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An *In Vitro* Approach Investigating the Gut Microbial Metabolite Differences after Using Probiotics and Prebiotics

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ABSTRACT

The human gut microbiota generate numerous metabolites via fermentation reactions that are known to play significant role in host homeostasis. The use of omics approaches to analyse gut microbiome has generated a lot of attention as a way of identifying metabolite biomarkers for treating and diagnosing gut diseases. The Nuclear Magnetic Resonance (NMR) metabolomic techniques can be used to investigate the influence of probiotics and prebiotics in gut metabolite generation and how the activity of certain microbes might be modulated. This study aimed to employ metabolomic techniques in faecal samples from five individuals at three different.

Keywords: Gut microbiota, *In vitro* gut models, Metabolomics, Multivariate statistical analysis, Prebiotic, Probiotic, Gut modulation

INTRODUCTION

The Gastrointestinal (GI) tract serves the niche for numerous microbial species interacting with each other, called symbiosis [1]. The gut micro biome is highly dynamic and versatile from birth till ageing. If this interaction loses its modulation, then disease is manifested. Gene sequencing technologies allow to investigate the identity and function of gut micro flora with the aim to maintain metabolic homeostasis. Gut micro biota play a crucial role in determining intestinal function and metabolism. They provide host nutrients and ferment certain products that are undigestible to humans including the conversion of dietary fibre to Short Chain Fatty Acids (SCFAs); leading to the activation of intestinal transporters such as the peroxisome proliferator receptor (PPAR-γ) and the Foresaid X Receptor (FXR) [2]. Numerous analytical techniques are used for understanding the microbial metabolic processes including DNA base methods of sequencing the 16S ribosomal RNA (rRNA) whereas metabolic products are measured in the stool, urine and serum using metabolomics [3]. Omits data reflect large amounts of butyrate, acetate and propionate in healthy individuals since they are the preferred microbial energy source [4]. Gut diseases including ulcerative colitis, irritable bowel syndrome and colorectal cancer have shown a reduction in butyrate and acetate production due to oxidation defects from microbial symbiosis [5]. Moreover, lactate which is not a metabolite of the healthy gut microbes appears in the NMR patient stool samples with colitis due to a decrease in coccids species [6]. The use of metabolomics allows non-invasive gut disease diagnosis which is clinically beneficial.

Diet can significantly alter gut microflora with probiotics and prebiotics being in the spotlight as intervention candidates for treating gut and chronic diseases. Probiotics are considered viable bacteria/yeasts that beneficially affect health, can survive the acidic gastric environment and are not fermented by the upper GI tract [7]. Although systematic reviews from Cochrane library have demonstrated beneficial effects in health, there are concerns regarding probiotic strain efficacy. There are methodological problems in the conducted clinical studies with some demonstrating that not all probiotic strains are beneficial for decreasing disease risk and there is individual variability in probiotic responses [8]. Prebiotics are ingredients selectively fermented by microbes already residing in the gut; resulting in changes in gut composition and metabolite release [9]. Prebiotics are classified according to their polymerization

as fructans-inulin oligosaccharides (FOS), xylo-oligosaccharides and galacto-oligosaccharides (GOS). Numerous studies [10] have demonstrated prebiotic benefits in blood lipid profiles, inflammatory status and a reduction in insulin resistance. Moreover, prebiotics regulate the lipogenic enzymes for propionate and butyrate SCFA production [11]. Furthermore, by-products of dietary fibre fermentation including lactate and succinate, make the intestinal pH more acidic preventing pathogen growth [12]. Clinical studies have shown that the low fibre Western diet reduces the production of SCFAs by commensal microbes and increases the release of harmful metabolites [13]. Current research is focused in the development of synergistic formulas containing prebiotics and probiotics due to their profound stimulatory effect [11].

In vitro fermentation models in contrast to in vivo studies aim to investigate the prebiotic and probiotic microbial therapeutic effects under controlled conditions; mimicking the different colonic areas and measuring the metabolites being produced [14]. The in vitro model approach is very cheap, and no ethical approval is needed. Currently, there are two in vitro models being used; the batch fermentation which is used in short experiments due to quick nutrient reduction and drop in pH and the continuous culture which is used in long experimental trials [15]. Main limitations of the batch fermentation include the short fermentation time, metabolite acquisition and pH decrease which alter microbial activities. Furthermore, the in vitro models lack the host response. Moreover, due to individual variability in terms of dietary patterns, ethnicity and age, the faecal samples once centrifuged, are pooled in different categories. Future perspectives to overcome these difficulties include the standardisation of the in vitro intestinal fermentation procedure that would allow a better comparison of the probiotic/prebiotic strain and its influence on gut microflora metabolic activity. Major aim of this study was to investigate the impact of prebiotic and probiotic dietary interventions in gut metabolites in human laboratory gut models using metabolomic techniques. In fulfilling the aim, omics data analysis was performed to the faecal fermentation data to investigate the gut metabolite differences in the probiotic and prebiotic intervention in the gut models. Further investigation is needed for clarifying the prebiotic and probiotic strains with additional sample testing in the long-term. Overall, omics techniques have shed some light for the significance of prebiotics and probiotics in gut health used for the prevention and treatment of gut and chronic diseases.

MATERIALS AND METHODS

Data collection

In this study, faecal samples from five different volunteers were used to prepare five in vitro gut models. Individuals either consumed a probiotic, prebiotic strain or nothing (control) and their faces were collected at three different time periods (0, 8 and 24 hours). Since the present study was based on data generated as part of an existing clinical intervention study, no further information was provided for the prebiotic/probiotic strain being used and of the anthropometric characteristics of the recruited individuals. Informed consent was obtained from all individuals in the beginning of the study.

Multivariate analysis

The faecal extract gut metabolites generated after using the Nuclear Magnetic Resonance (NMR) spectroscopy were analysed by using the SIMCA software package (Umetrics). The multivariate analysis workflow protocol is summarized in Table 1.

Table 1: Multivariate analysis workflow protocol of the faecal extract gut metabolites

	Stepwise work flow
STEP 1	The 43 faecal extract data were imported and the principal component analysis (PCA) model was performed to observe the pattern of the generated NMR data set and possibly look for any outlier values. This was the unsupervised analysis. The PCA analysis allowed to analyse the microbial metabolites from each intervention group by generating the score plot. At this point, the score and the model residual (DModX) plot were reviewed for possible outliers. The cross-val idation graph allowed to measure the predictive ability (Q2) as well as the actual variation in the data (R2). To identify the impact of time on the metabolites produced, a new PCA model was generated excluding the control data. This allowed to spot the time influence on the metabolite group differences.

STEP 2	In addition to the PCA analysis, the orthogonal projection to latent structure discriminant analysis (OPLS-DA) model was used to reveal the metabol ic differences between groups. By using the OPLS -DA a supervision (filter) was placed in studying two groups at a time. Three supervised gut models (prebiotic/control, probiotic/control and prebiotic/probiotic) were prepared by selecting the class differences and then OPLS-DA from the work set tab option.
STEP 3	For each of the three supervised gut models generated above, the OPLS-DA S-line plot was generated (including the time periods 0, 8 and 24 hours) in order to observe the differences in the chemical shifts (ppm) of the gut metabolites generated by NMR. This step was replicated three times thus ending up with the OPLS-DA score plot and S-line for the prebiotics/control , probiotics/control and prebiotics/probiotics groups. The S-line line plot allowed to point out the intensity and the co-variance of the gut metabol ites in each group. Each peak indicated the abundance of this metabolite in one group of the gut model group while the colour reflected the difference of this metabolite within that group. Ifthe colour was red, this indicated that there were differences within the group while green corresponded to no differences in the metabolites. In order to compare whether there are any differences in the S-line plots in the three generated model groups, another three OPLS-DA S-line plots were prepared by excluding the time period 8 hours. Further zooming in the peak metabolites was performed in each generated S-line in order to measure their appearance (singlet, duplet, triplet or multiple) and their chemical shifts (ppm). This allowed to identify from literature the gut metabolite being produced.
STEP 4	A summary table of all the gut metabolites was prepared with their associated chemical shifts (ppm) in order to spot the gut metabolite differences between the three studied groups.

RESULTS

Data analysis

The Principal Component Analysis (PCA) model was selected in the SIMCA software to import the data. The PCA gut model plot (Figure 1A) allowed to predict how close the faecal extract data were for each individual at each intervention group and to spot the influence of time (0, 8 and 24 hours) in the data pattern. Each dot represented one variable and there was variation within and across individuals. For instance, the dot point with 1_Pro_T0 corresponded to the fermentation extract sample from a gut model having faeces from the volunteer 1 taking the probiotic intervention at 0 hours. Generally, the data of individuals consuming the prebiotic intervention after 24 hours showed a shift to the right of the plot compared to those consuming the same product at 0 hours. Those taking the prebiotic at 8 hours appeared to be placed in the bottom horizontal line plot indicating the intermediate metabolite time period. This effect also appeared for the probiotic, but the shift was rather small. No big shift difference for the control group was observed except for the data from individual 3 being placed on the left area plot. To further examine whether the time factor resulted in metabolite differences between the intervention groups, a PCA model was prepared (Figure 1B) with data from the control group being excluded.

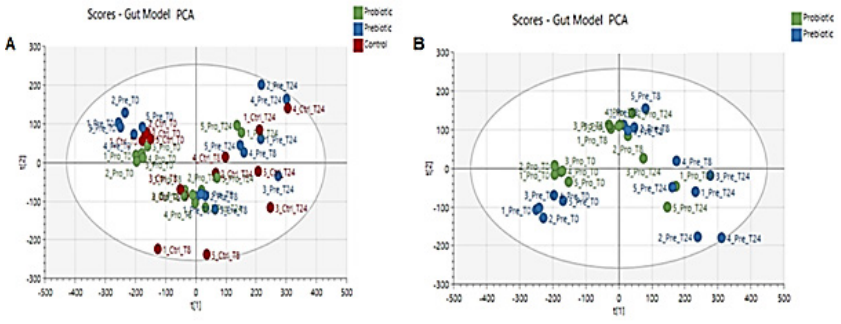

Figure 1: Unsupervised Principals Component Analysis (PCA) score plots of fermentation extract samples from the human gut models including

A: faces from 5 individual taking either the probiotic, prebiotic or no intervention- control o.8 ad 24 hours

B: faces from individuals taking only the probiotic prebloticat0.8and 24 hours R1=0.665, 0'=0.503,N=4l

C'l1_Pro_ fermentation extract sample from a gut model having faces from volunteer 1taking the probiotic intervention hours

It is evident that for the prebiotic and probiotic groups the time period 8 hours did not result in significant changes in gut metabolites since the data were clustered on the right side of the plot compared to the 0 and 24 hours where data

mainly positioned on the left and the right respectively. Generally, at time 0 hours as predicted there was not a big increase in the gut metabolite production (left shift on Figure 1). The time 8 hours was regarded the intermediate gut metabolite step with no major differences between groups and at time 24 hours there was an increase in gut metabolites particularly in the prebiotic group (right shift on Figure 1). It should be mentioned that the PCA gut model's predictive ability (Q2) was slightly increased from individual 1-3 and then remained constant whereas the real model's ability (R2) was cumulatively increased till individual 5. The values for both R2=0.665 and Q2=0.503 (reflected in the PCA residual DModX plot) were slightly different and not as high as expected which might relate to the small data sample used and some variation present with respect to the time periods being studied.

To further determine the actual differences between the intervention groups, the OPLS-DA supervised model was applied to uncover the gut metabolite differences for each gut model. The OPLS-DA models were prepared with one orthogonal and one PLS component for each gut model. Both the score plot and the OPLS-DA S- line plots were selected with the latter reflecting the range of endogenous metabolites generated in the NMR spectra with their peak size and color mode. Figure 2 reflects the OPLS-DA and the corresponding S-line plot when supervised for the prebiotic/control group. The OPLS-DA plot separated the samples with individuals' faces consumed the prebiotic on the right plot side and the control samples on the left plot side. The horizontal separation reflected less variation in the control data group compared to the prebiotic group with its points being further dispersed. The S-line plot showed that the prebiotic group generated ethanol, formate, butyrate, propionate and acetate metabolites compared to the control. The green color code reflected no significant changes in the gut metabolites within the probiotic group at the three different time periods.

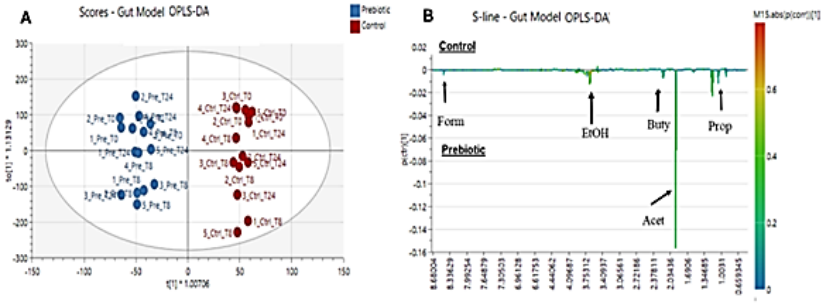

Figure 2: Orthogonal Projections to Latent Structures Discriminant analysis (OPLS-DA) model and the corresponding S-llne plot for the fermentation extract sample from the human gut models assigned to tile prebiotic and control groups at o. 8 and 24 hours

A: OPLS-DA score plot or the prebiotic and control groups shown as a two-way separallon of the fascal samples

B : OPLS-DA $- in plot reconnecting differences In the NMR spectra or the gut metabolites for the prebiotic and controling models. The downside peaks reconect the difference In gut metabolites between the two groups whereas the color or the line peaks Indicates the abundance of those metabolites In the particular group. Acet: Acetate ;Buty: Butyrate; EIOH: Ethanol; Form: formate, Prop: Propionate. R2=0.602.Q2=-0.567,N=29.

(•) 1_Pre_ TO: fermentation extract sample from a gut model having\ faces from volunteer 1 taking the prebiotic intervention at 0 hours

Further attempt was made to examine any association between the three OPLS-DA generated models in relation to time. For this, the OPLS-DA S-line plots were prepared for each case excluding the time period 8 hours. Figure 3 shows the OPLS-DA S-line plots when supervised for the probiotic/ control groups for the three time periods studied (Figure 3A) and when the time period 8 hours was excluded (Figure 3B). The probiotic group generated ethanol, butyrate, propionate and acetate metabolites compared to the control. The red color of those metabolites in the probiotic group in Figure 3A compared to the (Figure 3B) indicated the difference in gut microbes at the time period 8 hours. Also, formate and lactate metabolite products were only generated in the control group when the time period of 8 hours was excluded (Figure 4).

The OPLS-DA S-line plots were shown for all the three time periods studied (Figure 5A) and when the time period of 8 hours was excluded (5B). The prebiotic group generated ethanol, butyrate, propionate and acetate metabolites compared to the probiotic one. The green color in the prebiotic gut metabolites indicated no major differences within the prebiotic group across 24 hours. However, in the probiotic group lactate was produced but not when the time

period of 8 hours was excluded, which might relate to the microbes not fermenting this metabolite at that period.

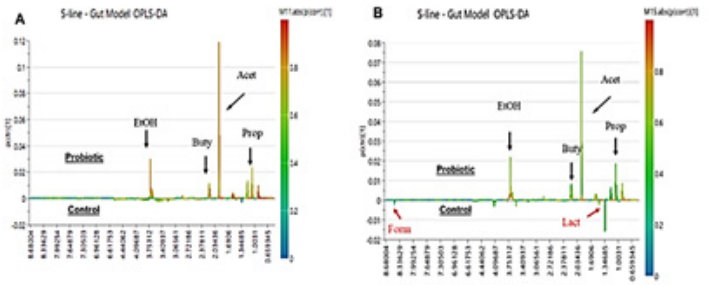

Figure 3: OOPLS-DA S-line plot reflecting differences in the NMR spectra of the gut metabolites for the probiotic (top) and control (bottom) gut models OPLS-OAS-in plots

A:NMR metabolic data of the probiotic and control groups generated at 0,8 and 24 hour$,

B:NMR metabiolic data of the two above groups excluding the time period or 8 hours. The downside or upside peaks reflect the difference in gut metabolic between the two groups whereas the color or the tine peaks Indicates the abundance or !hose metabolites In the particular group. Acer Acetate : Buty: Butyrate; EIOH• Ethanol; Form: Formate; Laci: Lactate; Prop: Propionate. R2=0 335,02=0.415,N= 28

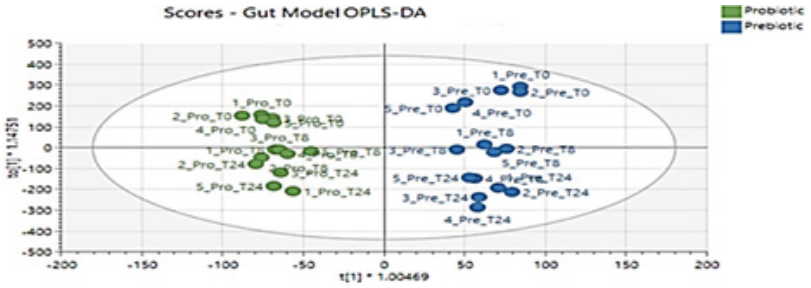

Figure 4: Shows the OPLS-DA S-score plot separating the samples with individuals' faces consumed the prebiotic on the right side of the plot and probiotic samples on the left side of the plot

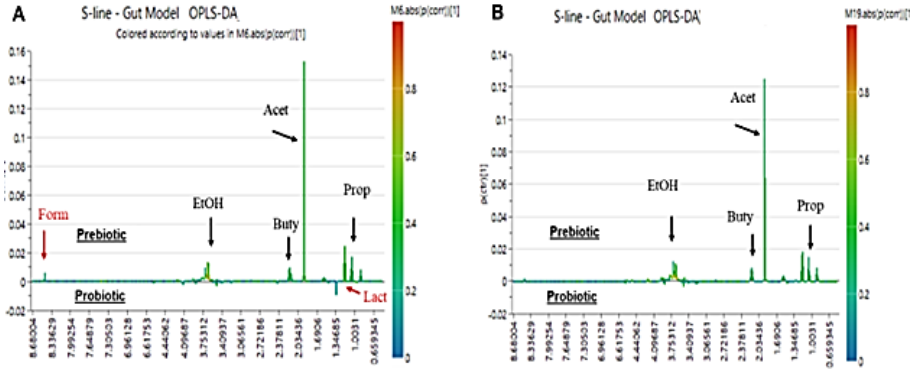

Figure 5: OPLS-DA S-line plots for the fermentation extract samples from the human gut models assigned to the probiotic (bottom) and prebiotic (lop) groups with

A: NMR metabolite data or 1he probiotic and prebiotic groups generated at 0,8 and 24 hours.

B: NMR metabolic data or the two above group excluding the time period of 8houf.The downside or upside peaks reflect 1he difference In gut metabiotics between the two groups whereas the Color or the line peaks indicates abundance or Choose metabolites in the particular group. Acet Acetate; Buty. &Butyrate, EIOH. Ethanol, Form. Formate; Lact: Lactate; Prop: Ploplonafe.R'>0 594.0'>0.665.N• 28

DISCUSSION

In this study, the prebiotic and probiotic dietary interventions were investigated using in vitro gut models to assess their impact in gut metabolite generation from the faeces of five volunteers at three different time periods (0, 8 and 24 hours) using NMR spectroscopy. Unsupervised and supervised principal component multivariate analyses (PSA and OPLS-DA) were performed to generate the gut models and S-line plots. Although the results of gut metabolites (butyrate, acetate, ethanol and propionate) when comparing both prebiotics and probiotics with the control (no intervention) group did not have any differences, there was a significant increase in those metabolites in the prebiotic group when compared with the probiotic one after 24 hours. This can be explained by the prebiotic's role to increase carbohydrate metabolism and thus the activity of the gut microbes to ferment carbohydrate and fibre by-products. Also, a decrease in lactate and format was present except the probiotic group. The time period did not seem to have a major influence in gut metabolites except when the time period 8 hours was excluded resulting in a fluctuation in formate and lactate metabolites as shown from the size and colour peaks.

The use of in vitro batch cultures provided a fast and inexpensive way to test gut metabolite quantities after dietary intervention. However, they did not fully recapitulate the *in vivo* effects due to the lack of host response which might relate to the standardized nature of the model in terms of nutrient, oxygen availability and fixed pH values. Such limitation might be overcome by the design of more humanised in vitro models and preferring continuous rather than batch cultures that will allow long-term experimentation; better reflecting the probiotic and prebiotic effects on gut microflora [15]. Although there was a slight difference between the predictive and actual PCA model values (R2=0.665 and Q2=0.503 respectively) from the PCA residual DModX plot, it would be wise to choose a much higher cohort sample size with 15-20 individuals and run the study longitudinally (more than 24 hours). The small variation in the pattern might relate to inter-individual differences in the gut generated metabolites due to age, genetic make-up, diet, disease and sociocultural context [16]. Moreover, by pooling donor samples and then stratify them according to disease and healthy would allow a better comparison of the dietary interventions being studied.

The use of OPLS-DA to distinguish the intervention groups was very beneficial in evaluating the faecal extract metabolome. Specifically, the S-line plots generated for each group reflected the gut metabolite variances. In both probiotics and prebiotics when compared with the control group, an increase in ethanol, acetate, butyrate and propionate was present as shown in Table 2 which is Short Chain Fatty Acids (SCFAs) produced as energy gut metabolites. However, these products were only present in the prebiotic when compared with the probiotic group, indicating the efficacy of prebiotics to increase the microbial activity for generating SCFAs in carbohydrate metabolism. This is expectable since prebiotics increase the activity of gut microbes already residing in the gut rather than introducing new species that probiotics do. Similar metabolomic studies have shown that prebiotic supplementation to humanised mouse models caused an increase in methylamines and SCFAs and a shift to Proteobacteria rather Firmicutes and Bacteroides phyla [17]. The significance of prebiotics' role to increase the activity of the SCFA producing gut microbes is further explored in the development of faecal transplants for treating diseases arising from gut dysbiosis [18].

Table 2: Summarizes the gut metabolite changes and their respective NMR chemical shifts (ppm) generated from the OPLS-DA line plots shown above. Ethanol, butyrate, acetate and propionate were produced both by the probiotic and prebiotic groups when compared with the control with minimal differences across the three time periods. However, lactate and formate were specifically produced at certain time periods (excluding time period of 8 hours). In comparison of the metabolites produced in the prebiotic/ probiotic groups, the prebiotic group was far more efficient in generating the above metabolites

Group	**Prebiotie/Control (R2•0.602, Q2•0.567,N•29)**		**Probiotie/Control(R2•0.415, 02•0.335,N•28)**		**PrebiotictProbiotic (R2•0.665, Q2•0.594,N•28)**	
Metabotites	Change	Chemicalstin (ppm)	Change	Chemicalsrin (ppm)	Change	Chemical stin (ppm)
Acetate	↑	1.91	↑	1.96	↑	1.91
Butyrate	↑	2.17	↑	2.16	↑	2.15
Camitine				-	-	
Ethanol	↑	3.65	↑	3.65	↑	3.71
formate	-	-	!(')	8.45	!(")	8.4
Lactate	-	-	!	1.34	!(..)	1.32
Propionate	↑	1.06	↓	1.03	t	1.06
Succinate	-	-	-	-	-	-
Tlimetllylamine	-	-	-	-	-	-

I: Increase or decrease in gut metabolices compared to the controlgroup(first two blocks) or compared to the probiotic (last block).
(*): Present in the OPLS-DAS –line plot generated without time period of 8 hours.
(**): Not present in the OPLS-DAS-line generated without time period of 8 hours.

Another possible explanation for probiotics not showing those metabolites when compared with prebiotics might relate to the limited time period studied (0-24 hours) that could not cause an increase in the activity of the SCFAs gut microbes. An interesting point is the production of lactate and formate when the time period 8 hours was excluded due to microbes using these as energy source. Furthermore, citrate which is a by-product of the glycolysis pathway, certain amino acids (alanine, glutamate) and phenol compounds (known to be carcinogenic) were not detected in this study. This reflects the benefit of dietary intervention since many studies have detected increased levels of alanine and phenols in patients' stool samples with irritable bowel syndrome and colorectal cancer. Those patients had increased activity of Lactobacilli and Clostridia [17]. Metabolomic studies using mouse models with acute colitis had shown an increase in butyrate and trimethylamine after probiotic consumption; leading to gut modulation [19]. Future research might include the use of faecal samples from people with gut diseases in exploring the potential of the prebiotic and probiotic interventions in minimising or curing gut diseases. Finally, metabolomic data obtained in this report might be compared with 16S rRNA and sequence metagenomics analysis that will better reflect the significance of dietary intervention in gut health [15]. Improvements in the quantitative analysis techniques will not only reinforce gut models' predictive ability but will better reflect the gut metabolite biosynthesis pathways.

CONCLUSION

This study used multivariate statistical analysis to investigate the differences of probiotic and prebiotic dietary interventions in gut metabolites by using in vitro human gut models. Although the study showed an increase in SCFAs metabolites for prebiotics after 24 hours, further experimentation is needed to overcome the associated challenges. Clarification in study parameters and the use of additional metabolomic and metagenomic techniques will better clarify the probiotic/prebiotic influence in gut microflora. Overall, the significant knowledge obtained from the omics' approaches used in the present study, can be effectively employed in the medical field to explore the microbial metabolic and molecular mechanisms involved in gut disease pathogenesis in order to design effective treatment strategies.

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