected for MS/MS and dynamically excluded for 60 seconds with 100 ppm mass tolerance.

ProID software (Applied Biosystems) was used to identify proteins from the mass spectrometric datasets according to SwissProt database. Mass tolerance was set to 0.15 Da (MS) and 0.1 Da (MS/MS) and carboxamidomethylation and methionine oxidation were chosen as modifications for database search.
Appendix 5

Results

Influence of different carbohydrates on the growth of Bifidobacterium

Based on the growth curves of the different bifidobacterial strains on the three carbohydrates, it can be noticed that the growth of B. animalis (DSM 20104) on glucose and lactose was similar (Fig. 1 A), whereas B. longum (NCC 2705) grew relatively better on lactose than on glucose (Fig. 1 B). B. breve (DSM 20213) showed the best growth on lactose, whereas its growth on glucose and galactose was similar (Fig. 1 C).

Reproducibility of the protein profiles as analysed with SELDI-TOF MS

Four cultures were inoculated for each stain grown on each carbohydrate. The quadruple cultures were harvested independently, cytoplasmic proteins extracted and applied to the proteinchips independently. The quadruple cultures showed similar spectra in terms of the number of peaks detected and the intensity of the peaks, with a few proteins of which peak intensity varied among replicates. Figure 2 shows spectra of the quadruple cultures of B. breve grown on glucose as an example of this observation. Peaks with signal-to-noise ratio > 5 which were present in all these four spectra were identified with proteinchip software 3.1 with the integrated Biomarker Wizard™ cluster analysis software (Ciphergen Biosystems, Inc.). For the 13 peaks identified, the average CV of the peak intensity of the four quadruple cultures was 28.4%.
Figure 1. Growth of *Bifidobacterium animalis* DSM 20104 (A), *Bifidobacterium longum* (NCC 2705) (B) and *Bifidobacterium breve* DSM 20213 (C) on different carbohydrates in mLAPT medium. ○, glucose; □, galactose; Δ, lactose.
Appendix 5

Figure 2. SELDI-TOF MS (WAX2 chips) spectra of cytoplasmic proteins (molecular weight range: 3000-20000 kDa) of the quadruple cultures of *Bifidobacterium breve* DSM 20213 grown on glucose. The spectra peaks are transformed into a ‘gel view’ using the software from the manufacturer.

**SELDI-TOF MS spectra of cytoplasmic proteins of B. animalis, B. breve and B. longum grown on different carbohydrates**

For the three strains, most peaks detected were in the molecular weight range of 1-20 kDa. Only a few peaks were detected in the range of > 20 kDa (data not shown). Grown on the same carbohydrate, *B. animalis, B. breve* and *B. longum* showed considerable differences in protein expression (Fig. 3). Some peaks were detected in one strain but not in other two strains. Most of the peaks found in all three strains varied in intensity. One strain grown on different carbohydrates showed similar spectra. However, the intensity of certain peaks was significantly different among different carbohydrates (Table 1). All the differences showed in Table 1 were significant and with ≥ 2-fold changes. When *B. animalis* was grown on lactose, the intensity of seven peaks increased and one decreased compared to that when grown on glucose. The intensity of two peaks, ~4757 Da and ~9275 Da, decreased in both *B. breve* and *B. longum* grown on glucose compared to on lactose. In *B. breve*, the intensity of three peaks increased when grown on glucose compared to on lactose.
Protein expression of Bifidobacterium grown on different carbohydrates

**Figure 3.** SELDI-TOF MS (WAX2 chips) spectra of cytoplasmic proteins (molecular weight range: 3000-20000 kDa) of *Bifidobacterium breve* DSM 20213 grown on glucose (A) and lactose (B), *Bifidobacterium animalis* DSM 20104 grown on glucose (C) and lactose (D), *Bifidobacterium longum* (NCC 2705) grown on glucose (E) and lactose (F). Each spectra is a representative of the quadruple cultures.
Appendix 5

Table 1. Differences in SELDI-TOF protein profiles of *Bifidobacterium* grown on glucose and lactose. The intensity of the peaks were significantly different between glucose and lactose with at least 2-fold differences in their intensity.

<table>
<thead>
<tr>
<th>Molecular weight (Da)</th>
<th>Intensity ratio (glucose/lactose)</th>
<th>Molecular weight (Da)</th>
<th>Intensity ratio (glucose/lactose)</th>
<th>Molecular weight (Da)</th>
<th>Intensity ratio (glucose/lactose)</th>
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<tr>
<td>B. animalis (DSM 20104)</td>
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<td>B. breve (DSM 20213)</td>
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<tr>
<td>3442.8</td>
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Table 2. Proteins identified in the band of *B. breve* grown on glucose in the rectangle in Figure 5.

<table>
<thead>
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<td>1.7</td>
<td>AAN25299</td>
<td>AE014295 NID: - <em>Bifidobacterium longum</em> NCC2705</td>
</tr>
<tr>
<td>12</td>
<td>1.6</td>
<td>AAN24609</td>
<td>AE014295 NID: - <em>Bifidobacterium longum</em> NCC2705</td>
</tr>
</tbody>
</table>
Protein expression of Bifidobacterium grown on different carbohydrates

SDS-PAGE analysis of cytoplasmic proteins and identification of differentially expressed proteins with LC-MS/MS

Differences existed in cytoplasmic proteins of *B. breve* grown on glucose, galactose and lactose as analyzed with SDS-PAGE (Fig. 4). The molecular weight of most of the differentially expressed proteins that could be discerned on the SDS-PAGE gel were above 28 kDa. Bands showing different intensities among the three different carbohydrates were processed for protein identification by LC-MS/MS. In total, 8 bands (indicated with arrows in Fig. 4) from each carbohydrate were excised, respectively. In the 8 bands from glucose, galactose and lactose, 78, 77 and 59 proteins were identified, respectively (> 95% confidence). We present results of three bands from glucose, galactose and lactose which were excised from the 38 kDa area (Fig. 4, in the rectangular) as an example of protein identification. More than one protein was identified in each of the three bands. As shown in Table 2, 12 proteins were identified in the band from glucose (Fig. 4, lane B), nine of which were directly related to *Bifidobacterium* in the database search results. Three of these nine proteins are involved in carbohydrate metabolism. In Table 3, identified proteins in these three bands were grouped according to the carbohydrate lane where they have been detected. Of the proteins identified, some were present in all three bands, some in two and others only in one of the three bands.

Discussion

During bacterial fermentation of lactose, lactose is first hydrolyzed into glucose and galactose. β-galactosidase is the enzyme that catalyzes this hydrolytic step. In recent studies, we observed that the degree and rate of this hydrolysis was not different between lactose tolerant and intolerant subjects, whereas the intolerant subjects had a higher and faster production of short-chain fatty acids and lactate (1). Hydrolysis of lactose may not be a rate-limiting step in lactose fermentation as bacterial β-galactosidase activity is abundant in the colon (13). These observations suggest that the subsequent fermentation process, rather than hydrolysis of lactose
itself, may be the crucial steps in lactose intolerance. Proteomic techniques can be helpful in studying metabolism of substrates by the colonic microbiota. In this study, we aimed to explore proteomic methodologies for differential proteomic analysis of lactose metabolism by the colonic bacteria. Understanding bacterial metabolism of lactose would help to clarify the possible role of colonic metabolism in lactose intolerance.

The major metabolic pathways for glucose, galactose and lactose are similar as lactose is first hydrolyzed to glucose and galactose, galactose is subsequently transformed to glucose (14,15). Therefore, differential proteomic analysis of bacteria grown on these carbohydrates provides a good model system for identification of proteins specifically involved in lactose degradation and for developing proteomic techniques which are expected to discern subtle changes in protein expression profiles.

In a pilot study, a stool sample was cleaned with 24% Polyethylene Glycol and 33% Dextran. Cytoplasmic proteins obtained by sonication were applied on SELDI-TOF MS Proteinchip. However, no useful spectra could be obtained, suggesting that stool samples are too complex in their protein composition and need extensive pre-treatment before they can be used for proteomic analysis (data not shown). In this study, a model system of Bifidobacterium was chosen for studying fermentation of lactose by the colonic microbiota. We have chosen Bifidobacterium because of its claimed health-promoting effects and the knowledge of its genome. The genome of B. longum strain NCC2705 was sequenced in 2002 (16). Recently, a comprehensive proteomic study was carried out to identify and characterize proteins expressed by the same strain (17).

Currently, 2D-PAGE is the technique extensively used in proteomic studies. 2D-PAGE can be a choice as a methodology for identification of proteins involved in lactose metabolism. However, considering its time-consuming procedure and difficulties in resolving some proteins, such as proteins with extreme pI and Mr (18), other approaches were explored for our purposes. We chose the SELDI-TOF MS Proteinchip array and SDS-PAGE for differential analysis of Bifidobacterium grown on different carbohydrates. SELDI-TOF MS has certain advantages over other methods such as 2D-PAGE, though proteins cannot be directly identified.
Figure 4. SDS-SDS-PAGE analysis of cytoplasmic proteins of *Bifidobacterium breve* DSM 20213 grown on (A) glucose, (B) galactose and (C) lactose. (M), markers for molecular weight of proteins. The arrows indicate the positions of the 8 bands from each sugar which were excised. The three bands in the rectangle are examples of the bands of which the intensity was different among the three carbohydrates and which were excised for analysis with LC-MS/MS.
Table 3. Proteins identified in the three bands of *B. breve* grown on glucose, galactose and lactose in the rectangle in Figure 5.

<table>
<thead>
<tr>
<th>Carbohydrates</th>
<th>Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose &amp; Glucose &amp; Lactose</td>
<td>UDP-glucose 4-epimerase. - <em>Bifidobacterium longum</em>&lt;br&gt;AE014295 NID: - *Bifidobacterium longum NCC2705</td>
</tr>
<tr>
<td>Galactose &amp; Lactose</td>
<td>Transaldolase. - <em>Bifidobacterium longum</em>&lt;br&gt;DTDP-glucose 4,6-dehydratase enzyme involved in rhamnose biosynthesis. - <em>Bifidobacterium longum</em></td>
</tr>
<tr>
<td>Galactose</td>
<td>(12 proteins), e.g.: Cystathionine beta-synthase. - <em>Bifidobacterium longum</em>&lt;br&gt;Aspartate-semialdehyde dehydrogenase. - <em>Bifidobacterium longum</em>&lt;br&gt;Glyceraldehyde 3-phosphate dehydrogenase C. - <em>Bifidobacterium longum</em></td>
</tr>
<tr>
<td>Glucose</td>
<td>L-lactate dehydrogenase (EC 1.1.1.27) mutant (C199S) (mixed t- and r- state tetramers), chain T – Bi&lt;br&gt;Probable 50S ribosomal protein L25. - <em>Bifidobacterium longum</em></td>
</tr>
<tr>
<td>Lactose</td>
<td>L-lactate dehydrogenase (EC 1.1.1.27) - <em>Bifidobacterium longum</em>&lt;br&gt;L-lactate dehydrogenase (Fragment). - <em>Bifidobacterium longum</em> bv. <em>Infantis</em>&lt;br&gt;Hypothetical protein. - <em>Bifidobacterium longum</em></td>
</tr>
</tbody>
</table>

This technique has higher throughput capability, has subfemtomole range sensitivity, offers higher resolution at the low mass range (*i.e.* <20kDa), and is relatively easy to use (19,20). In our study, with SELDI-TOF MS, differences in protein expression of different bifidobacterial strains and one bifidobacterial strain on different sugars could be detected in the low molecular weight range (<20kDa). The measurement with SELDI-TOF-MS in our experimental setting was reproducible. Therefore, SELDI-TOF MS can be used as a complementary approach to 2D-PAGE or SDS-PAGE in detecting peptides and proteins of low molecular weight (<20kDa) involved in lactose metabolism from defined bacterial or human populations. Furthermore, with its properties of good reproducibility and

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high throughput, it can be used for screening of sample quality and for optimization of techniques, for instance, culturing of bacteria and sample preparation.

In addition to SELDI-TOF MS, we also used SDS-PAGE for differential analysis of bifidobacterial proteins in a wider mass range, esp. the higher mass range (>20kDa). Differences were detected in protein expression of B. breve grown on glucose, galactose or lactose. With LC-MS/MS, proteins that were possibly responsible for these differences were identified. Most of the proteins were related to Bifidobacterium, including enzymes for metabolism of certain sugars. However, as one band on the SDS-PAGE gel contained more than one protein and analysis with LC-MS/MS was not quantitative, differences in protein expression could not be directly related to certain proteins. 2D-PAGE would probably be helpful in discriminating which of these proteins are differentially expressed.

For future studies on lactose metabolism by the colonic microbiota with proteomic techniques, we propose the following strategy: labeling of bifidobacteria grown on different carbohydrates with stable isotopes, followed by fractionation by SDS-PAGE and finally identification by LC-MS/MS. With the stable isotope labeling technique (21,22), proteins can be identified in a quantitative manner, which will facilitate identification of proteins of which expression is induced by specific substrates.

Acknowledgments

This study was partly financed by the Wageningen Centre for Food Sciences (WCFS), Wageningen, The Netherlands and also partly financed by the Commission of the European Communities, and specifically the RTD programme 'Quality of Life and Management of Living Resources', QLK 1-2001-00431 'Stable isotope applications to monitor starch digestion and fermentation for the development of functional foods' (EUROSTARCH). We gratefully acknowledge the Nestlé Research Center (Lausanne, Switzerland) for kindly providing Bifidobacterium longum (NCC 2705).
Appendix 5

References


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Appendix 6

The research protocol for an *in vivo* study on colonic fermentation of lactose using stable isotopes
Appendix 6

Background:

We recently observed *in vitro* that a faster and higher production of microbial intermediate and end metabolites during colonic fermentation of lactose may be related to the onset of lactose-induced symptoms. *In vitro* systems may not be a perfect reflection of the *in vivo* situation. *In vivo* studies are needed to verify the *in vitro* observations.

Objective:

To investigate *in vivo* whether fermentation of lactose by the colonic microbiota plays a role in the onset of lactose intolerance.

General design:

\(^{13}\)C-lactose and \(^2\)H-acetate will be delivered quantitatively via a catheter to the colon. \(^2\)H-acetate serves as an internal reference for the absorption rate of the colon and the Splanchnic first-pass retention of acetate. The kinetics of \(^{13}\)C-acetate in peripheral blood will be compared between lactose tolerant and intolerant subjects.

Protocol:

1. Classification of lactose tolerant (n=6) and intolerant subjects (n=6)
   1.1 ~30 Chinese subjects will undergo one glucose (placebo control) and one lactose challenge in two single-blinded tests. A 6-h symptom score will be recorded and breath samples will be collected for measurement of hydrogen.
   1.2 Criteria for classification: (i) breath hydrogen test positive; (ii) no symptoms after glucose, (iii) tolerant: no symptoms after lactose, intolerant: symptoms after lactose.

2. Introduction of the catheter
A protocol for studying colonic fermentation of lactose in vivo

3. Introduction of substrates via the catheter
   3.1 Substrates: 500 mg $^{13}$C-lactose + 19.5 g lactose + 200 mg $^2$H-acetate,
      dissolved in 204 ml of sterile water (37°C)
   3.2 Infusion rate: 4.5 ml/min

4. Collection of samples
   4.1 Blood: from an antecubital vein, 6 ml every 15 min from -45 min till 240 min
   4.2 Breath: every 15 min from -45 min till 240 min
   4.3 Stool: 24 h stool after infusion starts

5. Measurement
   5.1 Plasma: $^{13}$C-acetate (Gas chromatography/combustion/ isotope ratio mass spectrometry, GC-/C/IRMS) and $^2$H-acetate (GC-MS)
   5.2 Breath: $^{13}$C-CO$_2$ (GC/C/IRMS)
   5.3 Stool: $^{13}$C-enrichment

6. Data analysis
   6.1 Calculate ratio $^{13}$C / $^2$H-acetate
   6.2 Compare production rate of $^{13}$C-acetate (with ratio $^{13}$C / $^2$H-acetate) between lactose tolerant and intolerant subjects
   6.3 Calculate cumulative $^{13}$C-enrichment in total (blood, breath, stool)