RESEARCH ARTICLE

The prebiotic, oligofructose-enriched inulin modulates the faecal metabolite profile: An *in vitro* analysis

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Scope: Health benefits of prebiotic administration have been judged mainly from the increased numbers of bifidobacteria and the enhanced production of short-chain fatty acids in the colon. Only a few studies have focused on the capacity of prebiotics to decrease the proteolytic fermentation, which might contribute to health as well.
Methods and results: The influence of the prebiotic oligofructose-enriched inulin (OF-IN) on the pattern of volatile organic compounds was characterized using an *in vitro* faecal model. Faecal slurries, obtained from healthy subjects, were anaerobically incubated at 37°C with and without different doses of OF-IN (2.5, 5, 10, or 20 mg) and changes in the metabolite pattern and pH were evaluated. A total of 107 different volatile organic compounds were identified and classified according to their chemical classes. The concentration of esters and acids significantly increased with increasing doses of OF-IN. Similar effects were observed for some aldehydes. To the contrary, OF-IN dose-dependently inhibited the formation of S-compounds. Also, the generation of other protein fermentation metabolites such as phenolic compounds was inhibited in the presence of OF-IN.
Conclusion: Our results confirmed a clear dose-dependent stimulation of saccharolytic fermentation. Importantly, a significant decrease in toxic protein fermentation metabolites such as sulphides attended these effects.

Keywords:
Metabolomics / Prebiotic / Protein fermentation / SCFA

1 Introduction

Health benefits of prebiotic administration have been judged mainly from the increased numbers of bifidobacteria and the enhanced production of short-chain fatty acids (SCFAs) in the colon [1]. Inulin-type fructans are considered as model prebiotics because of the vast amount of research performed on their regulatory and health-promoting properties [2–4]. Several *in vitro* and *in vivo* (animal and human) trials have demonstrated their stimulating effect on the colonic bifidobacterial populations [5–8]. Besides the bifidogenic effect, fermentation of inulin-type fructans by the colonic microbiota enhances the production of SCFAs [3, 9]. In particular, an increase in butyrate formation may be observed, the so-called butyrogenic effect [10]. SCFAs are essential for the metabolism of the colonic epithelial cell and maintenance of normal mucosal function. Especially, butyrate is considered as the major energy source for the colonic mucosa [11].

In contrast to the extensive evidence confirming the bifidogenic and butyrogenic effects, only a few studies have

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Abbreviations: BCFA, branched-chain fatty acids; **OF-IN,** oligofructose-enriched inulin; **PCA,** principal components analysis; **PLS-DA,** partial least square discriminant analysis; **SCFA,** short-chain fatty acid; **UC,** ulcerative colitis; **VIP,** Variable Importance Plot; **VOCs,** volatile organic compounds

focused on the capacity of inulin-type fructans to decrease the proteolytic fermentation in the colon. Protein putrefaction is often regarded as detrimental to host health, in particular with respect to colon toxicity, mutagenicity, and carcinogenicity [12]. Anaerobic metabolism of proteins and peptides by the microbiota generates, besides a limited amount of SCFAs, a series of potentially toxic substances including ammonia, sulphur-containing compounds, and phenolic and indolic compounds. Several protein metabolites are associated with diseases such as the uremic syndrome and bladder and bowel cancers [13–16]. Sulphur-containing compounds have been shown *in vitro* to act as damaging agents in inflammatory bowel diseases [17]. Therefore, decreased proteolytic fermentation might contribute to health as well.

The aim of the present study was to characterize the influence of the prebiotic substrate oligofructose-enriched inulin (OF-IN) on the pattern of volatile organic compounds (VOCs) using an *in vitro* faecal model. Special attention was paid to its influence on the colonic proteolytic activity.

2 Materials and methods

2.1 Chemicals

Dimethyl sulphide, diethyl sulphide, *p*-cresol, and 2,6-dimethylphenol were supplied by Sigma-Aldrich (Steinheim, Germany), propionate by UCB (Leuven, Belgium), butyrate by Devos-Francois (Charleroi, Belgium), and acetate and 2-ethylbutyrate by Merck (München, Germany). All standards were of analytical quality (at least 99% purity). Diethyl sulphide, 2,6-dimethylphenol, and 2-ethylbutyrate were used as internal standards. Sodium sulphate (99%) was purchased from Acros Organics (Geel, Belgium) and sulphuric acid (99%) from Sigma-Aldrich.

2.2 Prebiotic

OF-IN was a commercially available 1:1 (w/w) mixture of oligofructose (average degree of polymerization or $DP_{av} = 4$) and long-chain inulin ($DP_{av} = 25$) (ORAFTI® Synergy-1, BENEO-Orafti, Tienen, Belgium).

2.3 Faecal samples

Ten healthy subjects (5 female/5 male, age range 18–33 years) provided a faecal sample. None of the subjects had used antibiotics or undergone any other medical treatment influencing gut transit or intestinal microbiota during the preceding month. Samples were collected in dedicated recipients containing an Anaerocult® strip (Merck, Darmstadt, Germany) to create anaerobic conditions during transport to the laboratory. Faecal samples were immediately used for the analysis. We prepared a 10% m/V faecal slurry from each sample using oxygen-free phosphate-buffered saline (1 ×). The samples were homogenized using a vortex.

2.4 *In vitro* fermentation

2.4.1 Influence of OF-IN on metabolic activity as a function of time

Of each faecal slurry, 21 aliquots of 4870 μL were transferred in dedicated 25-mL purge vials (Interscience, Louvain-la-Neuve, Belgium). The vials were immediately flushed with gaseous nitrogen to eliminate oxygen and keep anaerobic conditions. Three aliquots were analysed immediately (*i.e.* before incubation or supplementation with OF-IN) to assess the baseline metabolic activity. Subsequently, 10 mg of OF-IN was added to nine other aliquots of the faecal slurry followed by incubation at 37°C for 3, 6, and 24 h, respectively. The remaining nine aliquots were incubated simultaneously at 37°C without the addition of OF-IN and analysed at the same time points (3, 6, and 24 h). These samples served as negative controls to quantify the metabolic activity produced by substrate present in the faecal sample.

2.4.2 Effect of different doses of OF-IN on metabolic activity

Of each faecal slurry, 20 aliquots were prepared as described above. To 16 aliquots, different doses of OF-IN (2.5, 5, 10, and 20 mg; four aliquots *per* dose) were added, followed by incubation at 37°C for 24 h. Negative controls (four aliquots) were incubated simultaneously at 37°C. All incubations were performed in quadruplicate: three samples were used for GC-MS analysis and one sample was used for the determination of faecal pH.

2.5 GC-MS analysis

After incubation, samples were placed on ice to cease fermentation. Three internal standards (50 μL diethyl sulphide (2.5 mg/100 mL), 40 μL 2,6-dimethylphenol (25 mg/100 mL), and 40 μL 2-ethylbutyrate (250 mg/100 mL)) were added to each vial (final volume 5 mL). Sodium sulphate and sulphuric acid were added to salt out and acidify the solution, respectively.

The metabolites were analysed on a gas chromatograph–mass spectrometer type time of flight (Trace GC, Thermoquest, Rodano, Italy and Tempus II, Thermo Electron, San Jose, CA, USA, respectively), which was coupled on-line to a purge-and-trap system (Velocity, Teledyne Tekmar, Mason, OH, USA). The optimal conditions

for analysis of faecal metabolites have previously been described [18]. Each incubation was performed in triplicate and only those compounds identified in at least two out of three incubations were used for further evaluation. Relative indices of all VOCs *versus* 2-ethylbutyrate 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 their chemical nature, the number of identified compounds in all samples was summed up and the number of compounds in each chemical class was calculated.

2.6 Quantification of the hypothesis-driven metabolites

A number of VOCs were selected as markers for saccharolytic fermentation (SCFA (acetate, propionate, and butyrate)) and proteolytic fermentation (dimethyl sulphide, *p*-cresol, and indole). They were absolutely quantified with appropriate calibration curves obtained using internal standard quantitation. The SCFAs were quantified using 2-ethylbutyrate as internal standard, whereas *p*-cresol and indole as well as dimethyl sulphide were quantified *versus* 2,6-dimethylphenol and diethyl sulphide, respectively. The results were expressed as mmol/L or μmol/L for dimethyl sulphide.

2.7 pH measurements

The pH in the faecal slurries was measured at room temperature with a pH meter (Knick type 751, Elscolab, Kruibeke, Belgium) equipped with an Inlab 412 electrode (Mettler-Toledo, Zaventem, Belgium).

2.8 Statistics

The multivariate statistical analysis consisted of principal components analysis (PCA) and partial least square discriminant analysis (PLS-DA). These analyses were performed to cluster the metabolite profiles according to similar metabolite patterns. The data were weighed by their standard deviation to give them equal variances. A PCA analysis was carried out for first data exploration and outlier detection using the 95% Hotelling's T^2 limit [19]. PLS-DA is a supervised technique, which is a compromise between discriminant analysis and PLS. It can be regarded as a refinement of PCA 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 different VOCs were considered as *X*-variables and the categorical variable dose was considered as *Y*-variable. The correlation loading plots were used to identify the metabolites that contributed most to pattern distinction. VOCs with a high correlation loading (>70%) for a certain PLS component contributed significantly to the class distinction along that PLS component. The Variable Importance Plot (VIP) was used as a format tool to rank the different VOCs in order of importance based on the correlation loadings [19]. All calculations were performed using Unscrambler Version 9.7 (CAMO A/S, Trondheim, Norway).

Univariate statistical analysis was used to test significant changes in absolute concentrations of hypothesis-driven

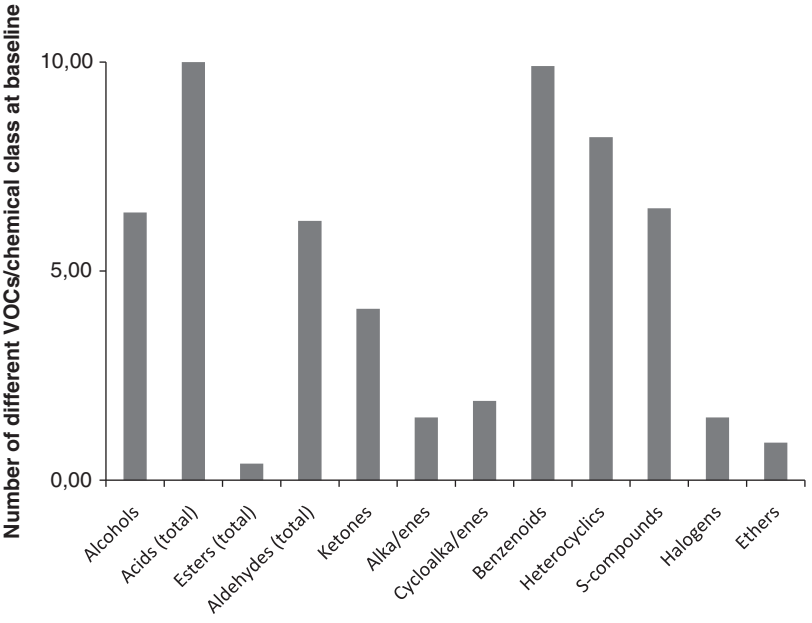

Figure 1. Classification of VOCs retrieved in the faecal samples of ten healthy subjects according to chemical classes.

metabolites. Concentrations are expressed as median plus interquartile range. Figures are shown as median plus 25th and 75th percentiles. The statistical analysis was performed with SPSS software (SPSS 17.0 for Windows; SPSS, Chicago, IL, USA). Given the low number of subjects, non-parametric statistical analysis was used regardless of the distribution of results (Friedman analysis of variance and/or Wilcoxon test (with Bonferroni correction)). The level for statistical significance was set at $p < 0.05$.

3 Results

3.1 Profiles of VOCs at baseline

A total of 107 different VOCs was identified in the faecal slurries with a median of 46 (interquartile range 23) *per* sample. Fifty compounds were present in at least 50% of the slurries, among which 13 VOCs were found in all faecal slurries, indicating that many compounds were shared by healthy subjects taking an *ad libitum* diet (Supporting Information Table 1). Twenty-three compounds were person specific.

When classifying the VOCs according to chemical classes (Fig. 1), acids and benzenoids appeared as the largest groups of VOCs. Among the acids, SCFAs are fermentation products of undigested carbohydrate, while branched-chain fatty acids (BCFAs) arise from dissimilation of amino acids. Other acids included longer chain fatty acids. The class of benzenoids consisted of a diverse range of mono-, di-, and tri-substituted benzenoids. Of these, phenolic and indolic compounds are typical compounds arising from the bacterial metabolism of aromatic amino acids. Heterocyclics, like furan derivatives, formed the third

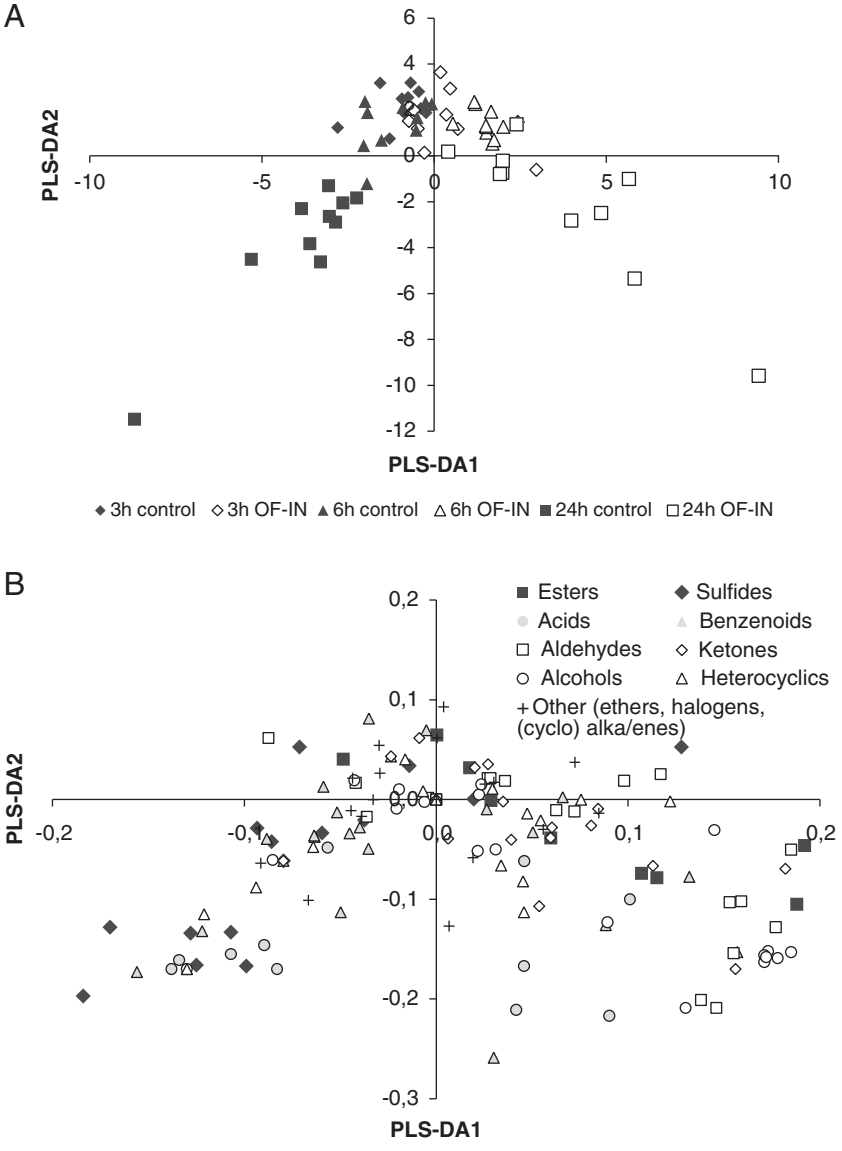

Figure 2. PLS-DA analysis on VOCs represented as different chemical classes after 3, 6, and 24 h of incubation with and without 10 mg OF-IN: PLS-DA score plot (A) and loading plot (B).

largest group. Furthermore, alcohols and S-containing compounds were found. Most commonly found alcohols were phenols and primary and secondary alcohols. Methanethiol, dimethyl sulphide, as well as di- and trimethyl disulphide have previously been identified in faecal samples. Also, aldehydes and ketones were commonly found in the faecal slurries. Alka/enes, cycloalka/enes, halogens, and ethers were less common and esters were scarce (<1%).

3.2 Influence of OF-IN on metabolic activity as a function of time

3.2.1 Metabolite profile

PCA and PLS-DA were used to analyse the impact of addition of OF-IN on the metabolite patterns as a function of time. No outliers were detected on the PCA score plots (data not shown). A PLS-DA analysis of all VOCs of the metabolite profile gave interesting information about the differences between the negative controls and the OF-IN supplemented samples at different incubation times. In this plot, each data point represents a metabolite profile. The score plot (Fig. 2A) showed that after 3 h of incubation, the negative control samples were not separated from samples incubated with OF-IN, but were separated from the samples obtained after 6 and 24 h of incubation. The negative control samples after 6 and 24 h incubation were separated from the OF-IN supplemented samples along the PLS-DA2 axis. The corresponding loading plot (Fig. 2B), depicts the correlation of the original VOCs with the PLS-DA1 and PLS-DA2 variable. In this way, we were able to discriminate and identify the VOCs, which were differentially metabolized in different test conditions. A more extreme loading of a VOC implies a larger contribution to the variation. VOCs with a high positive or negative correlation with PLS-DA1 explain the discrimination between the different incubation times. Similarly, VOCs with a high correlation with PLS-DA2 are important in discriminating negative control samples from OF-IN supplemented samples after 6 and 24 h of incubation. The VIP procedure yielded those VOCs that contributed most to the discrimination of the metabolite profiles (data not shown). Discrimination appeared to be mainly due to S-compounds (carbon disulphide, methanethiol, dimethyl sulphide, dimethyl disulphide, and dimethyl trisulphide), which occurred in higher concentrations in the negative control samples, and due to esters, which occurred in higher concentrations in the OF-IN incubated samples. Besides, benzenoid compounds, especially 3-methyl-1H-indole and phenol, and the BCFAs 3-methyl butyrate and 2-methyl propionate, were more abundantly present in the negative control samples, whereas samples with OF-IN were characterized by enhanced relative concentrations of acids. Relative concentrations of aldehydes, ketones, alcohols, heterocyclics, ethers, (cyclo-) alka/enes, and halogens were not affected by the addition of OF-IN.

3.2.2 Semi-quantitative characterization of S-compounds and esters

Because S-compounds and esters emerged as discriminating compounds between samples with and without OF-IN, we looked into more detail at the different compounds belonging to those classes (Fig. 3). The relative amounts of esters gradually increased over time and this increase was much more pronounced in the samples incubated with OF-IN than in the control samples. On the contrary, addition of OF-IN to the faecal slurry clearly inhibited the formation of S-containing compounds.

3.2.3 Absolute concentrations of the hypothesis-driven metabolites

SCFAs, dimethyl sulphide, and *p*-cresol were absolutely quantified in the samples at baseline and after incubation with and without OF-IN (Fig. 4). Acetate concentrations were higher in the OF-IN-supplemented samples after 3 h ($p = 0.039$), 6 h ($p = 0.015$), and 24 h ($p = 0.015$) incubation as compared to the baseline concentration (Friedmand $p < 0.0001$). As compared to the negative controls, acetate

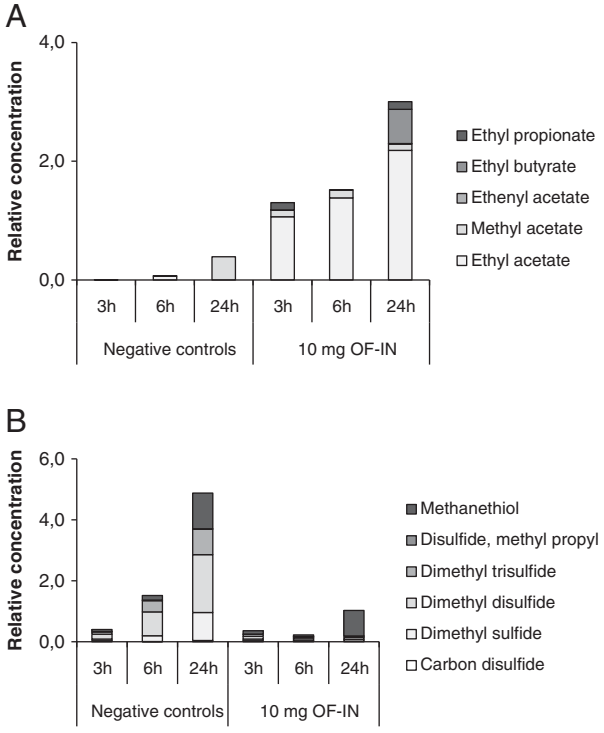

Figure 3. Relative indices of different sulphides (A) and esters (B) after 3, 6, and 24 h of incubation with and without 10 mg OF-IN.

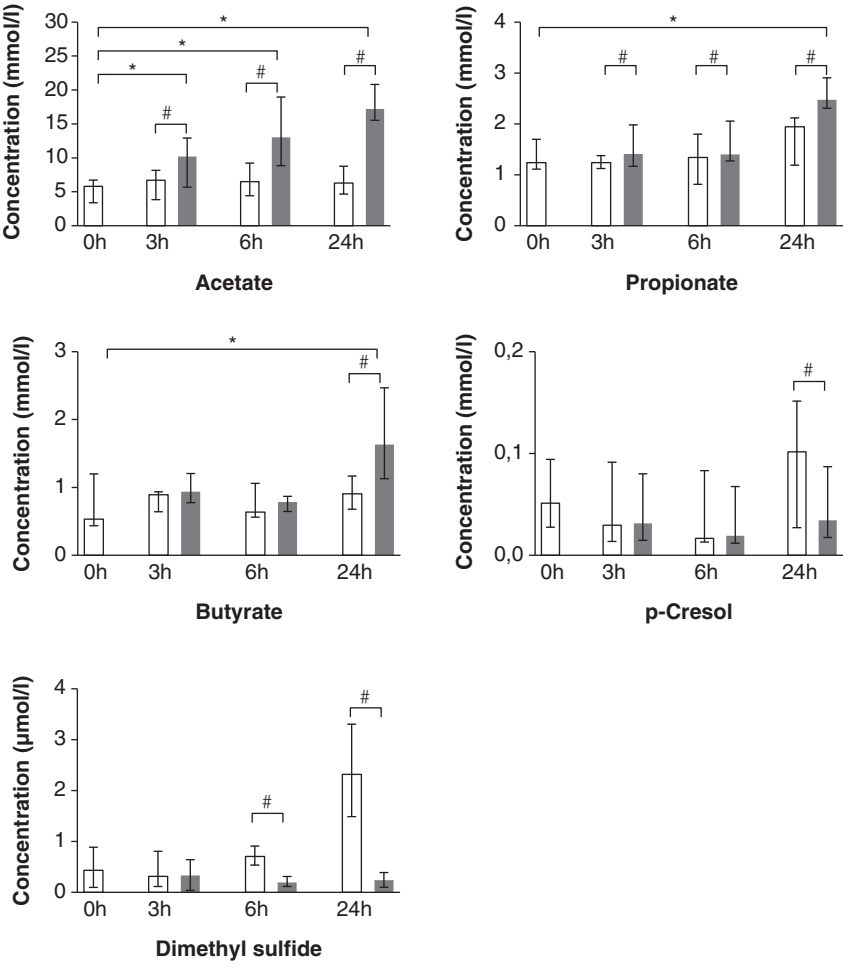

Figure 4. Metabolite concentrations of the VOCS at baseline (0 h) and after 3, 6, and 24 h of incubation with (■) and without (□) 10 mg OF-IN (Friedman ANOVA for comparison of baseline samples *versus* incubations with OF-IN (∗) and Wilcoxon test for within time comparisons between the negative control samples and OF-IN-supplemented samples (♯); median (25th to 75th percentile); $\alpha = 0.05$).

was significantly more increased in the OF-IN supplemented sample after 3 h of incubation ($p = 0.013$), after 6 h of incubation ($p = 0.005$), and with a maximum difference after 24 h of incubation ($p = 0.005$). OF-IN addition also affected the propionate concentrations as compared to baseline (Friedman $p = 0.008$) with a significantly higher propionate concentration after 24 h of incubation ($p = 0.021$). Comparison of the negative controls with the OF-IN-supplemented samples showed an increase after 3 h ($p = 0.017$), 6 h ($p = 0.007$), and 24 h ($p = 0.005$) of incubation. After 24 h of incubation, the butyrate concentration was significantly higher when compared with baseline (Friedman $p = 0.015$) and the negative control ($p = 0.005$).

Furthermore, addition of OF-IN significantly inhibited the formation of dimethyl sulphide after 6 h ($p = 0.007$) and 24 h of incubation ($p = 0.005$) when compared with the negative control. The *p*-cresol formation decreased in the 24-h incubation upon OF-IN supplementation when compared with its negative control ($p = 0.024$). Addition of OF-IN did not affect the concentration of dimethyl sulphide and *p*-cresol over time as compared to baseline.

3.3 Effect of different doses of OF-IN on metabolic activity

3.3.1 Metabolite profile

PCA revealed no outliers using the 95% Hotelling's T^2 limit (data not shown). PLS-DA analysis enhanced the discrimination among the different samples and resulted in clustering of the samples *per* dose OF-IN along the PLS-DA2 (score plot: Fig. 5A). To identify the important VOCs involved in discriminating the different samples, the analysis was focused on the correlation loading plot (Fig. 5B), which represents the correlation between the VOCs and the class membership for the samples. Using the VIP procedure, 15 VOCs were yielded that contributed most to class separation (data not shown). Among the selected VOCs, five occurred in higher relative concentrations for the negative control samples (dimethyl sulphide, dimethyl di- and trisulphide, methanethiol, and carbon disulphide). On the contrary, samples incubated with OF-IN were characterized by higher amounts of SCFAs (acetate and butyrate), esters (methyl and ethyl acetate and ethyl butyrate), some aldehydes (acetaldehyde, 5-methyl-2-furancarboxaldehyde, propanal, and pentanal), and an alcohol (ethanol).

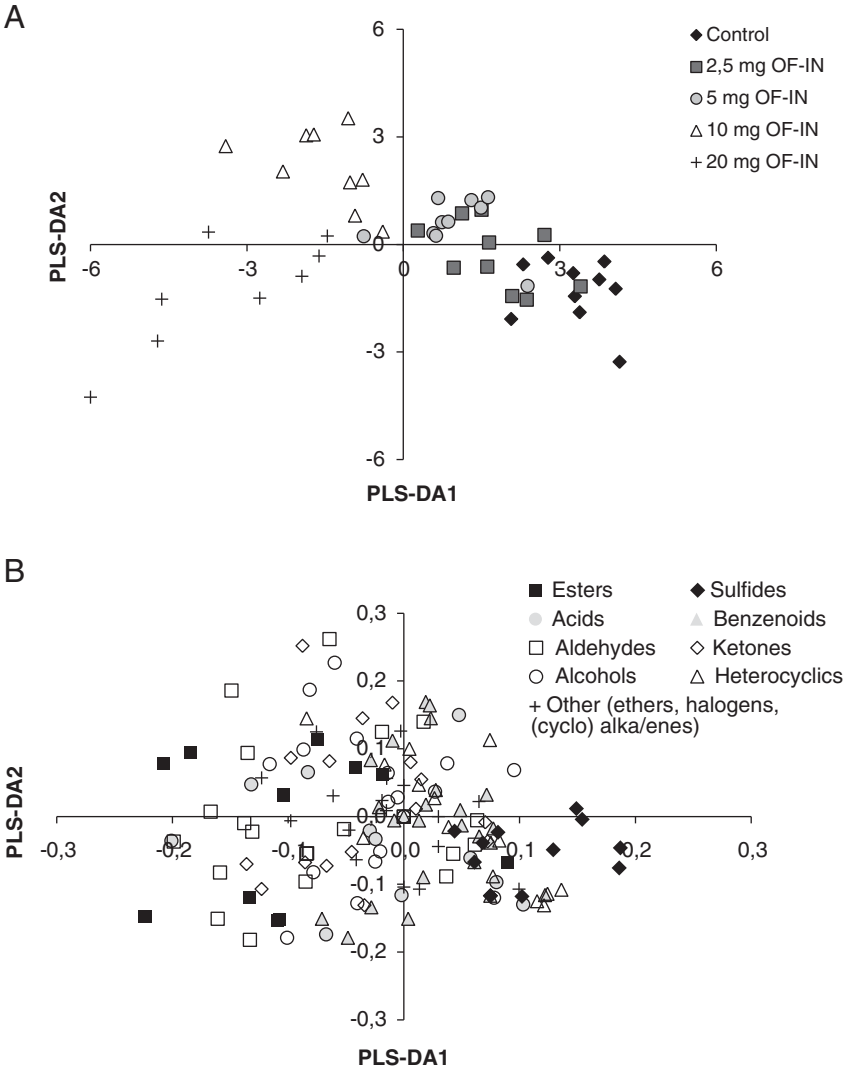

Figure 5. PLS-DA analysis on VOCs represented as different chemical classes after 24 h of incubation with and without different doses of OF-IN: PLS-DA score plot (A) and correlation loading plot (B).

3.3.2 Semiquantitative characterization of S-compounds and esters

Figure 6 shows the proportion of relative amounts of S-compounds and esters after 24 h of incubation with different doses of OF-IN. With increasing dose of OF-IN, esters increased. This was due to a higher prevalence of methyl and ethyl esters of the SCFAs acetate, propionate, butyrate, and pentanoate, whereas the formation of S-containing compounds was inhibited.

3.3.3 Absolute concentrations of hypothesis-driven metabolites

The influence of OF-IN on the absolute concentrations of selected metabolites after 24 h of incubation is shown in Fig. 7. Acetate concentrations significantly increased with increasing doses of OF-IN (Friedman $p<0.0001$). As compared to baseline, all doses of OF-IN resulted in a significantly higher acetate concentration ($p<0.05$), whereas compared to the negative control only a significant increase was observed with 10 and 20 mg OF-IN (both $p=0.02$). Supplementation with 10 mg OF-IN increased the propionate concentration significantly as compared to baseline ($p=0.045$) and the negative control ($p=0.020$). Butyrate concentrations were not significantly different, although butyrate formation increased with increasing doses of OF-IN. *p*-Cresol formation dose-dependently decreased, although not significant. Dimethyl sulphide formation was significantly increased in the negative control as compared to baseline ($p=0.028$). Addition of OF-IN inhibited this formation in a dose-dependent way ($p=0.032$ for 2.5 mg and $p=0.020$ for 5g, 10, and 20 mg OF-IN, as compared to the negative control).

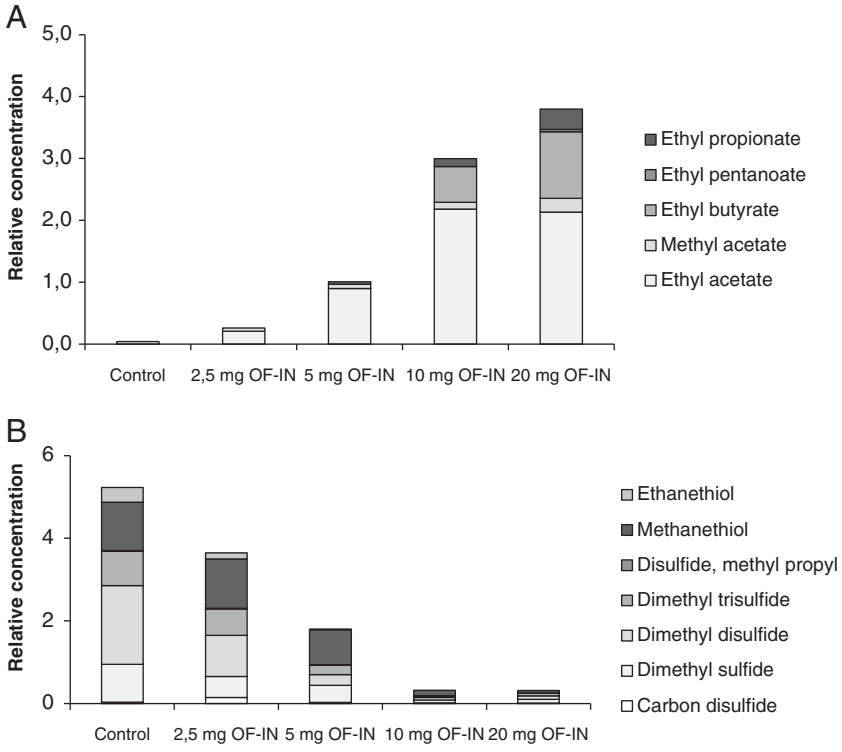

Figure 6. Relative indices of different sulphides (A) and esters (B) after 24 h of incubation without and with different doses of OF-IN (2.5, 5, 10, or 20 mg).

3.4 Effect of different doses of OF-IN on the pH

In the absence of the prebiotic, the pH was relatively constant and varied between 6.8 and 7.4 (Fig. 8). Upon addition of increasing doses of OF-IN to the faecal slurries, the pH progressively decreased (Friedman $p<0.0001$). The pH was significantly decreased in the OF-IN-supplemented samples as compared to baseline ($p=0.025$ for all doses) and to the negative control ($p=0.020$ for all doses). Between the different doses, the pH was also significantly different ($p<0.05$).

4 Discussion

To date, only a few studies have attempted to generate an overall metabolite profile in faeces and to evaluate the impact of pre- and/or probiotic intervention on this profile. Geypens *et al.* investigated the metabolite profile in faeces before and after a high-protein diet [20]. More recently, the potential of faecal VOCs patterns has been explored to diagnose gastrointestinal disease [21, 22]. Stool samples from patients with infectious diarrhoea, ulcerative colitis (UC), and *Clostridium difficile* and *Campylobacter jejuni* infections display characteristic metabolic fingerprints that allow diagnosis. In the present study, we characterized the impact of prebiotic supplementation on the faecal VOC profile. Our results confirmed the well-established observation that oligofructose and/or inulin increase SCFAs concentrations [23–27]. Besides, we found a significant increase in esters and a decreased generation of S-compounds.

At baseline, total acids, including linear-chain fatty acids and BCFAs, constituted a predominant class of VOCs. SCFAs are the principal fermentation products of non-digestible carbohydrates, whereas BCFAs are produced from the colonic degradation of amino acids. Other common acids included medium-chain fatty acids, such as heptanoic acid and octanoic acid. SCFAs form an important energy source for the colonocytes and influence colonic function by stimulating water and sodium absorption and modulating motility [28]. Especially, butyrate is considered as the major energy source for the colonic mucosa providing up to 70% of the energetic needs [11]. Addition of OF-IN clearly stimulated SCFA production in a time-dependent and dose-dependent way. Butyrate is produced through a butyryl-coenzyme A (CoA):acetate transferase pathway or the butyrate kinase pathway, the former being the most common pathway among butyrate-producing members of the colon microbiota [29–31]. Increased SCFAs synthesis also creates a more acidic environment in the gut [32], which is important *in vivo* in terms of colonization resistance against pathogens [33].

Surprisingly, changes in SCFAs concentrations were not the key features that distinguished negative control samples from faecal samples incubated with OF-IN. A more discriminatory factor for the prebiotic effect was a time- and dose-dependent increase in the relative concentration and number of esters. Until now, few reports of prebiotics have been associated with ester production. Recently,

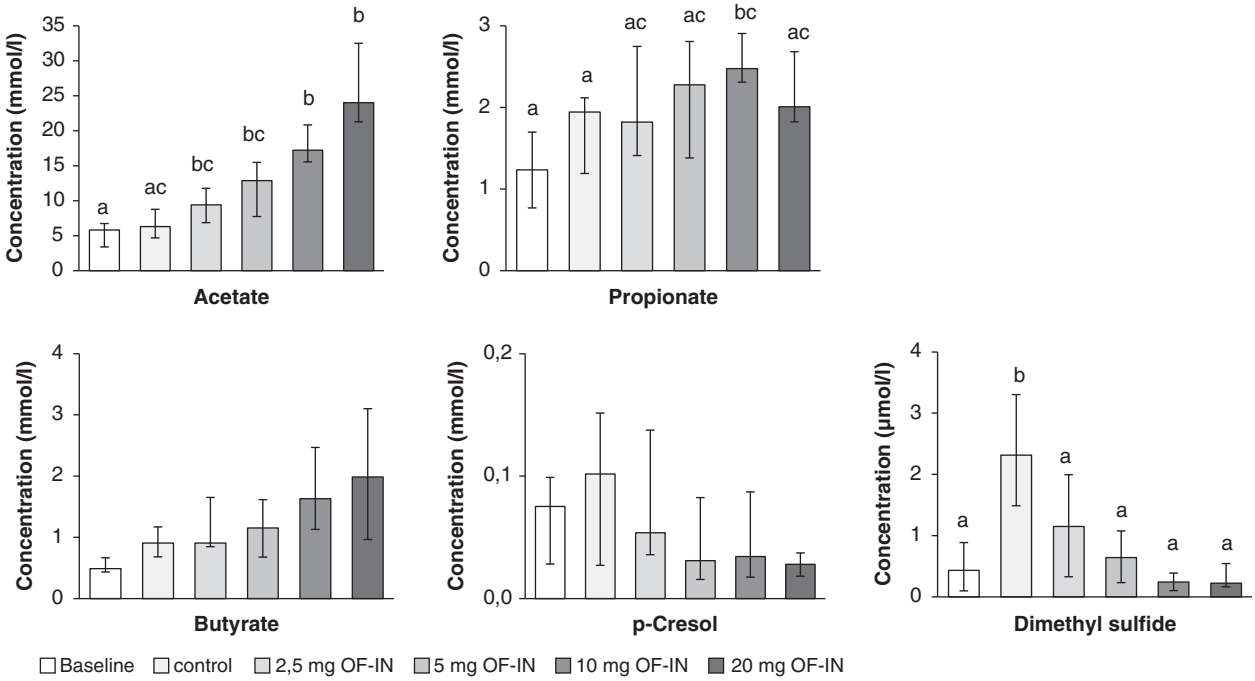

Figure 7. Absolute metabolite concentrations of VOCs at baseline and after incubation without and with different doses of OF-IN (2.5, 5, 10, and 20 mg) for 24 h (Friedman ANOVA $\alpha = 0.05$; median (25th to 75th percentile)). Different letters indicate significantly different results ($p < 0.05$).

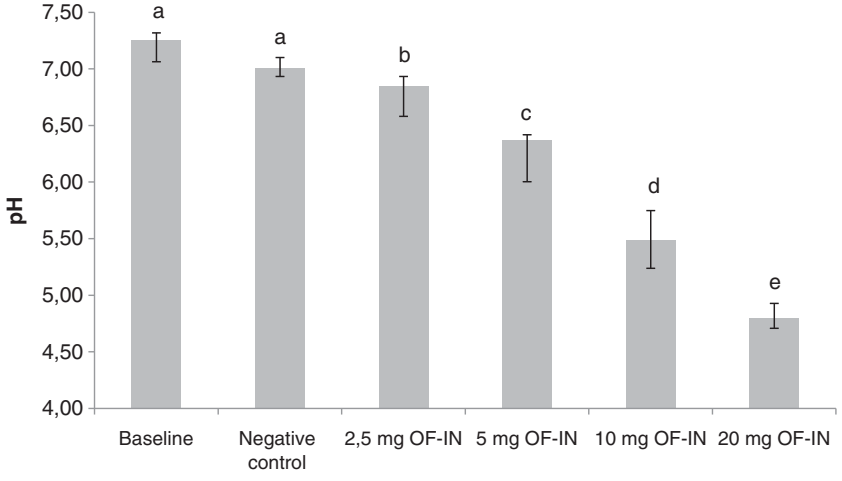

Figure 8. Effect of different doses of OF-IN on pH in faecal slurries before and after 24 h of incubation (Friedman ANOVA $\alpha = 0.05$; median (25th to 75th percentile)). Different letters indicate significantly different results ($p < 0.05$).

Garner *et al.* demonstrated bacterial synthesis of esters from acids using ^{13}C-labelled butyrate [21]. Hence, the higher presence and concentration is probably due to an increased acid production. At this moment, it is not known whether these esters are relevant to health.

S-containing compounds constituted another class of VOCs that allowed discrimination between faecal samples incubated with OF-IN and negative control samples. They originate from microbial degradation of dietary and mucinous sulphate and sulphur-containing amino acids, such as cysteine and methionine, by sulphate-reducing bacteria [34]. Sulphides have been shown to inhibit butyrate oxidation in rat colonocytes [35] and have been suggested to play a role in the aetiology and/or risk of relapse in UC [36, 37]. Data that would imply sulphide in colon carcinogenesis are scarce. However, Ramasamy *et al.* recently reported that thiosulphate sulphurtransferase, an enzyme with sulphide-detoxifying capacities in the colonic epithelium, is markedly reduced in colon cancer [38]. Methanethiol, dimethylsulphide, as well as dimethyl- and trimethyldisulphide are commonly found VOCs and have previously been identified in faecal samples [39]. In the present study, the decreased generation of S-compounds upon incubation with OF-IN is probably not due to an alteration of the microbiota

composition in the faecal slurries since incubations were performed for only 24 h. It is hypothesized that a reduction in pH reduced the bacterial proteolysis of S-amino acids, since proteases typically have a neutral to alkaline pH optimum [40]. Additionally, carbohydrate availability is also involved in the regulation of metabolic pathways. In the presence of carbohydrates, the transcription of genes involved in the metabolism of amino acids is repressed in certain bacteria and the corresponding genes are inactivated, a process called catabolite repression [41]. Other studies on the effects of prebiotics on faecal sulphides are scarce. Lewis *et al.* found a reduced hydrogen sulphide concentration after oligofructose supplementation [42]. In another study, Swanson *et al.* reported on the effect of fructooligosaccharides on volatile sulphur compounds, but could not quantify most of the sulphur compounds due to methodology limitations [43]. The effect of the prebiotic on sulphides may be relevant for the treatment of UC, in which elevated levels of hydrogen sulphide (and possibly other S-containing compounds) as well as higher counts of sulphate-reducing bacteria in faeces have been demonstrated [36, 37, 44].

Also, our results showed a consistent inhibition of OF-IN on the formation of other protein fermentation metabolites. Phenol and 3-methylindole, as well as BCFAs emerged as VOCs contributing to the discrimination of negative control samples and samples with 10 mg OF-IN. Phenol and 3-methylindole arise from the bacterial metabolism of aromatic amino acids tyrosine, and tryptophan, respectively, and have been studied as possible agents involved in colon and bladder cancers [45]. *p*-Cresol is another bacterial metabolite from tyrosine, which is relevant to health status, since it is considered as the prototype of protein-bound uremic toxins in chronic kidney disease. We have shown previously that lower serum *p*-cresol levels are correlated to a lower mortality in uremic syndrome [13] and that serum *p*-cresol levels can be decreased by administration of OF-IN to those patients [16]. Nevertheless, we did not observe a significantly reduced *p*-cresol generation after incubation with OF-IN. Possibly, this might be attributed to the relatively low concentrations of *p*-cresol found at baseline.

In conclusion, our results confirmed a dose-dependent stimulation of saccharolytic fermentation upon addition of OF-IN. Importantly, a significant decrease in toxic protein fermentation metabolites such as S-compounds attended these effects. Dietary intervention studies in healthy subjects and patients with pathologies such as UC should follow to confirm these effects *in vivo*.

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The authors have declared no conflict of interest.

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