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RESEARCH ARTICLE

# Impact of the synbiotic combination of *Lactobacillus casei* shirota and oligofructose-enriched inulin on the fecal volatile metabolite profile in healthy subjects

*Vicky De Preter[1], Aklilu Habteab Ghebretinsae[2], José Cortinas Abrahantes[2,3], Karen Windey[1], Paul Rutgeerts[1] and Kristin Verbeke[1]*

[1] Translational Research Center for Gastrointestinal Disorders (TARGID) and Leuven Food Science and Nutrition Research Centre (LFoRCe), University Hospital Gasthuisberg, KULeuven, Herestraat, Leuven, Belgium
[2] Center for Statistics, Hasselt University, Diepenbeek, Belgium and Leuven Food Science and Nutrition Research Centre (LFoRCe), University Hospital Gasthuisberg, KULeuven, Herestraat, Leuven, Belgium
[3] EFSA, European Food Safety Authority, Parma, Italy

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**Scope:** Hypothesis-driven approaches have mainly focused on the quantification of SCFAs as mediators of beneficial effects of synbiotics. However, the emergence of metabolite profiling strategies allows to evaluate the colonic metabolism from a top-down approach. In the present study, we evaluated the impact of a synbiotic combination on fecal metabolite profiles.
**Methods and results:** A synbiotic combination (*Lactobacillus casei* Shirota cells+oligofructose-enriched inulin) was evaluated in nine healthy volunteers. Before the start, during and after 4-wk treatment, fecal samples were obtained. GC-MS technology was applied to analyze the volatile metabolites. Application of a Type III test revealed that the metabolite profiles from the three conditions were significantly different. We identified three volatile organic compounds, acetate, dimethyl trisulfide and ethyl benzene, which were significantly affected. The acetate levels increased, whereas the dimethyl trisulfide levels decreased during and after the intervention. For ethyl benzene only an effect during the synbiotic intervention period was observed.
**Conclusion:** We report a detailed analysis of the influence of *L. casei* Shirota combined with oligofructose-enriched inulin on fermentation metabolites. Our results indicated a stimulation of saccharolytic fermentation and, importantly, a reduction of potentially toxic protein fermentation metabolites dimethyl trisulfide and ethyl benzene attended these effects.

**Keywords:**
GC-TOF-MS / Metabolomics / Purge-and-trap / Synbiotic / Volatile organic compounds

## 1 Introduction

Administration of pre- and/or probiotics has been associated with various health benefits such as inhibition of pathogenic microorganisms, improvement of gastrointestinal transit, enhanced mineral absorption, improvement of lactose digestion, reduction of serum triglyceride and cholesterol levels and reduction of colon cancer risk [1–5]. Most often, these health advantages have been attributed to a beneficial modulation of the colonic microbiota composition, in particular, an increase in bifidobacteria and/or lactobacilli numbers. For many years, the efficacy of pre- and probiotic substrates has been judged largely on its ability to increase the numbers of bifidobacteria and/or lactobacilli [6, 7].

More recently, analysis of the microbial diversity of the intestinal ecosystem has been complemented with the functional analysis of the colonic microbiota. Functional

**Correspondence:** Dr. Kristin Verbeke, Department of Gastrointestinal Research, University Hospital Leuven Herestraat 49, 3000 Leuven, Belgium
**E-mail:** Kristin.Verbeke@uz.kuleuven.ac.be
**Fax:** +32-16-34-43-99

**Abbreviations: DGGE,** denaturing gradient gel electrophoresis; **DP,** degree of polymerization; **OF-IN,** oligofructose-enriched inulin; **PLS,** partial least square; **VOCs,** volatile organic compounds

 

analysis can be performed on DNA level (metagenomics), RNA level (metatranscriptomics), protein level (metaproteomics) or on the metabolite level (metabolomics) [8]. Gene expression and protein data mainly indicate the potential for specific metabolic functions but do not always reflect the effective physiological processes. In contrast, metabolites represent the end products of metabolic activity and changes in metabolite concentrations might reflect changes in metabolic activity.

Targeted, hypothesis-driven approaches have mainly focussed on the quantification of SCFAs in fecal samples as mediators of beneficial effects of pre- and probiotic administration [9]. However, the emergence of metabolomics defined as ''the quantitative measurement of the multiparametric metabolic responses of a living system to pathophysiological stimuli or genetic modification'' allows to evaluate the colonic metabolism from a top-down approach bypassing the need for an *a priori* hypothesis [10, 11]. Recently, we have developed and validated a simple and fast GC-MS-based screening method for metabolite profiling in fecal samples [12].

In the present study, we applied this analysis technique to evaluate the impact of a synbiotic combination on fecal metabolite profiles in healthy subjects. We hypothesized that changes in the metabolic activity could be due to either direct fermentation of the prebiotic component of the synbiotic or indirectly, to changes in the composition of the colonic microbiota. To differentiate these direct and indirect effects, fecal samples were collected at different time points during the study. On the first day of the intervention period, a fecal sample was collected to investigate the direct effect of the synbiotic combination on the colonic metabolism. Then, no changes in the microbial composition could have occurred. After the intervention, another fecal sample was collected to investigate the indirect effect. In this test, we evaluated whether synbiotic intervention had changed the composition of the fecal microbiota and whether possible changes in composition were related to changes in colonic metabolic activity.

## 2 Materials and methods

### 2.1 Subjects

Nine healthy subjects (5 female/4 male, age range 19–23 years, BMI range from 18.5 to 26.5 $kg/m^2$) participated in the study. None of the subjects had a history of gastrointestinal or metabolic disease or previous surgery (apart from appendectomy) nor had they used antibiotics or any other medical treatment influencing gut transit or intestinal microbiota during the preceding three months. The Ethics Committee of the University of Leuven approved the study protocol (ML2253) and written informed consent was obtained from all participants.

### 2.2 Pre- and probiotic substrate

Oligofructose-enriched inulin (OF-IN) (ORAFTI® Synergy-1, Orafti, Tienen, Belgium) was chosen as prebiotic substrate [13, 14]. Inulin is a mixture of β-(2-1)-linear fructans obtained from chicory root with a degree of polymerization (DP) ranging between 2 and 60 (average DP of 12). Oligofructose (DP 2–8; $DP_{av} = 4$) is obtained by partial enzymatic hydrolysis of inulin, whereas a long-chain inulin known as inulin HP (DP 10–60; $DP_{av} = 25$) is produced by applying specific physical separation techniques. OF-IN is obtained by combining inulin HP and oligofructose. In the present study, a 1:1 w/w mixture has been used. As probiotic substrate, *Lactobacillus casei* Shirota, incorporated in a single strain fermented milk product (provided by Yakult®, Yakult Honsha, Tokyo, Japan) was chosen [15].

### 2.3 Experimental design

During 4 wk, each subject consumed a synbiotic consisting of $6.5 \times 10^9$ *L. casei* Shirota and 10 g OF-IN twice a day. Throughout the study, the volunteers maintained their usual diet, taking care that the diet remained as stable as possible over the treatment period. The intake of food substances containing pre- and/or probiotics was forbidden. At the time of inclusion, information about pre- and probiotics and food products containing pre- and/or probiotics was provided.

Before the start of the study, a fecal sample was obtained from all volunteers (T1). A second fecal sample was collected on the first day of the dietary intervention (T2). This test was aimed at investigating the direct effect of the synbiotic on the colonic metabolism. A third fecal sample (T3) was collected on the first day after the intervention period. In this test, we evaluated whether synbiotic intervention had changed the composition of the fecal microbiota and whether possible changes in composition were related to changes in colonic metabolic activity. An aliquot of the fecal samples was immediately frozen at −20°C until for volatile organic compounds (VOCs) analysis and DNA extraction.

### 2.4 VOC analysis

#### 2.4.1 Chemicals

2-Ethylbutyrate, supplied by Merck (München, Germany), was of analytical quality (at least 99% purity) and was used as an internal standard. Sodium sulfate (99%) was purchased from Acros organics (Geel, Belgium) and sulfuric acid (99%) from Sigma-Aldrich (Steinheim, Germany).

#### 2.4.2 Experimental procedure

Immediately before analysis, fecal aliquots were thawed and 0.25 g fecal sample was suspended in 4870 μL water. 2-

 

Ethylbutyrate (40 μL; 250 mg/100 mL) was added as internal standard. A magnetic stirrer, a pinch of sodium sulfate and 130 μL sulfuric acid were added to the sample to salt out and acidify the solution, respectively. To prevent cross-over from one sample to another, water samples were extracted after each triplicate analysis of the same sample.

The VOCs were analyzed on a GC-MS type TOF (Trace GC, Thermoquest, Rodano, Italy and Tempus II, Thermo Electron, San Jose, CA, USA), which was coupled on-line to a purge-and-trap system (Velocity, Teledyne Tekmar, Mason, OH, USA). The optimal procedure for analysis of fecal VOC has been described previously [12].

Briefly, the VOCs were purged out the sample for 20 min with a helium flow-rate of 40 mL/min, carried over a dry flow column (Trap Tenax tbv Velocity, Interscience, Louvain-la-Neuve, Belgium) for 3 min to control moisture transfer, and concentrated on a second polar trap column (Trap Vocarb tbv Velocity Interscience, Louvain-la-Neuve, Belgium). Consequently, the VOCs were desorbed from the trap by raising the trap temperature to 250°C for 5 min. After desorption, the trap temperature was further raised to 270°C for 10 min to remove any contamination of tailing compounds. The desorbed compounds were conducted *via* the transfer line (175°C) to the injector of the gas chromatograph to the GC column. The analytical column was a 30 m × 0.25 mm internal diameter, 0.25 μm film thickness, AT$^{TM}$ Aquawax DA (Grace, Deerfield, IL, USA). Helium GC grade was used as the carrier gas with a constant flow of 10 mL/min. The oven temperature was kept at 35°C (isothermal for 2 min) and increased with 5°C/min to 100°C and to 240°C with 10°C/min. This final temperature was held for 5 min. TOF-MS was performed in full scan mode from $m/z$ 30 to $m/z$ 500 at 2 scans/s. Xcalibur$^{TM}$ software was used for the automatization of the GC-MS and for data acquisition.

The resulting chromatograms were processed using AMDIS (Automatic Mass Spectral Deconvolution and Identification Software version 2.1) provided by the National Institute of Standards and Technology (NIST, CA, USA). This software provides quality matching using advanced spectral algorithms, adjacent peak deconvolution and background subtraction, which enables an unambiguous identification and quantification of the metabolite levels. Identification of the analytes in the samples was achieved by comparing the mass spectra of unknown peaks with the NIST library. Compounds showing mass spectra with match factors ≥90% in the NIST library were positively identified.

All samples were analyzed in triplicate and only those components identified in at least two of the three analyses were used for further evaluation. Relative indices of all VOCs *versus* 2-ethylbutyrate acid as internal standard were calculated. A data matrix containing the relative indices of all VOCs *per* sample was generated and used for statistical analysis. To classify the VOCs according to chemical nature, the number of identified compounds in all samples was summed.

### 2.5 Analysis of predominant microbiota

#### 2.5.1 DNA extraction

Total bacterial DNA was extracted from the fecal samples using the method of Pitcher and co-workers with slight modifications [16].

#### 2.5.2 Denaturing gradient gel electrophoresis (DGGE)

Community PCR was conducted using universal primers F357+GC clamp and R518 targeting the hypervariable V3 region of the 16S rRNA gene. The resulting 16S rRNA gene amplicons were analyzed with DGGE using a 35–70% gradient as previously described [16]. On each DGGE gel, a standard home-made reference consisting of an amplicon mix of 12 different bacterial species was included to allow digital gel normalization. DGGE profiles were digitally processed with BioNumerics software version 4.6 (Applied Maths, St-Martens-Latem, Belgium) in a multistep procedure following the manufacturer's instructions. Next, the quantitative information derived from relative band intensities *per* subject and *per* band-class was exported as a data matrix [17].

### 2.6 Statistics

#### 2.6.1 Statistical processing of the VOCs profiles

The data matrix displayed a paired data structure as the measurements were repeated on the same individuals before, during and after the treatment period. First, data were analyzed dichotomously whereby each VOC in the fecal samples was coded as ''1'' when it was present or ''0'' when it was absent. The McNemar test for correlated proportions was used to evaluate differences in the presence/absence of VOCs between baseline and respective treatments. This statistical analysis was performed with the SPSS software (SPSS 16.0 for Windows; SPSS, Chicago, IL, USA).

#### 2.6.2 Multivariate statistical analysis

##### 2.6.2.1 Exploratory data analysis

Partial least square analysis (PLS) was performed to evaluate clustering of similar metabolite profiles. PLS is a supervised learning technique in which the correlation between variables is maximized. This statistical technique allows clustering and grouping of observations with similar metabolite profiles and identification of the VOCs responsible for this discrimination. The relative indices of the different VOCs

 

were considered as *X*-variables and the categorical variable intervention was considered as *Y*-variable. The correlation loading plots were used to identify the metabolites that contributed most to pattern distinction. The variable importance plot was used as a format tool to rank the different VOCs in order of importance based on the correlation loadings [18]. Calculations were performed using Unscrambler Version 9.7 (CAMO A/S, Trondheim, Norway).

#### 2.6.2.2 Statistical analysis

To investigate whether the clustering patterns observed in the PLS analysis were due to statistically significant differences in overall metabolite profiles, a multivariate mixed effect model was applied.

Linear mixed models are commonly used to model longitudinal/repeated measures as they can appropriately account for within and between subject sources of variability [19]. Multivariate mixed effect model is an extension of the linear mixed model to jointly model multiple longitudinal variables. It allows the consideration of all the VOCs simultaneously and helps to see if there is overall dietary effect, *i.e.* if the intervention affects at least some of the VOCs. The model takes into account that the dietary intervention can have different effect on the evolution of the different metabolites of the same individual. A Type III test was performed to test the overall effect of the intervention. Briefly, a Type III estimable function for an effect is a linear function of the model parameters that involves the parameters of the effect and any interactions with that effect. This was implemented in SAS 9.2 using MIXED procedure [20].

To further see which of the compounds were affected by the dietary intervention, the evolution of each compound was analyzed using a linear mixed model separately. The model used is a subject-specific model that considers the natural variation among the individuals in baseline measurement as well as the evolution over time. It takes into account that individuals could behave differently to the treatment though they are exposed to the same dietary intervention. In modelling, unstructured covariance matrix was assumed for the random effect.

When a large number of tests are done simultaneously, incorrectly rejecting the null hypothesis is likely to occur. Thus, stronger evidence is required in order for an individual comparison to be deemed significant. The false discovery rate using Benjamini and Hochberg method for independent test was used to correct for such multiple testing issues [21].

Finally, subject-specific VOCs, *i.e.* compounds that were detected in only one person, were discarded to evaluate whether these compounds exert any discriminatory power due to their low occurrence rate and might introduce noise if implemented into the classification model. In addition, the effect of age, BMI and gender on the metabolite profile was investigated.

### 2.6.3 Statistical processing of the DGGE profiles

Changes in the profiles before and after synbiotic intake *per* band-class were assessed with SPSS Statistics 17.0. using Wilcoxon signed-rank test *per* band-class. *p*-Values below 0.05 were considered significant.

## 3 Results

### 3.1 Assignment of VOCs

A total of 139 different VOCs was identified in the present study, with an average of $58 \pm 4$ VOCs *per* sample (Table 1). The number of VOCs identified was relatively constant within an individual at each stage during the intervention study. There was no difference in the presence/absence of VOCs between baseline and synbiotic intervention (McNemar, $p = \text{NS}$). For further evaluation, relative indices of VOCs were used. All VOCs identified are presented in descending order of occurrence in Supporting Information Table 1. Seventeen compounds were found in all subjects at each test occasion.

Benzenoid and heterocyclic compounds were the most abundant chemical classes (Fig. 1). The class of benzenoids consisted of a diverse range of mono-, di- and tri-substituted benzenoids, including phenolic and indolic compounds. Furan derivatives are typical heterocyclic compounds. Furthermore, acids, alcohols, aldehydes, ketones, cycloalka/enes and S-containing compounds were commonly found. We identified 11 different acids (SCFAs and branched-chain fatty acids). Alcohols were mainly phenols and, primary and

**Table 1.** Number of VOCs identified at baseline and after either synbiotic (SYN) intervention

| | Baseline | Direct effect SYN | Indirect effect SYN |
|---|---|---|---|
| Total VOCs | 115 | 115 | 123 |
| Average VOCs/sample | $59 \pm 5$ | $58 \pm 3$ | $58 \pm 5$ |
| VOCs present in all samples | 21 | 22 | 23 |
| VOCs present in at least 50% of samples | 52 | 53 | 49 |
| Person-specific VOCs | 29 | 27 | 33 |

 

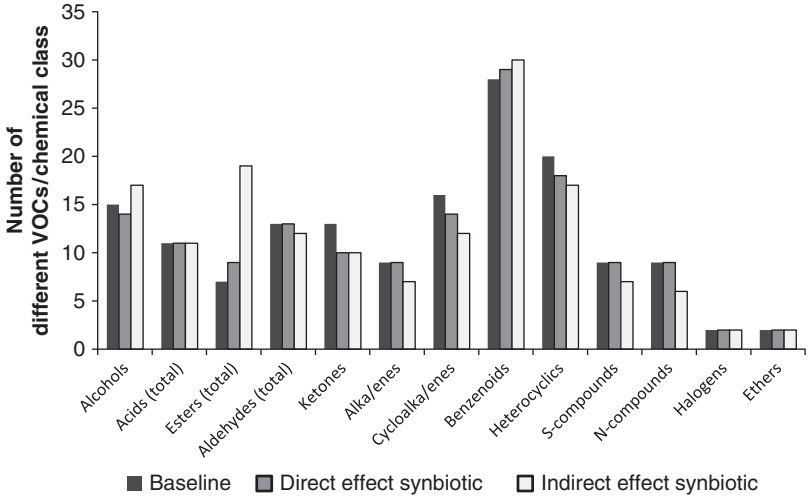


**Figure 1.** Classification of the VOCs, retrieved in all fecal samples at baseline, during and after synbiotic intervention, according to chemical classes.

secondary alcohols. A diverse range of S-containing compounds was identified. Less common chemical classes were esters, alka/enes, N-containing compounds, halogens and ethers. Esters were scarce at baseline, but increased during and after synbiotic intervention. We identified 19 different esters of which 7 were found at baseline, whereas 9 and 19 different esters were found during and after synbiotic treatment, respectively.

### 3.2 Metabolite profile

PLS analysis revealed the impact of the synbiotic intervention on the fecal metabolite profiles. The VOC fingerprints clustered in three groups, discriminating between baseline conditions, direct and indirect effects of synbiotic treatment (Fig. 2). Baseline samples were characterized by higher levels of S-containing compounds (carbon disulfide, methanethiol, dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide), while samples obtained after the synbiotic intervention (direct and indirect stimulation) contained higher relative levels of acetate and butyrate, as well as esters (Supporting Information Fig. 2B). Relative concentrations of aldehydes, ketones, alcohols, heterocyclics and benzenoids did not contribute to the clustering.

#### 3.2.1 Semi-quantitative characterization of VOCs: Efficacy of synbiotic treatment

PLS-analysis is an interesting graphical and descriptive tool to visualize clustering between samples, but this analysis does not demonstrate whether the observed clusters of samples are statistically different. The use of mixed models by means of the type III test to assess the VOCs levels between the test conditions revealed that the metabolite profiles from the three conditions were significantly different ($p = 0.0001$) which means that at least one VOC was

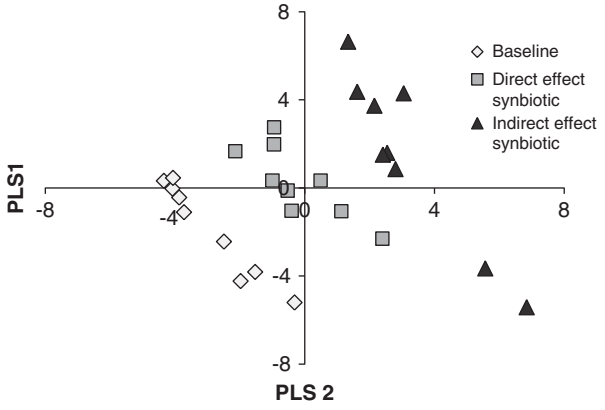


**Figure 2.** PLS analysis on relative levels of VOCs represented as different chemical classes for baseline, the direct and indirect effect of synbiotic intervention: score plot.

significantly different between the different test conditions. The metabolite profile could also depend on individual characteristics such as age, gender and BMI. To take this into account, sources of variation (age, gender and BMI) were included into the model.

#### 3.2.2 VOCs affected by synbiotic intervention

In the next step, we investigated which compounds were significantly influenced during and after the intervention and were responsible for the significantly different profiles. Fifteen VOCs were significantly affected by the synbiotic treatment (Table 2). After correction for multiple testing, three VOCs, acetate, dimethyl trisulfide and ethyl benzene, remained significantly affected (Table 2). Acetate levels were increased at both time points as compared to the initial levels (Fig. 3). In addition, the indirect effect was more pronounced than the direct effect ($p = 0.005$), suggesting that changes in the colonic microbiota contributed more to

 

**Table 2.** Compounds significantly affected by synbiotic (SYN) intervention as compared to baseline

| Compound | *p*-Value without correction | Adjusted *p*-value (Benjamini and Hochberg) | Direct effect SYN | Indirect effect SYN |
|---|---|---|---|---|
| Ethyl benzene | <0.0001 | <0.01 | Decrease | = |
| Acetate | 0.0003 | 0.021 | Increase | Increase |
| Dimethyl trisulfide | 0.0004 | 0.019 | Decrease | Decrease |
| Butyrate | 0.003 | NS | Increase | Increase |
| Furan, 2-ethyl-5-methyl- | 0.006 | NS | Decrease | Decrease |
| *o*-Xylene | 0.008 | NS | Decrease | = |
| α-Pinene | 0.009 | NS | Increase | = |
| Acetophenone | 0.027 | NS | = | Increase |
| Benzene acetaldehyde | 0.030 | NS | = | Decrease |
| 2-Acetyl-5-methylfuran | 0.033 | NS | Increase | = |
| Phenol | 0.034 | NS | Decrease | = |
| Oxepine, 2,7-dimethyl- | 0.038 | NS | Increase | = |
| Pentanoate | 0.044 | NS | Decrease | = |
| Phenol, 4-methyl- | 0.044 | NS | Decrease | Decrease |
| Carbon disulfide | 0.045 | NS | Increase | = |

the rise in acetate levels than the actual presence of the synbiotic. The relative levels of dimethyl trisulfide were significantly decreased to a similar degree during and after synbiotic intervention as compared to baseline, indicating that the direct and indirect effects of the synbiotic were comparable. In contrast, only the actual presence of the synbiotic in the colon reduced the levels of ethyl benzene. Initials levels were restored after cessation of treatment.

#### 3.2.3 Exclusion of subject-specific compounds from statistical analysis

Thirty-one compounds were only found in one sample at one time point. Exclusion of these subject-specific VOCs from the statistical analysis did not result in a higher number of significantly affected compounds.

### 3.3 DGGE profiles

On a total of 18 PCR-DGGE profiles at baseline and after the synbiotic intervention period, 58 band-classes were identified. Statistical comparison of the intensities of all band-classes from DGGE profiles at baseline samples and after synbiotic intervention revealed no differences.

## 4 Discussion

By analyzing metabolite profiles, we showed that a synbiotic combination of *L. casei* Shirota and OF-IN modifies the metabolic activity of the colon microbiota in healthy subjects. Despite no changes in the predominant microbiota, both direct fermentation of this synbiotic combination in the colonic lumen as well as after 4 wk synbiotic intake influenced the microbial metabolic processes. We used a metabolomics approach that circumvented the need for an *a priori* hypothesis and allowed us to detect impact of the dietary intervention on previously unknown pathways. Our results confirmed that the combination of *L. casei* Shirota and OF-IN stimulates the saccharolytic activity and reduces the proteolytic activity of the colonic microbiota. Furthermore, we demonstrated the impact on new interesting targets such as S-containing compounds and esters.

The fecal metabolite profiles of healthy subjects were characterized by inter- and intra-individual variation at each time point. PLS analysis showed clustering of the samples according to dietary intervention and not according to individual. These results suggest that the impact of the dietary intervention outweighs the inter-individual variation in metabolite profiles. Similar effects were previously observed by Jacobs *et al.* [22] who applied $^{1}$H NMR profiling as a tool to assess the impact of grape juice alone or combined with a wine extract on fecal metabolites in healthy subjects.

Stimulation of the saccharolytic fermentation was evident from the significantly increased levels of acetate both in the presence of the synbiotic as well as after the intervention period, and from the general increase in butyrate. Other synbiotic combinations also showed increases in fecal acetate levels [23, 24]. Increased SCFAs synthesis creates a more acidic environment in the gut [25], which enhances the colonization resistance against pathogens [26] and reduces the formation of secondary bile acids [27]. Furthermore, acidification of the colonic lumen may

 

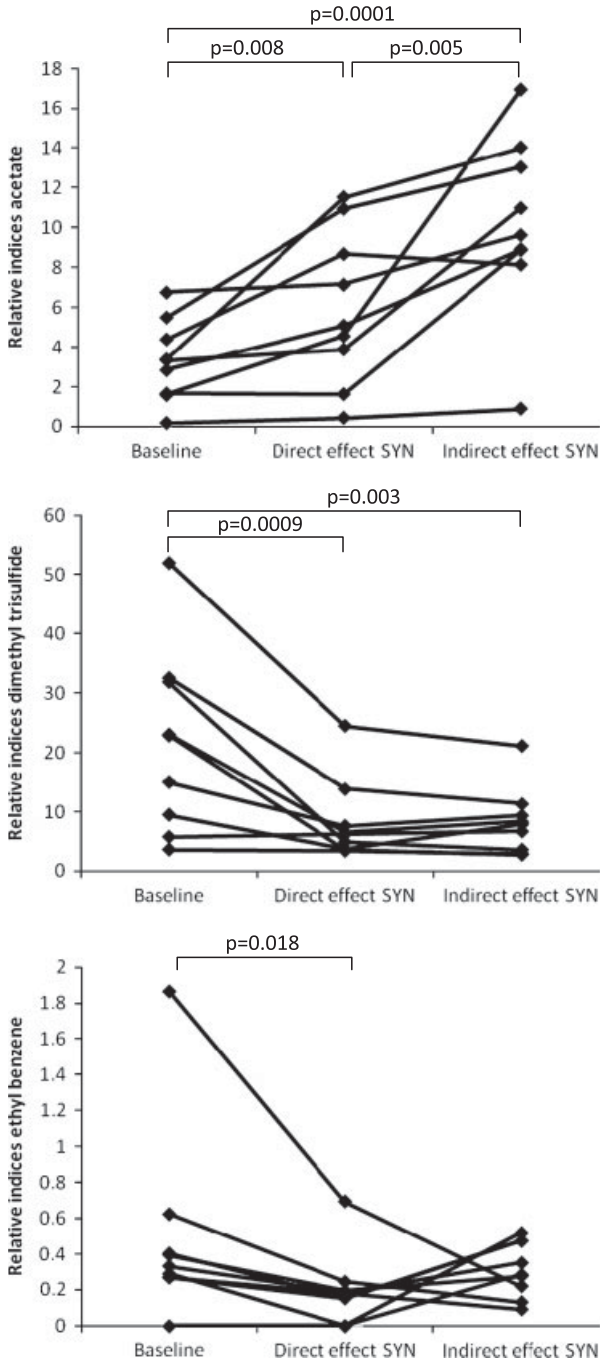


**Figure 3.** Boxplots of the compounds, acetate, dimethyl trisulfide and ethyl benzene, significantly affected by synbiotic (SYN) intervention.

impair the activity of specific enzymes such as proteases and, in this way, contribute to a reduction in protein fermentation [28]. The production rate of SCFAs is often considered as an indication of the beneficial bacterial activity in the colon [29].

Increased acid production might also be the origin of a higher presence of esters. Previously, we already demonstrated an increase in the number and relative concentration of esters in an *in vitro* model using fecal slurries after the addition of the prebiotic OF-IN [30]. Also in this study, we found more esters in the presence and after synbiotic intake in the metabolite profiles. Recently, Vitali *et al.* also demonstrated a significant increase of the ester methyl acetate after 1-month synbiotic administration (500 mg fructooligosaccharides combined with *B. longum* Bar33 and *L. helveticus* Bar 13) in healthy subjects using GC-MS/SPME technology [23]. Garner *et al.* demonstrated bacterial synthesis of esters from acids using $^{13}$C-labelled butyrate [31]. Besides, esters derived from methyl to hexyl alcohol and from acetic to hexanoic acid were found. Methanol was not found as a free alcohol, although it was extensively found as methyl ester. Given the toxicity of methanol, the ease with which an individuals' gut microbiota can trap methanol as an ester may have important health considerations. Interestingly, Garner *et al.* found that a number of esters were consistently absent in preterm infants developing necrotising enterocolitis, while present in non-necrotising enterocolitis infants [32]. Other reports on esters associated with stool samples are scares. As a consequence, the relevance of esters to health needs to be further explored in future studies.

Besides an increase in saccharolytic fermentation, a reduction in protein fermentation was evident from reduced levels of dimethyl trisulfide and ethyl benzene. Proteins that escape digestion in the human small intestine undergo in the colon intense proteolysis resulting in peptides and amino acids, which are further fermented to bacterial metabolites such as ammonia, S-containing and phenolic compounds.

The mechanisms leading to reduced protein can be explained by (i) the rapid fermentation of the substrate in the colon resulting in a lower colonic pH which reduces the bacterial proteolysis of proteins and peptides [25], (ii) a process of so-called catabolite repression which results in an inhibition of the deamination of amino acids [33] and (iii) an enhanced uptake of amino acids and intermediary metabolites for bacterial biosynthesis [34]. For the test performed in the absence of the synbiotic, an alteration of the microbiota may be responsible for a decreased generation of protein fermentation compounds.

A broad range of volatile sulfur compounds (hydrogen sulfide, methanethiol, dimethyl sulfide, dimethyl disulfide and dimethyl trisulfide) has been identified in fecal samples [31, 35, 36]. They have a bad reputation because of their toxicity and bad odor. These S-containing compounds typically originate from microbial degradation of sulfur-containing amino acids and, dietary and mucinous sulfate by sulfate-reducing bacteria [36, 38]. In this study, lower levels of the S-containing compound dimethyl trisulfide were found during and after dietary intervention as compared to baseline. These results corroborate our previous *in vitro* results. Incubation of fecal slurries with OF-IN prevented in a dose- and time-dependent way the

 

production of S-containing compounds [30]. However, the level of carbon disulfide tended to increase during the synbiotic intervention. Previously, Vitali *et al.* also found increased levels of carbon disulfide after intake of a synbiotic substrate and hypothesized that carbon disulfide was produced by carbonation of hydrogen sulfide as a detoxification mechanism exerted by colonic bacteria [23]. The reduced concentrations of the S-compounds after synbiotic intake *in vivo* may be of importance in the treatment of ulcerative colitis. Hydrogen sulfide and possibly also other S-containing compounds have been implicated in the etiology and/or risk of relapse of this disease [39, 40]. Increased levels of hydrogen sulfide and sulfate-reducing bacteria in feces of ulcerative colitis have previously been demonstrated [37–39].

Another indication for reduced protein fermentation was found in the lower levels of ethyl benzene. This benzenoid compound has previously been described in fecal samples and was thought to arise from air pollution [31]. However, in a study investigating the influence of increased dietary protein intake on colonic bacterial metabolism, a 2- to 12-fold increase in ethyl benzene levels after protein supplementation was observed, suggesting that ethyl benzene originates from protein metabolism [35]. Indeed, ethyl benzene can be formed by hydroxylation of styrene, a decarboxylation product of cinnamic acid, which is produced by L-phenylalanine deamination [42]. In our study, ethyl benzene levels were significantly lower in the presence of the synbiotic, which could be a result of the former explained mechanisms leading to reduced protein fermentation. Once the administration of the synbiotic has been ceased, the effect readily disappears suggesting that a continuous stimulation of the microbiota might be necessary to maintain effects.

In conclusion, administration of a synbiotic combination of *L. casei* Shirota with OF-IN leads to an altered metabolite profile. Our results demonstrated an *in vivo* stimulation of SCFA production, in particular acetate. More importantly, a decrease in protein fermentation metabolites dimethyl trisulfide and ethyl benzene attended these effects. It must be emphasized that the effects observed with the synbiotic used in the present study cannot be extrapolated to other synbiotic combinations since it has been shown previously that different pre- and probiotics have specific substrate- and strain-dependent effects.

Using the metabolite profiling methodology, we have revealed new pathways affected by dietary intervention. However, an important challenge for current and future research is to relate changes in bacterial metabolism to concrete health benefits.

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*The authors have declared no conflict of interest.*

## 5 References

[1] Beylot, M., Effects of inulin-type fructans on lipid metabolism in man and in animal models. *Br. J. Nutr.* 2006, *93*, S163–S168.

[2] Cummings, J. H., Macfarlane, G. T., Gastrointestinal effects of prebiotics. *Br. J. Nutr.* 2002, *87*, S145–S151.

[3] Delzenne, N. M., Williams, C. M., Prebiotics and lipid metabolism. *Curr. Opin. Lipidol.* 2002, *13*, 61–67.

[4] Guarner, F., Inulin and oligofructose: impact on intestinal diseases and disorders. *Br. J. Nutr.* 2006, *93*, S61–S65.

[5] Scholz-Ahrens, K. E., Ade, P., Marten, B., Weber, P. *et al.*, Prebiotics, probiotics, and synbiotics affect mineral absorption, bone mineral content, and bone structure. *J. Nutr.* 2007, *137*, 838S–846S.

[6] Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A. *et al.*, Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.* 2004, *17*, 259–275.

[7] Kolida, S., Tuohy, K., Gibson, G. R., Prebiotic effects of inulin and oligofructose. *Br. J. Nutr.* 2002, *87*, S193–S197.

[8] Zoetendal, E. G., Rajilic-Stojanovic, M., de Vos, W. M., High-throughput diversity and functionality analysis of the gastrointestinal tract microbiota. *Gut* 2008, *57*, 1605–1615.

[9] Falony, G., De Vuyst, L., Ecological interactions of bacteria in the human gut. Chapter 22. Charalampopoulos, Rastall, R. A. (Ed.), *Prebiotics and Probiotics Science and Technology*, Springer, Heidelberg, Germany 2009, pp. 641–682.

[10] Nicholson, J. K., Use of metabolomics to study gut microorganisms. *Annu. Rev. Nutr.* 2008, DOI: 10.1146/annurev-nutr-080508-141243.

[11] Nicholson, J. K., Lindon, J. C., Systems biology: metabonomics. *Nature* 2008, *455*, 1054–1056.

[12] De Preter, V., Van Staeyen, G., Esser, D., Rutgeerts, P. *et al.*, Development of a screening method to determine the pattern of fermentation metabolites in faecal samples using on-line purge-and-trap gas chromatographic-mass spectrometric analysis. *J. Chromatogr. A* 2009, *1216*, 1476–1483.

[13] Jenkins, D. J. A., Kendall, C. W. C., Vuksan, V., Inulin, oligofructose and intestinal function. *J. Nutr.* 1999, *129*, 1431S–1433S.

[14] Roberfroid, M. B., Introducing inulin-type fructans. *Br. J. Nutr.* 2006, *93*, S13-S25.

[15] Spanhaak, S., Havenaar, R., Schaafsma, G., The effect of consumption of milk fermented by *Lactobacillus casei* strain Shirota on the intestinal microflora and immune parameters in humans. *Eur. J. Clin. Nutr.* 1998, *52*, 899–907.

[16] Vanhoutte, T., Huys, G., De Brandt, E., Swings, J., Temporal stability analysis of the microbiota in human feces by denaturing gradient gel electrophoresis using universal and group-specific 16S rRNA gene primers. *FEMS Microbiol. Ecol.* 2004, *48*, 437–446.

[17] Joossens, M., Van Steen, K., Cnockaert, M., Vermeire, S. *et al.*, High-throughput method for comparative analysis of denaturing gradient gel electrophoresis profiles from

 

human fecal samples reveals significant increases in two bifidobacterial species after inulin-type prebiotic intake. *FEMS Microbiol. Ecol.* 2011, *75*, 343–349.

[18] Pedreschi, R., Vanstreels, E., Carpentier, S., Hertog, M. *et al.*, Proteomic analysis of core breakdown disorder in Conference pears (*Pyrus communis* L.). *Proteomics* 2007, *7*, 2083–2099.

[19] Verbeke, G., Molenberghs, G., *Linear Mixed Model for Longitudinal Data*, Springer, New York 2000.

[20] SAS Institute, *Chapter 15: The Four Types of Estimable Functions. SAS/STAT® 9.2 User's Guide*, SAS Institute, Cary, NC 2008, pp. 271–288.

[21] Benjamini, Y., Hochberg, Y., Controlling the false discovery rate – a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B Methodol.* 1995, *57*, 289–300.

[22] Jacobs, D. M., Deltimple, N., van Velzen, E., van Dorsten, F. A. *et al.*, (1) H NMR metabolite profiling of feces as a tool to assess the impact of nutrition on the human microbiome. *NMR Biomed.* 2008, *21*, 615–626.

[23] Vitali, B., Ndagijimana, M., Cruciani, F., Carnevali, P. *et al.*, Impact of a synbiotic food on the gut microbial ecology and metabolic profiles. *BMC Microbiol.* 2010, *10*, 4.

[24] Ndagijimana, M., Laghi, L., Vitali, B., Placucci, G. *et al.*, Effect of a synbiotic food consumption on human gut metabolic profiles evaluated by (1)H Nuclear Magnetic Resonance spectroscopy. *Int. J. Food Microbiol.* 2009, *134*, 147–153.

[25] Scheppach, W., Luehrs, H., Menzel, T., Beneficial health effects of low-digestible carbohydrate consumption. *Br. J. Nutr.* 2001, *85*, S23–S30.

[26] Topping, D. L., Fukushima, M., Bird, A. R., Resistant starch as a prebiotic and synbiotic: state of the art. *Proc. Nutr. Soc.* 2003, *62*, 171–176.

[27] Zampa, A., Silvi, S., Fabiani, R., Morozzi, G. *et al.*, Effects of different digestible carbohydrates on bile acid metabolism and SCFA production by human gut micro-flora grown in an *in vitro* semi-continuous culture. *Anaerobe* 2004, *10*, 19–26.

[28] Macfarlane, G. T., Allison, C., Gibson, G. R., Effect of Ph on protease activities in the large-intestine. *Lett. Appl. Microbiol.* 1988, *7*, 161–164.

[29] Cummings, J. H., Short chain fatty-acids in the human-colon. *Gut.* 1981, *22*, 763–779.

[30] De Preter, V., Falony, G., Windey, K., Hamer, H. M. *et al.*, The prebiotic, oligofructose-enriched inulin modulates the faecal metabolite profile: an *in vitro* analysis. *Mol. Nutr. Food Res.* 2010, *54*, 1791–1801.

[31] Garner, C. E., Smith, S., Costello, B. D., White, P. *et al.*, Volatile organic compounds from feces and their potential for diagnosis of gastrointestinal disease. *FASEB J.* 2007, *21*, 1675–1688.

[32] Garner, C. E., Ewer, A. K., Elasouad, K., Power, F. *et al.*, Analysis of faecal volatile organic compounds in preterm infants who develop necrotising enterocolitis: a pilot study. *J. Pediatr. Gastroenterol. Nutr.* 2009, *49*, 559–565.

[33] Vince, A. J., Burridge, S. M., Ammonia production by intestinal bacteria: the effects of lactose, lactulose and glucose. *J. Med. Microbiol.* 1980, *13*, 177–191.

[34] Cummings, J. H., Bingham, S. A., Dietary fiber, fermentation and large bowel-cancer. *Cancer Surv.* 1987, *6*, 601–621.

[35] Geypens, B., Claus, D., Evenepoel, P., Hiele, M. *et al.*, Influence of dietary protein supplements on the formation of bacterial metabolites in the colon. *Gut* 1997, *41*, 70–76.

[36] Tangerman, A., Measurement and biological significance of the volatile sulfur compounds hydrogen sulfide, methanethiol and dimethyl sulfide in various biological matrices. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 2009, *877*, 3366–3377.

[37] Gibson, G. R., Macfarlane, G. T., Cummings, J. H., Sulfate reducing bacteria and hydrogen metabolism in the human large-intestine. *Gut* 1993, *34*, 437–439.

[38] Le, P. D., Aarnink, A. J., Ogink, N. W., Becker, P. M. *et al.*, Odour from animal production facilities: its relationship to diet. *Nutr. Res. Rev.* 2005, *18*, 3–30.

[39] Levine, J., Ellis, C. J., Furne, J. K., Springfield, J. *et al.*, Fecal hydrogen sulfide production in ulcerative colitis. *Am. J. Gastroenterol.* 1998, *93*, 83–87.

[40] Pitcher, M. C. L., Cummings, J. H., Hydrogen sulphide: a bacterial toxin in ulcerative colitis? *Gut* 1996, *39*, 1–4.

[41] Marquet, P., Duncan, S. H., Chassard, C., Bernalier-Donadille, A. *et al.*, Lactate has the potential to promote hydrogen sulphide formation in the human colon. *FEMS Microbiol. Lett.* 2009, *299*, 128–134.

[42] Sabatini, N., Perri, E., Marsilio, V., An investigation on molecular partition of aroma compounds in fruit matrix and brine medium of fermented table olives. *Innovat. Food Sci. Emerg. Tech.* 2009, *10*, 621–626.

 