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Metabolic Footprinting of Fermented Milk Consumption in Serum of Healthy Men

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Abstract

Background: Fermentation is a widely used method of natural food preservation that has consequences on the nutritional value of the transformed food. Fermented dairy products are increasingly investigated in view of their ability to exert health benefits beyond their nutritional qualities.

Objective: To explore the mechanisms underpinning the health benefits of fermented dairy intake, the present study followed the effects of milk fermentation, from changes in the product metabolome to consequences on the human serum metabolome after its ingestion.

Methods: A randomized crossover study design was conducted in 14 healthy men [mean age: 24.6 y; mean body mass index (in kg/m^2): 21.8]. At the beginning of each test phase, serum samples were taken 6 h postprandially after the ingestion of 800 g of a nonfermented milk or a probiotic yogurt. During the 2-wk test phases, subjects consumed 400 g of the assigned test product daily (200 g, 2 times/d). Serum samples were taken from fasting participants at the end of each test phase. The serum metabolome was assessed through the use of LC-MS–based untargeted metabolomics.

Results: Postprandial serum metabolomes after milk or yogurt intake could be differentiated [orthogonal projections to latent structures discriminant analysis (OPLS-DA) Q2 = 0.74]. Yogurt intake was characterized by higher concentrations of 7 free amino acids (including proline, $P = 0.03$), reduced concentrations of 5 bile acids (including glycocholic acid, $P = 0.04$), and modulation of 4 indole derivative compounds (including indole lactic acid, $P = 0.01$). Fasting serum samples after 2 wk of daily intake of milk or yogurt could also be differentiated based on their metabolic profiles (OPLS-DA Q2 = 0.56) and were discussed in light of the postprandial results.

Conclusions: Metabolic pathways related to amino acids, indole derivatives, and bile acids were modulated in healthy men by the intake of yogurt. Further investigation to explore novel health effects of fermented dairy products is warranted. This trial was registered at clinicaltrials.gov as NCT02230345. *J Nutr* 2018;148:1–10.

Keywords: metabolomics, dairy, yogurt, milk, postprandial, indole derivatives, bile acids, fermentation, gluconic acid, healthy men

Introduction

Fermentation of milk was first used by cattle herders as a way to extend storage life, facilitate transportability, and, by reducing lactose content, improve digestibility (1). Today, ~20–40 kg of fermented dairy products are consumed per person each year in Western countries, of which ~40% is represented by yogurt (2, 3). During fermentation, the proteolytic and lipolytic activities of lactic acid bacteria (LAB), as well as the conversion of milk carbohydrates to alcohols, carbon dioxide, and organic acids, cause major changes in the product's final composition and organoleptic properties. Consequently, these foods have been extensively evaluated for their role in health. In particular, the beneficial effects of yogurt have been investigated for more than a century (4) and include immune function modulation, lowering of circulating cholesterol, and improvements in a range of gastrointestinal conditions such as lactose intolerance, constipation, diarrheal diseases, colon cancer, inflammatory bowel disease, *Helicobacter pylori* infection, and allergies (5–7). However, the mechanisms underpinning these potential properties are not yet well-established, although many compounds that result from the bioactivity of LAB have been associated with the reported health benefits of yogurt, such as conjugated linoleic acid (8), sphingolipids (9), or bioactive peptides (10, 11). Moreover, the health qualities of yogurt may rely not only on the production of bioactive metabolites during milk fermentation, but also on both the activity of the LAB in the gut and the modulation of the host intestinal microbiota (12).

The use of untargeted metabolomics in dietary interventions is a promising approach as the method can give a snapshot

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of the metabolic activity of a subject at a specific time point after the ingestion of a test food (13–15). However, to date, few nutritional intervention studies investigating fermented dairy intake have used metabolomics (16–21). The primary aim of the present study is to evaluate the metabolic response of healthy men to the ingestion of a fermented dairy product with the use of an untargeted metabolomics approach. The impact of fermentation on the milk metabolome is investigated, as is that of fermented milk ingestion on the serum metabolome, both postprandially and fasting after short-term daily intake.

Methods

Subjects. The subjects were healthy adult men (n = 14) aged 24.6 ± 4.7 y (mean ± SD) and with a BMI (in kg/m^2) of 21.8 ± 1.8 (mean ± SD) (**Supplemental Table 1**). None of the subjects had evidence of intolerance or adverse reactions to dairy products. Details regarding exclusion criteria and the inclusion visit are given by Burton et al. (22). One subject was excluded from all analyses due to suspected noncompliance with dietary instructions that was detected during microbiota analysis (22). Another subject withdrew before completing the final test day due to acute illness (**Supplemental Figure 1**). Before entering the study, all subjects provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki as revised in 1983 and received ethical approval from the Commission Cantonale d'Ethique de la Recherche sur l'Etre Humain (CER-VD, approval number 392/13, Vaud, Switzerland). The study was registered at clinicaltrials.gov as NCT02230345.

Test products. The fermented test product was a yogurt produced by fermentation of milk with classical yogurt starter cultures and the widely used probiotic *Lactobacillus rhamnosus* Gorbach-Goldin (LGG) (23). Starter cultures consisted of a mix of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* and were obtained from Chr. Hansen A/S, Denmark (Thermophilic Yoflex). The probiotic LGG was obtained from the Culture Collection of the University of Goteborg, Sweden (CCUG 34,291). The nonfermented dairy control was a chemically acidified milk obtained by the addition of 2% glucono-δ-lactone (GDL, ≥99.0%, Jungbunzlauer AG, Switzerland). The addition of 2% GDL to milk mimics the slow pH reduction occurring during fermentation and allows the final product to have a color, texture, and pH similar to a mild semiliquid yogurt. All the milk used in the study was provided by Emmi (Mittelland Molkerei AG, Switzerland) from a single production batch. Details of product preparation, nutritional composition, and biochemical analyses are given in the **Supplemental Methods** and **Supplemental Table 2**.

Study design. The intervention study used a randomized, double-blind, and crossover design (**Figure 1**). The postprandial response to the acute intake of milk or yogurt was assessed at the beginning of each intervention phase. During this test day, participants ingested a single 800-g dose of milk or yogurt within 15 min and serum was sampled postprandially up to 6 h (fasting, then 15, 30, 60, 90, 120, 180, 240, 300, and 360 min). The dose of 800 g, although uncommon in normal dietary situations, was chosen to amplify the postprandial effect of dairy intake, thereby facilitating the identification of the metabolites and metabolic pathways that are most likely to change after normal chronic intake. Such an acute approach has been previously used to characterize postprandial lipidemia after dairy intake (24) or to identify postprandial biomarkers of dairy intake (25). During the following 2 wk, subjects were instructed to consume 400 g of the assigned test product daily (200 g morning and evening) and fasting serum was taken at the end of the 2-wk period to evaluate the effect of short-term intake on the fasting serum metabolome. During the 3-wk run-in and 2-wk washout periods, participants consumed 400 g of nonacidified (normal) milk per day (200 g morning and evening). Dietary intake was semicontrolled during all phases of the study with specific guidance given on portions of fermented foods, alcohol intake, and coffee intake. Participants were instructed to exclude all dairy products from their diet except those provided. Before each test day, participants followed a 3-d controlled diet by only consuming food provided by the investigators (22).

Untargeted metabolomics. Serum and products samples were kept at −80°C before being analyzed with the use of the same protocol. To limit ion-suppression, phospholipids were removed from the samples by the Phree filter (Phenomenex Inc., Torrance, CA). An ultra-high-pressure LC system, coupled to a quadrupole time-of-flight mass spectrometer, was applied for untargeted metabolomics analysis (UltiMate 3000, Thermo Fisher Scientific, Waltham, MA, coupled to a maXis 4G+, Bruker Daltonik GmbH, Bremen, Germany). The mass spectrometer electrospray interface was operating in positive ion mode and spectra were recorded from m/z 75 to m/z 1500. In light of the large number of samples to be measured and the better coverage of the metabolome in the positive mode (26), positive mode was favored over negative mode. Details regarding sample preparation and LC-MS protocol are given in the Supplemental Methods. Mass-spectrometer signal drift was assessed with the regular injection of a quality control sample (QC) consisting of either a pool of all serum samples or all products samples. Contaminants were accounted for by the injection of a blank (ultrafiltered water). For metabolite identification, the Human Metabolome Database (27), the MassBank of North America (28), the National Institute of Standards and Technology database (NIST v14), and Metlin (29) were used with a 5 ppm accuracy threshold. Identities of selected metabolites were confirmed by performing collision-induced dissociation (5–70 eV collision energies) and/or with the injection of standards. Standards were either purchased separately or obtained from the Mass Spectrometry Metabolite Library kit (MSMLS, IROA Technologies, LLC, Bolton, MA; Gainesville, FL). If absent from the MSMLS kit, bile acid (BA) identities were confirmed with the injection of a bile salt extract solution. All solvent, reagents, and standards were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). As the analysis is semiquantitative, the concentrations mentioned in the text refer to relative concentrations, determined from the metabolites' peak area (arbitrary unit).

Data processing and statistical analysis. Progenesis QI (v.2.3.6198.24128, NonLinear Dynamics Ltd., Newcastle upon Tyne, United Kingdom) was used for retention time correction, peak-picking, deconvolution, and normalization with default settings (default automatic sensitivity and without minimum peak width). Signal drift correction was performed with R (v.3.1.2; R Foundation for Statistical Computing, Vienna, Austria) via the QC-based robust locally estimated scatterplot smoothing signal correction method (30). Metabolites with poor repeatability, i.e., detected in <50% of QCs and with a relative standard deviation (RSD) >30% in the QC samples, were removed. Also, features that had a median in the QC samples that was <3 times higher than the median calculated for the blanks were excluded. For each metabolite, nonparametric analysis of longitudinal data (nparLD) was performed to test the hypothesis that a time effect exists postprandially (nparLD R package, 0.05 as P significance cutoff) (31).

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Supplemental Methods, Supplemental Tables 1–3, and Supplemental Figures 1–5 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/jn/.
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Abbreviations used: AhR, aryl hydrocarbon receptor; BA, bile acid; BSH, bile salt hydrolase; GDL, glucono-δ-lactone; IAA, indole-3-acetic acid; IAAld, indole-3-acetaldehyde; ILA, indole-3-lactic acid; IPA, 3-indole propionic acid; LAB, lactic acid bacteria; LGG, *Lactobacillus rhamnosus* Gorbach-Goldin; nparLD, nonparametric analysis of longitudinal data; OPLS-DA, orthogonal projections to latent structures discriminant analysis; QC, quality control sample; Q2, predictive ability parameter; R2Y, goodness-of-fit parameter; VIP, variable importance in projection; VIP_{tot}_CI, 95% confidence interval for VIP_{tot}.

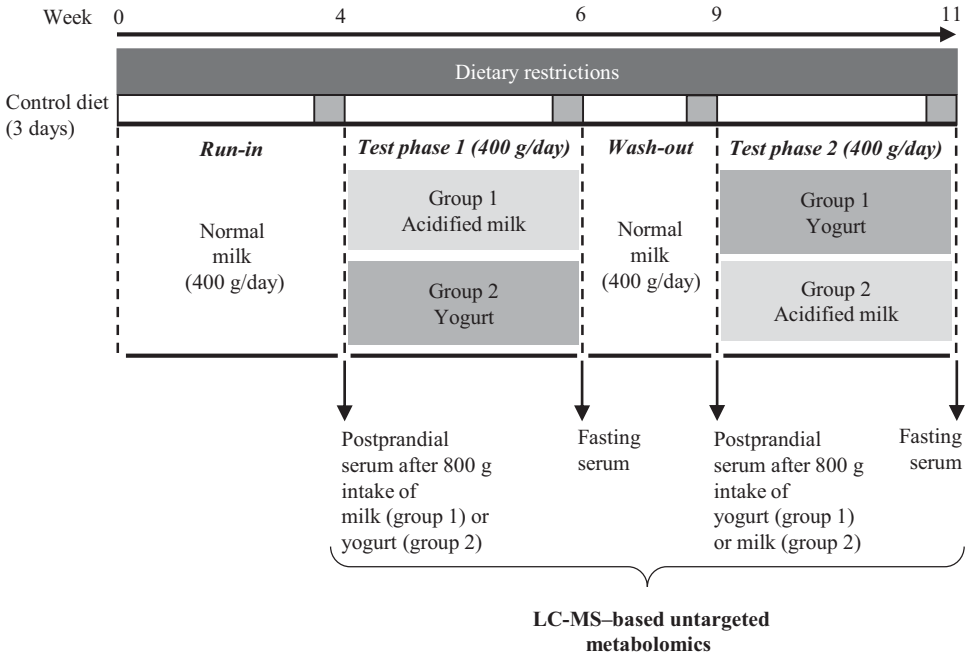

FIGURE 1 Overview of study design. Participants were assigned randomly to group 1 or group 2 in a crossover design to test the acute and short-term intake of acidified milk and yogurt. At the beginning of each test phase, serum samples were taken 6 h postprandially after the ingestion of 800 g of a nonfermented milk or a probiotic yogurt. During the 2-wk test phases, subjects consumed 400 g of the assigned test product daily (200 g, 2 times/d). Serum samples were taken from fasting participants at the end of each test phase. A washout period separated each test phase and a run-in preceded the beginning of the study. Subjects consumed 400 g of regular milk/d during the run-in and washout periods.

A hierarchical clustering analysis was then conducted on this final dataset to group metabolites based on their postprandial kinetics (amap and dendextend R packages, clustering by Spearman's distance measure and Ward linkage). Five clusters were chosen based on the visual differences in the postprandial responses.

Orthogonal projections to latent structures discriminant analysis (OPLS-DA, SIMCA-P software v.14.0, Umetrics, Umeå, Sweden) was conducted to differentiate milk from yogurt, or serum after milk intake from serum after yogurt intake. The dataset was scaled through the use of the unit variance method to make all metabolites equally important and to limit the over-influence of metabolites with manyfold changes. The incremental AUC was used for postprandial data (MESS R package). Quality of the models was evaluated by the goodness-of-fit parameter (R2Y) and the predictive ability parameter (Q2, calculated by 12-fold cross-validation). Subsequently, to rule out any random separation of the sample groups, permutation tests with 999 random permutations were carried out (32). Finally, the most discriminatory metabolites were selected based on variable importance in projection (VIP) scores. Both VIP_{tot} (VIP scores for the predictive and orthogonal components) and VIP_{pred} (VIP scores for the predictive component) were calculated (33). $VIP_{tot} > 1$ was used as the minimum threshold value for variable selection. VIP_{pred}, VIP_{tot}, as well as the Jack-Knife 95% confidence intervals (VIP_{tot}_CI) are indicated in **Supplemental Table 3**. In addition to the multivariate approach, a univariate analysis was performed, as the 2 methods have been shown to be complementary (34). A paired Wilcoxon's signed rank test was conducted on each metabolite to compare the products, the postprandial serum, and the fasting serum. Multiple comparisons were adjusted by Benjamini Hochberg's correction ($P = 0.05$ as the threshold for significance). The Kolmogorov-Smirnov test was used to compare the mass distributions of discriminating metabolites (R 3.1.2).

Results

A total of 2302 unique metabolites were detected in the test products or serum and used for further statistical analysis. Figure 2 summarizes the parts of the metabolome that are shared between the different types of sample.

Products metabolome. A higher number of metabolites was measured in yogurt compared with milk, in line with the expected release of new metabolites during fermentation. Sixteen percent of the milk metabolome (73 metabolites) was no longer detected in yogurt, whereas 24% of the yogurt metabolome was not present in milk (122 new metabolites). Nevertheless, 395 metabolites remained present in both products, representing 84% and 76% of the milk and yogurt metabolome, respectively. The multivariate analysis confirmed the clear difference

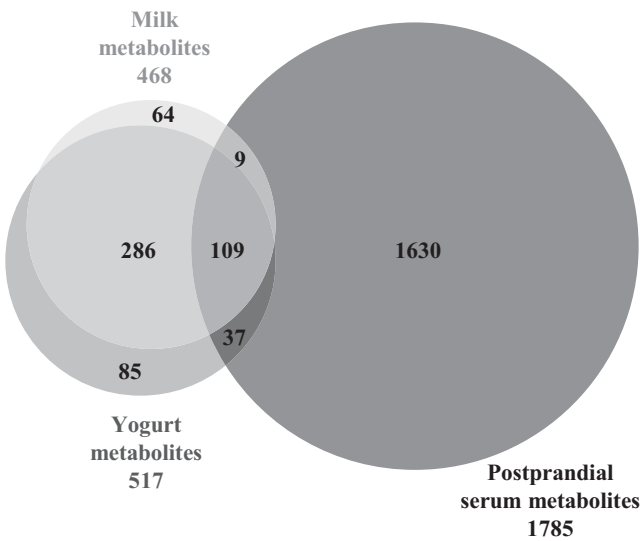

FIGURE 2 Shared metabolites between milk, yogurt, and postprandial serum of healthy men, assessed by LC-MS–based metabolomics.

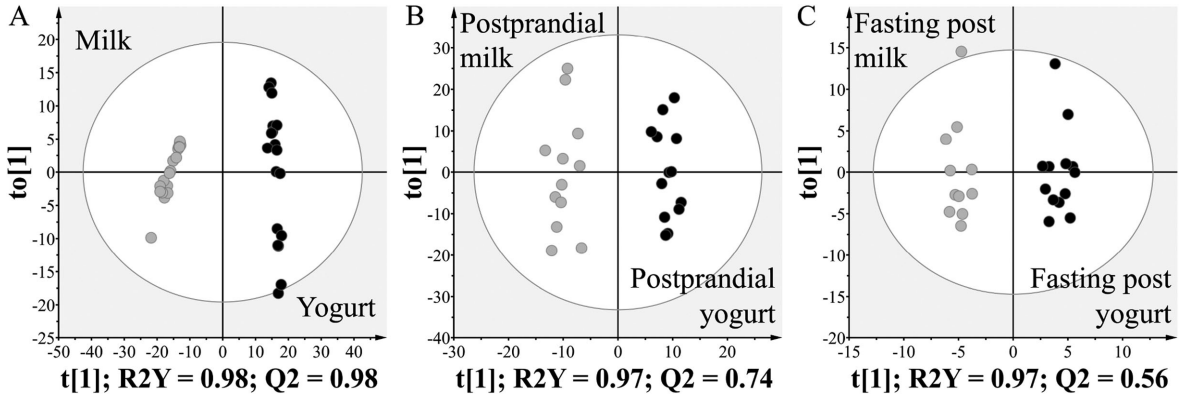

FIGURE 3 Differentiation of dairy products and serum samples of healthy men after milk or yogurt intake through the use of untargeted LC-MS metabolomics. OPLS-DA scores plot derived from (A) milk and yogurt samples, (B) postprandial serum after milk and yogurt intake (800 g), and (C) fasting serum after 2 wk of daily intake (400 g/d) of milk or yogurt. OPLS-DA, orthogonal projections to latent structures discriminant analysis; Q2, predictive ability parameter; R2Y, goodness-of-fit parameter; t[1], OPLS-DA predictive component; to[1], OPLS-DA orthogonal component.

between the 2 product metabolomes (Figure 3A). The OPLS-DA model showed goodness-of-fit and high prediction ability (R2Y = 0.98, Q2 = 0.98), both indicators being higher than the corresponding values from the permutation test (**Supplemental Figure 2**). Among the 590 metabolites measured in milk and/or yogurt, 432 (73%) could discriminate milk from yogurt ($VIP_{tot} > 1$ or $P < 0.05$). Comparison of the mass distribution of the discriminatory metabolites (*m/z*, Figure 4A) revealed that metabolites with higher concentrations in milk (i.e., decreased during fermentation) had a significantly lower mass distribution compared with metabolites with higher concentrations in yogurt (i.e., produced during fermentation) (one-sided Kolmogorov-Smirnov test, $P = 5.46 \times 10^{-3}$).

Postprandial serum metabolome: general trends. A total of 1785 metabolites were detected in serum. The nparLD analysis showed that approximately half of the serum metabolome (51%, 906 metabolites) showed a dynamic response to the acute intake of milk and/or yogurt, 29% (524 metabolites) after the intake of milk and yogurt, 13% (231 metabolites) after the intake of milk only, and 9% (151 metabolites) after the intake of yogurt only. The remaining metabolites (49%, 879 metabolites) did not display significant postprandial kinetics.

The 906 metabolites presenting a significant postprandial kinetic response were then used to build an OPLS-DA model to differentiate postprandial serum after milk intake from postprandial serum after yogurt intake. The model was shown to be reliable (R2Y = 0.97, Q2 = 0.74) with a clear separation between the 2 treatments (Figure 3B and **Supplemental Figure 3**). Based on VIP_{tot} scores and *P* values, 282 metabolites (31%) were selected as discriminant, i.e., presenting a different postprandial response depending on whether milk or yogurt was ingested ($VIP_{tot} > 1$ or $P < 0.05$). Figure 5 shows the postprandial kinetics of these 282 serum metabolites after the intake of milk and yogurt. Further details regarding their retention times, neutral masses, adducts, and identification suggestions are given in Supplemental Table 3. Hierarchical clustering analysis could distinguish 5 main groups of metabolites. Two hundred and thirty-six metabolites increased postprandially (clusters 1, 2, 4, and 5), including 116 with a higher response after yogurt intake (clusters 1, 2, and a few of cluster 4). A group of 46 features decreased postprandially (cluster 3), the decrease of these metabolites being less pronounced after yogurt intake with a return to baseline concentrations after 6 h. The Kolmogorov-Smirnov distribution test on mass distributions showed that the metabolites having a higher response after yogurt intake (clusters 1 and 2) had a significantly lower mass distribution than the metabolites having a higher response after milk intake (clusters 4 and 5) (*m/z*, $P = 4.41 \times 10^{-6}$, Figure 4B).

Fasting serum metabolomes after 2 wk of daily intake: general trends. Of the 1785 metabolites detected in serum, 185 (10%) were changed in fasting samples assessed after the 2-wk daily intake of milk and/or yogurt (paired Wilcoxon's signed rank test, $P < 0.05$). The 185 metabolites were then used to build an OPLS-DA model in order to differentiate fasting serum assessed after 2 wk of daily intake of milk from that assessed after 2 wk of daily intake of yogurt. The resulting model could separate the 2 groups of samples with a goodness-of-fit parameter R2Y = 0.97 and a predictive ability parameter Q2 = 0.56, slightly over the recommended threshold (Q2 > 0.50) (31) (Figure 3C and **Supplemental Figure 4**). As expected, the differentiation of fasting serum was less marked than that of the postprandial serum.

Based on VIP_{tot} scores, 81 metabolites were selected as discriminant for fasting serum samples ($VIP_{tot} > 1$). Among them, 31 metabolites were also discriminant for postprandial serum samples ($VIP_{tot} > 1$, both postprandially and fasting); their distribution in the 5 postprandial kinetic clusters and their direction of change under fasting conditions are indicated in Figure 5. Interestingly, 29 had a postprandial behavior that matched their concentration in fasting serum; i.e., when their serum concentrations were higher postprandially after yogurt intake, they remained higher in fasting serum after 2 wk of yogurt intake, and similarly for milk intake. In total, 36% of the changes observed in the fasting serum metabolome after 2 wk of daily intake of dairy could already be observed during the postprandial test.

Identification. Discriminatory metabolites were submitted to a multistep identification procedure, including database searching, collision-induced fragmentation, and the injection of standards. The identities of 18 discriminant features were confirmed with the injection of a standard and, if necessary, additional fragmentation pattern analysis. Identified discriminatory metabolites are listed in Table 1 with additional information concerning their concentrations in the test products and serum. Among the identified features, GDL and its hydrolyzed form, gluconic acid, were the most discriminatory

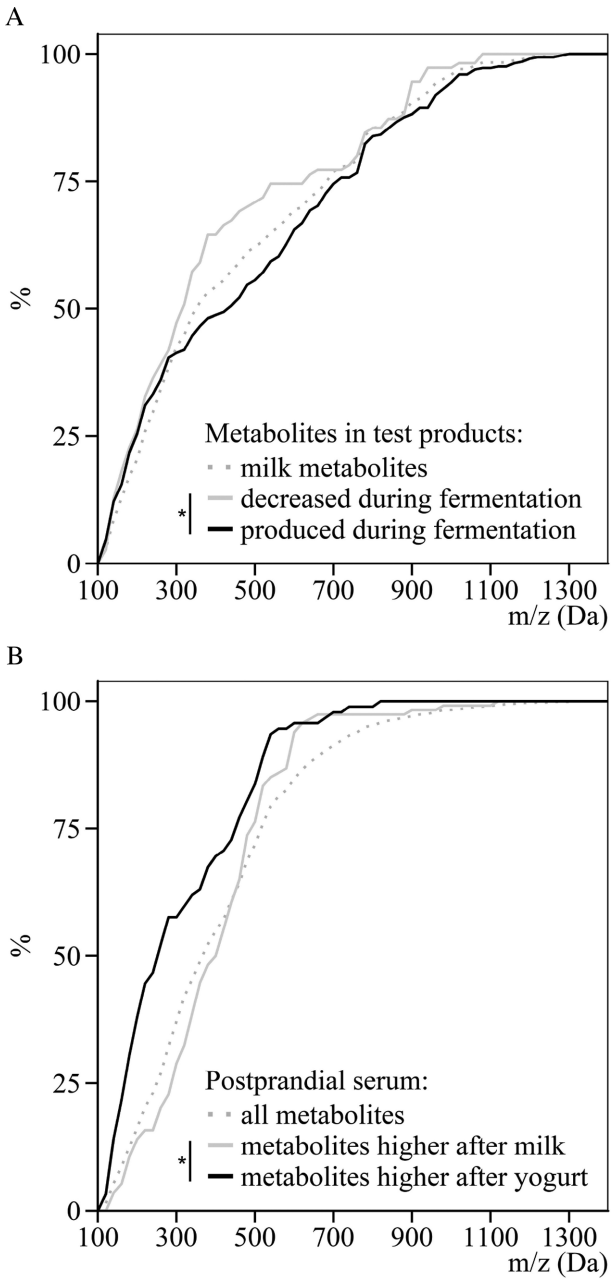

FIGURE 4 Differentiation of the size distribution of metabolites in dairy products and serum samples of healthy men after milk or yogurt intake through the use of untargeted LC-MS metabolomics. (A) Cumulative mass distribution of all milk metabolites, metabolites that decreased during fermentation, or metabolites that were produced during fermentation (OPLS-DA $VIP_{tot} > 1$ or $P < 0.05$). (B) Cumulative mass distribution of all serum metabolites, metabolites with higher postprandial serum concentration after milk intake, or metabolites with higher postprandial serum concentration after yogurt intake (OPLS-DA $VIP_{tot} > 1$ or $P < 0.05$). * $P < 0.01$, Kolmogorov-Smirnov test between the 2 indicated distributions. OPLS-DA, orthogonal projections to latent structures discriminant analysis; VIP_{tot}, variable importance in projection for the predictive and orthogonal components.

metabolites of the postprandial response to dairy products (**Supplemental Figure 5**A, cluster 4 in Figure 5). As explained, GDL was purposely added to milk for acidification and texture modification. GDL and gluconic acid were rapidly absorbed after milk ingestion with maximum concentrations being reached by 3 h and remaining higher than baseline concentrations after 6 h. Interestingly, concentrations of both GDL and gluconic acid remained higher in fasting serum after 2 wk of daily intake of acidified milk compared to yogurt intake ($VIP_{tot} = 1.12$, $VIP_{tot}\_CI < 1$ for both metabolites). OPLS-DA models were also tested without GDL and gluconic acid since GDL was purposely added to the milk, but the outcomes remained unchanged.

A group of 7 metabolites were identified as free amino acids. Their influence on the OPLS-DA model appeared to be limited ($VIP_{tot} > 1$, $VIP_{tot}\_CI < 1$, Supplemental Table 3); however, all of these metabolites showed a postprandial increase, with a higher response after yogurt intake (Table 1, Supplemental Figure 5A–C). The essential amino acids that were identified (threonine, lysine, and phenylalanine), as well as tyrosine that is derived from phenylalanine, were grouped in cluster 2, with a rapid increase (maximum serum concentration at 1 h) and a return to baseline value after 4 h. On the other hand, asparagine showed a slower increase (maximum serum concentration at 3 h) and returned to baseline at 6 h (cluster 4). Most of the postprandial concentrations of the amino acids were in accordance with their contents in the test products. This was the case for lysine, phenylalanine, asparagine, and tyrosine, which were present at higher concentrations in yogurt compared with milk. The metabolite identified as taurine presented higher postprandial concentrations after milk intake. A significant increase in free tryptophan was observed in postprandial serum after milk and yogurt intake (nparLD $P = 2.26 \times 10^{-7}$ and 5.08×10^{-12}, respectively). However, there was no marked difference between the 2 treatments ($VIP_{tot} = 0.34$) despite the higher tryptophan content in yogurt compared with milk (Table 1, Supplemental Figure 5C).

Conversely, a clear difference was seen postprandially for indole compounds that are products of tryptophan metabolism. Indole-3-lactic acid (ILA), indole-3-acetaldehyde (IAAld), indole-3-acetic acid (IAA), and 3-indole propionic acid (IPA) were among the most discriminatory metabolites postprandially, although with very different kinetic behaviors (Supplemental Figure 5E). ILA and IAAld clearly discriminated yogurt intake from milk intake. The differences observed between the products (higher concentration in yogurt) were also visible in postprandial serum (rapid increase after yogurt intake). Conversely, IPA could not be detected in either milk or yogurt. A significant postprandial increase was observed during the 6 h after milk intake (nparLD $P = 9.61 \times 10^{-6}$), whereas no significant postprandial response could be observed after yogurt intake (nparLD $P > 0.05$). The postprandial difference for IPA between milk and yogurt intake ($VIP_{tot} = 1.47$, $VIP_{tot}\_CI < 1$) was also apparent in fasting serum ($VIP_{tot} = 1.32$, $VIP_{tot}\_CI < 1$). IAA showed a significant postprandial increase after milk intake (nparLD $P = 1.72 \times 10^{-3}$) with a return to baseline after 6 h whereas a continuous decrease from 60 min to 6 h was observed after yogurt intake (nparLD $P = 6.12 \times 10^{-9}$). IAA showed some discrimination for postprandial serum ($VIP_{tot} = 1.62$, $VIP_{tot}\_CI < 1$) and was not discriminating for fasting serum after daily intake of yogurt and milk.

Among the most discriminant identified features with a significant response after milk or yogurt intake, 5 metabolites were identified as BAs (Supplemental Figure 5C, D). All the identified BAs presented a postprandial increase and were classified in cluster 5 with maximum concentrations between 60 and 180 min. In each case, the postprandial increase was lower after yogurt intake (Table 1). This difference was particularly marked for tauroursodeoxycholic acid and 3b-hydroxy-5-cholenoic acid ($VIP_{tot} = 2.01$ and 2.19, respectively, $VIP_{tot}\_CI > 1$). However, this effect of yogurt intake appeared to

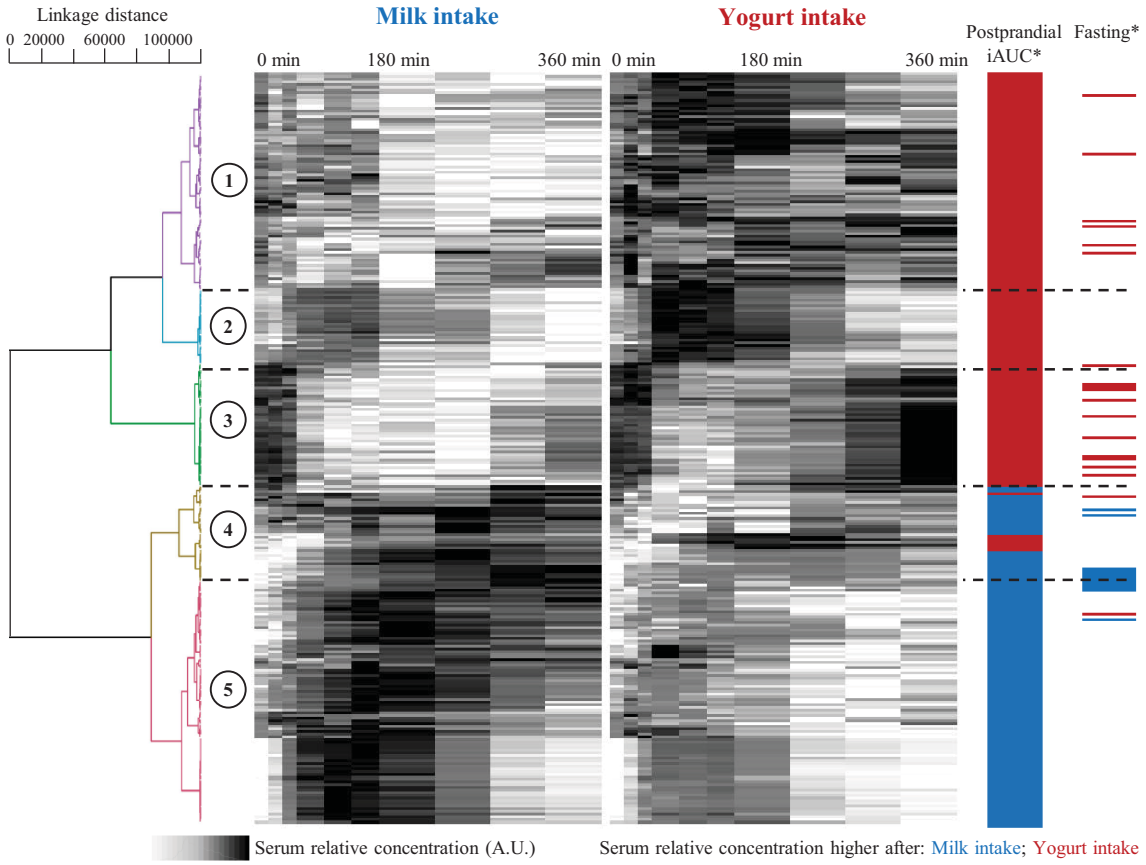

FIGURE 5 Serum metabolites discriminant for the postprandial response after milk or yogurt intake in healthy men, clustered by postprandial kinetics. Clustering by Spearman's distance measure and Ward linkage. Five main clusters are identified. Metabolites with higher serum concentrations after milk intake or after yogurt intake are identified for the postprandial test (800 g intake, iAUC) and fasting after 2 wk of daily intake (400 g/d). *OPLS-DA $VIP_{tot} > 1$ or $P < 0.05$. A.U., arbitrary units; iAUC, incremental AUC; OPLS-DA, orthogonal projections to latent structures discriminant analysis; VIP_{tot}, variable importance in projection for the predictive and orthogonal components.

be transitory since no difference could be seen in fasting serum after 2 wk of daily intake.

Discussion

Through the use of an untargeted LC-MS–based metabolomics approach, our study has characterized the metabolic footprint of fermentation, not only in the dairy metabolome but also in the serum metabolome after acute or short-term (2-wk) intake of milk and yogurt.

Products metabolome. Our comparative evaluation of milk and yogurt has shown a complexification of the milk metabolome during fermentation. There is little research that describes the milk fermentation process with the use of untargeted metabolomics. Among the existing studies in this field, free amino acids, peptides, and volatile compounds are the most frequently reported metabolites that are released in yogurt (35, 36), cheese (37), and various fermented milks (38). Our study indicates that globally, milk metabolites used by LAB during fermentation have a smaller mass than metabolites subsequently released in yogurt. This difference would reflect the presence in yogurt of oligopeptides from caseins hydrolysis (higher concentrations of low molecular weight peptides, Supplemental Table 2), of oligosaccharides (from bacterial exopolysaccharides) (39), or of other complex metabolites constitutive of the biomass (40). However, the results of such comparisons between products highly depend on the analytical method used. In our case, the removal of phospholipids and the use of the positive ionization mode might have influenced these results.

Postprandial serum metabolome: general trends. We have also shown that, despite milk and yogurt being similar in their composition, and despite the generic effects of digestion and intestinal transport on the macronutrients present in these products, the acute ingestion of milk or yogurt resulted in 2 different postprandial serum metabolic profiles, with 16% of the serum metabolome responding differently. The effect of yogurt intake on the serum metabolome was not limited to the exogenous metabolites that were absorbed from the food but also included changes relating to endogenous metabolic pathways, hence the variety of kinetics profiles observed among the discriminatory metabolites (Figure 5). It appeared that serum metabolites specific to yogurt intake (i.e., metabolites with a greater postprandial increase after yogurt intake) had a significantly lower mass compared with metabolites specific to milk intake. Such differences in mass distribution could be due to the presence in yogurt of fermentation products derived from LAB activity, such as volatile compounds (e.g., SCFAs) (35, 41), oligonucleotides (42), or free amino acids, and also to the fact that yogurt components of higher mass did not reach the circulation.

Amino acids. Native milk proteins have been described as a good source of free amino acids with respect to plant-derived proteins (43) and essential amino acids were shown to be preferentially released during milk in vitro digestion (44).

TABLE 1 Identified serum metabolites discriminant for the postprandial response after milk or yogurt intake in healthy men[1]

Retention time (min)_Adduct mass (Da/charge)	Measured neutral mass (Da)	Identification	PubChem CID	Adducts	Identification method[2]	Product with the highest content	Product causing the highest postprandial response	Postprandial kinetic cluster	Product causing the highest fasting serum concentration after 2 wk of daily intake
1.11_219.0470	196.0578	Gluconic acid	10,690	.+H; +Na	1,2,4	Milk*	Milk*	4	Milk*
1.11_179.0550	178.0478	Δ-Gluconolactone	736	.+H	1,4	Milk*	Milk*	4	Milk*
1.16_138.0528	115.0636	Proline	145,742	.+H; .+Na; +2Na-H; .x2+H; .x2+Na	1,4	Milk*	Yogurt*	1	Yogurt
0.92_147.1123	146.1051	Lysine	5962	.+H; .+Na; .+2Na-H; .+H-H2O	1,4	Yogurt*	Yogurt*	2	Yogurt
1.06_164.0290	119.0578	Threonine	6288	.+H; .+2Na-H; .+Na	1,4	Milk	Yogurt*	2	Yogurt
3.43_166.0861	165.0789	Phenylalanine	6140	.+H; .x2+H	1,4	Yogurt*	Yogurt*	2	Milk
1.28_174.0868	132.0534	Asparagine	6267	.+ACN+H; .+Na; .+H-H2O	1,4	nd	Yogurt*	4	Milk
2.31_182.0807	181.0734	Tyrosine	6057	.+H; .+Na	1,4	Yogurt*	Yogurt*	2	Milk
4.23_205.0972	204.0899	Tryptophan	6305	.+H; .+Na	1,2,3,4	Yogurt*	Yogurt	—	Milk
1.07_198.0846	175.0954	Citrulline	9750	.+H; .+Na; +2Na-H	1,4	nd	Yogurt*	1	Yogurt
1.07_126.0214	125.0142	Taurine	1123	.+H; +2Na-H; .+Na; .+H-H2O	1,4	Yogurt	Milk*	4	Milk
6.04_206.0807	205.0735	Indole-3-lactic acid	92,904	.+H; .+Na	1,2,3,4	Yogurt*	Yogurt*	1	Yogurt
6.04_160.0753	159.0680	Indole-3-acetaldehyde	800	.+H	1,2	Yogurt*	Yogurt*	1	Yogurt
6.67_176.0701	175.0629	Indole-3-acetic acid	802	.+H; .+Na	1,2,3,4	nd	Milk*	5	Milk
7.26_190.0858	189.0785	3-Indole propionic acid	3744	.+H; .+Na; .+H+H2O	1,2,4	nd	Milk*	4	Milk*
9.91_375.2887	374.2814	3b-Hydroxy-5-cholenoic acid	92,997	.+H; .+H-H2O; .+H-2H2O	1,2,3,4	nd	Milk*	5	Yogurt
10.10_434.3256	392.2920	(cheno)Deoxycholic acid	222,528	.+H; .+Na; .+NH4; .+H-H2O; .+H-2H2O; .x2+H	1,2,3,4	nd	Milk*	5	Yogurt
8.46_450.3205	449.3132	Glycoursodeoxycholic acid	12,310,288	.+H; .+NH4; .+Na; .x2+H; .x2+Na; .x2+NH4; .+H-H2O; .+H-2H2O	1,2	nd	Milk*	5	Milk
7.40_483.3415	465.3077	Glycocholic acid	10,140	.+H; .+NH4; .+Na; .+H-H2O; .+H-2H2O	1,2,4	nd	Milk*	5	Yogurt
9.51_500.3028	499.2956	Tauroursodeoxycholic acid	9,848,818	.+H; .+H-H2O; .+H-2H2O	1,2,3,4	nd	Milk*	5	Milk

[1] *OPLS-DA VIP_{tot} score > 1 or $P < 0.05$. CID, compound identifier; nd, not detected; OPLS-DA, orthogonal projections to latent structures discriminant analysis; VIP_{tot}, variable importance in projection for the predictive and orthogonal components.

[2] Identification methods: 1, mass database; 2, theoretical fragmentation; 3, fragment database; 4, standard injection.

Milk-derived free amino acids have been discussed in the context of postprandial skeletal muscle protein synthesis, after resistance exercise (45) and in elderly populations that exhibit low skeletal muscle mass (sarcopenia) (46). In this study, higher postprandial concentrations of 7 free amino acids were observed after yogurt intake with respect to milk intake, 3 being essential (phenylalanine, threonine, and lysine). These differences could be related to the prior hydrolysis of milk proteins during fermentation (Supplemental Table 2), with similar trends observed in postprandial urine after cheese intake compared with milk intake (25). Prior fermentation might therefore be considered as a means to increase circulating free amino acids after dairy intake. However, as suggested by the kinetics profiles (Supplemental Figure 5A–C), the differences in postprandial free amino acids between milk and yogurt intake appeared to be rather limited in intensity and in time (returning to baseline values after 4–6 h), which might explain why they were no longer discriminant in fasting serum after 2 wk of the daily interventions.

Indole derivatives. Indole compounds are known products of microbial metabolism of tryptophan via the "indole pathway". In the present study, probably owing to the proteolytic activity of LAB during fermentation, we report higher concentrations of free tryptophan in yogurt compared with milk. The subsequent use of free tryptophan by LAB during fermentation (47) explains the higher concentrations of ILA and IAAld in yogurt, resulting in higher postprandial serum concentrations after yogurt intake. In humans, ILA being an end-product of the tryptophan metabolic pathway (48), it is likely to accumulate in serum postprandially. In accordance with our observation, an increase in postprandial ILA has been reported in plasma after the ingestion of different whey protein fractions, the ILA concentrations positively correlating with the tryptophan content in the different fractions (49).

The use of dietary free tryptophan by the microbiota and the subsequent release of indole derivatives reaching the blood could also contribute to the presence of indole derivatives in postprandial serum. Such mechanisms have only been described in rodents to date (50, 51). Under high-tryptophan conditions, as is the case in our study after yogurt intake, a 2-fold increase in tryptophanase activity has been reported in murine microbiota, tryptophanase being a key enzyme in the indole pathway (52). Moreover, as described by Zelante et al. (51), in unrestricted tryptophan conditions intestinal lactobacilli favor tryptophan as a source of energy over carbohydrates. It is then likely that the excess in tryptophan provided by the yogurt is directly metabolized by the intestinal microbiota, especially via the indole pathway (51, 53).

As IPA and IAA were not detected in either milk or yogurt, their presence in postprandial serum could be of intestinal microbiota origin. IPA has been reported as being produced in the intestine specifically by *Clostridium sporogenes* (50, 54). Furthermore, a similar increase in IPA during the late postprandial phase has been previously described after the intake of 500 mL dairy shake (55). The lower IPA and IAA concentrations reached in blood after yogurt intake may appear to be contradictory considering the higher tryptophan content in yogurt. However, in a human intervention study investigating protein intake and tryptophan metabolites, no correlation could be observed between plasma tryptophan and plasma IAA, and in mice plasma IAA was lowered after a high-protein diet (56). The interactions of the microbiota with foods make any mechanistic explanation even more complex. For example, yogurt bacterial species have been shown to inhibit *C. sporogenes* growth (57) and its adhesion to intestinal epithelial cells (58), which could result in a lower intestinal production of IPA. Other mechanisms, such as the binding to the aryl hydrocarbon receptor (AhR) (59) or enzymatic degradation in the circulation, may also contribute to the postprandial responses observed here.

Indole derivatives have recently been investigated for their role on the epithelial barrier function and gut inflammatory homeostasis via their activation of the AhR signaling pathway, IAA and IAAld being ligands for this receptor (51, 54, 59, 60). Strikingly, the whole blood transcriptomic analysis of our postprandial samples independently identified the gene coding for AhR among the top genes showing significant change after yogurt intake (61).

BAs. Primary BAs are synthesized in the liver and are conjugated to glycine and taurine before excretion in the duodenum. The influence of the intestinal microbiota on the pool size and composition of BAs is well documented (62) and mainly relies on the expression of bile salt hydrolase (BSH) by colonic bacteria, hydrolyzed BAs being less efficiently reabsorbed into the enterohepatic recirculation (63–66). Consequently, dietary probiotics have been tested to modulate metabolism of BAs, with the aim of influencing the host metabolism including cholesterol and TG pathways (65). As a result, BSH activity has been proposed as a requirement for probiotic organisms (67). The presence of the BSH gene in the genome of LGG (LRHM_0484) and the known BSH activity of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* (68) could explain the lower postprandial concentrations observed here after yogurt intake. In addition, all 3 yogurt strains have the capacity to survive in the gastrointestinal tract after ingestion (22, 69). Furthermore, it is worth noting that taurine, which is used in the liver for de novo BA synthesis, tends to have a lower postprandial response after yogurt intake compared with milk intake. However, this change in serum BAs appeared to be transitory as no difference was observed in fasting serum after daily intake. In contrast, other intervention studies have reported an increase in circulating BA (conjugated or unconjugated) after chronic intake of the BSH-active *Lactobacillus reuteri* (70, 71). These studies do however differ in important aspects of study design, including mode of administration (capsules), choice of probiotic strains, as well as the duration of the studies (up to 6 wk). Interestingly, our group of discriminant BAs included conjugated (glycoursodeoxycholic acid) and unconjugated [(cheno)deoxycholic acid] BAs, as well as a primary BA synthesized by the liver (3b-hydroxy-5-cholenoic acid) and a secondary BA of microbial origin (glycocholic acid), reflecting the complexity of the mechanisms behind the regulation of BA metabolism.

Fasting metabolome after 2 wk of daily intake: general trends. Interestingly, more than a third of the changes detected in fasting assessments after 2 wk were already visible postprandially. Although most of the identities of these metabolites are not yet confirmed, these results nevertheless give an estimation of the ability of acute postprandial studies to predict changes that are likely to occur after chronic intake. The postprandial phase is generally less frequently studied in nutritional interventions, which tend to focus on fasting measurements after semichronic or chronic intake to identify long-term effects (16, 19, 21, 72). However, considering the typical meal frequency in Western countries, most individuals are in a postprandial state during the day and, therefore, looking at the postprandial phase may also help to identify dietary biomarkers as well as to understand the mechanisms underpinning long-term effects of diet.

Conclusion. To conclude, we firstly observed that milk and yogurt still share most of their metabolites. Nonetheless, fermentation left an identifiable footprint on the product metabolome, and more importantly, on the serum metabolome, postprandially after acute intake, as well as fasting after short-term daily intake. The postprandial modulation of circulating amino acids, indole derivatives, and BAs suggests that the metabolic footprint of fermented dairy intake does not only consist in the absorption of metabolites from the product, but also in the regulation of the endogenous metabolic activity, notably that of the intestinal microbiota. Such metabolites could be used as targets in observational studies to identify potential benefits of fermented dairy products on cholesterol and TG concentrations through the modulation of BAs, or on inflammatory status through the AhR signaling pathway. Moreover, assessing the expression of genes involved in these pathways could identify the molecular targets modulated by these metabolites as well as clarify the mechanisms underlying such effects. Finally, compounds like indole derivatives might be considered as biomarkers of fermented dairy intake in observational studies, with the limitation that other nondairy fermented food can also be a source of such metabolites [kimchi, sauerkraut, or pickles (73)].

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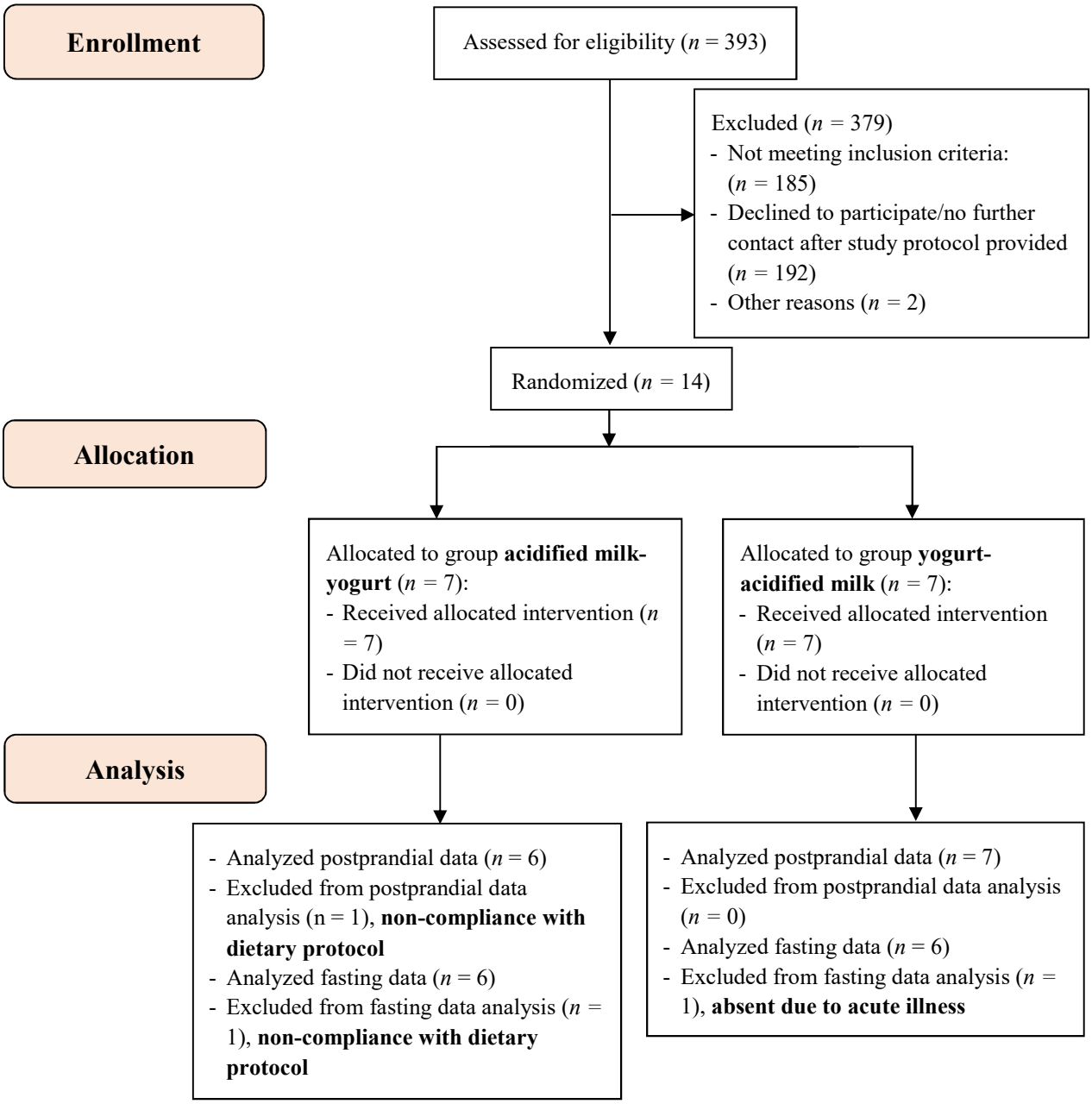
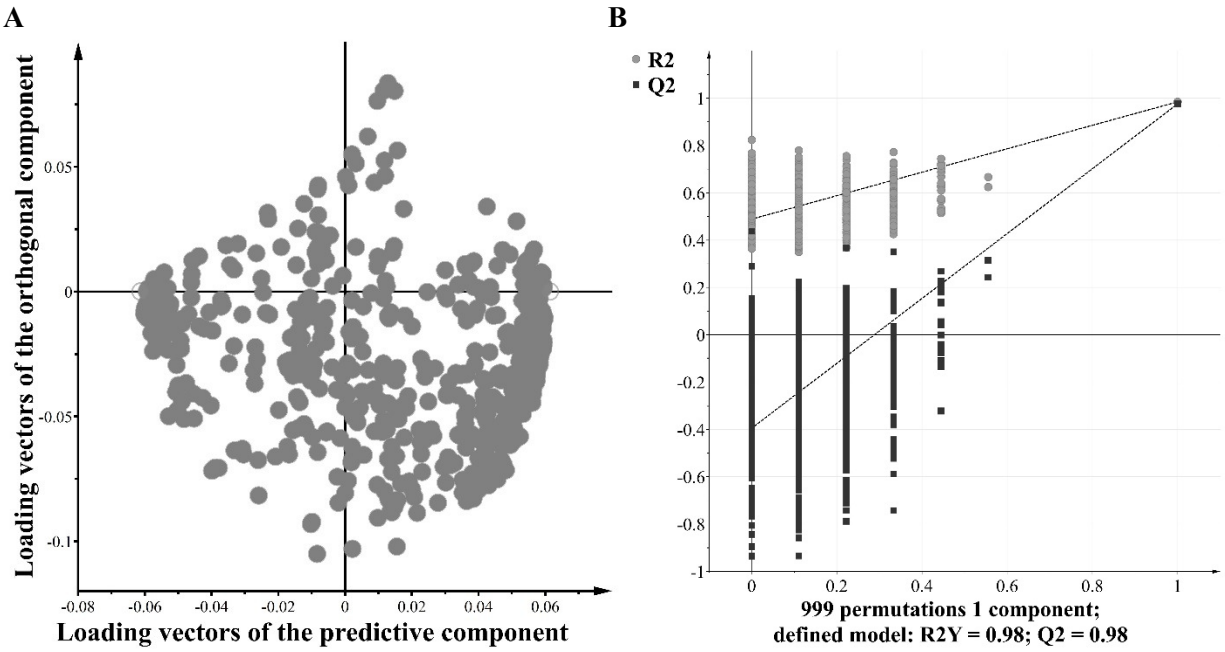
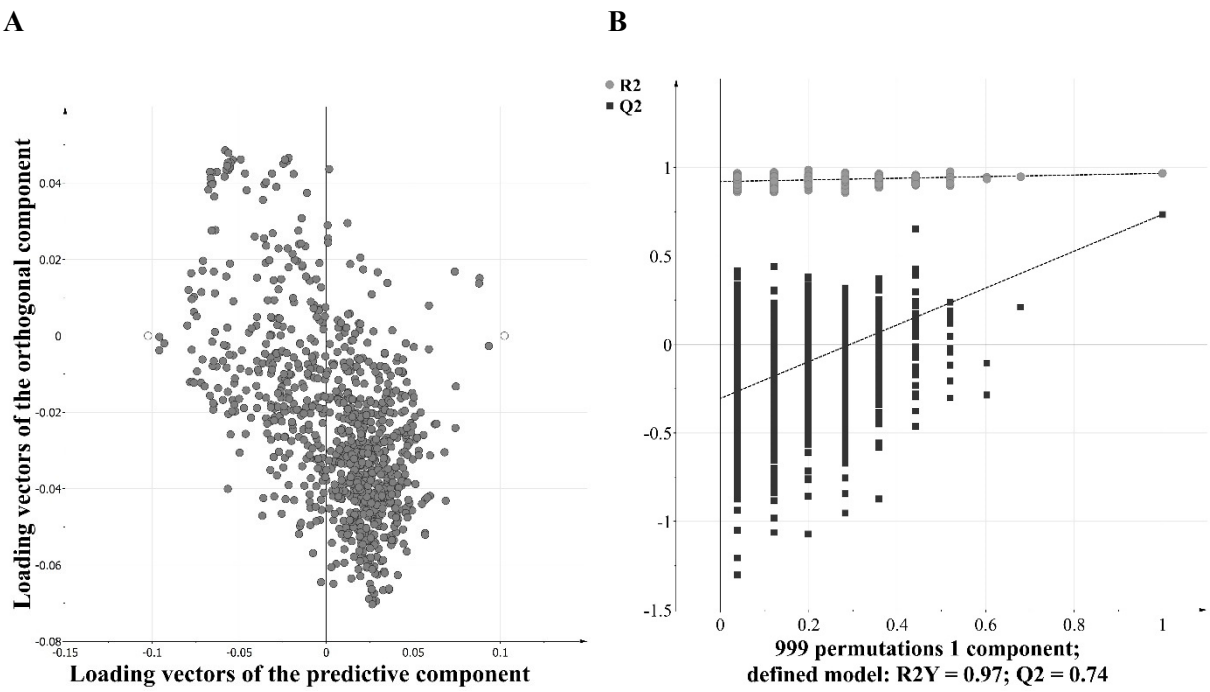


SUPPLEMENTAL FIGURE 1 Flow chart of the study.[1]

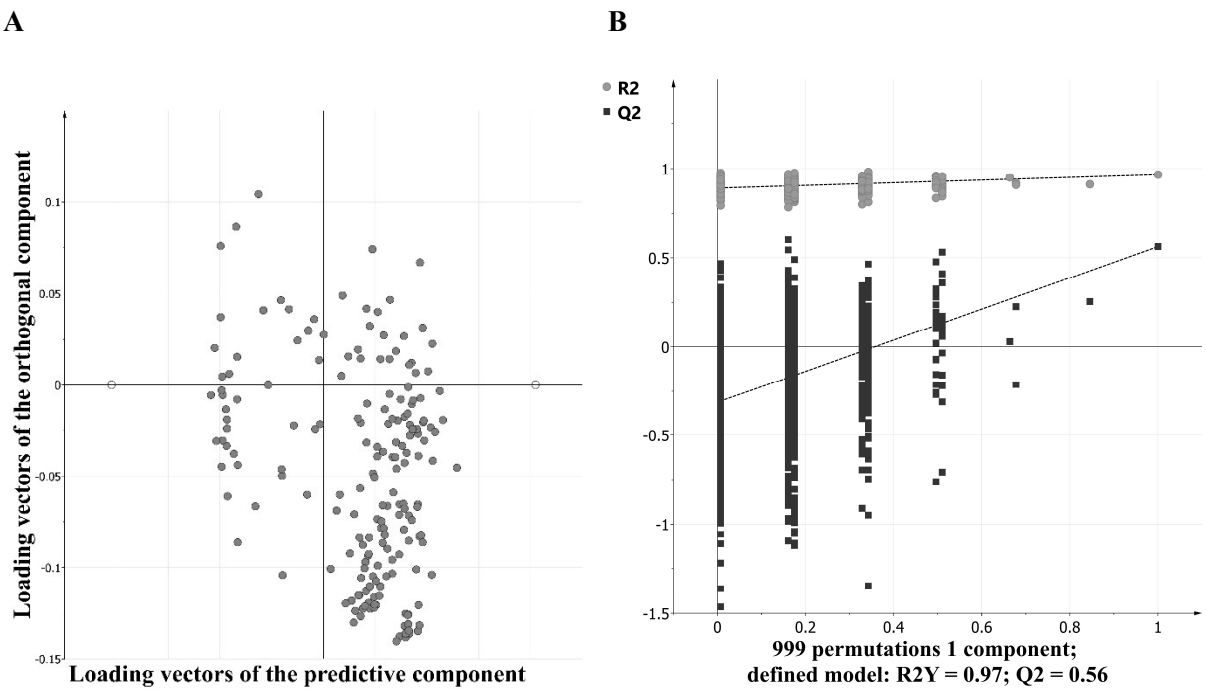
[1] Adapted with permission from Burton *et al.* (22)



SUPPLEMENTAL FIGURE 2 Orthogonal partial least squares discriminant analysis derived from milk and yogurt samples: loading plot and permutation test. Number of components: 1+1+0, R2X (cum) = 0.55, R2Y (cum) = 0.98, Q2 = 0.98. (A) Loading plot of the 590 metabolites detected in milk and/or yogurt and (B) permutation test with 999 permutations. The defined model was validated by higher original values of R2Y and Q2 than values obtained after permutations.

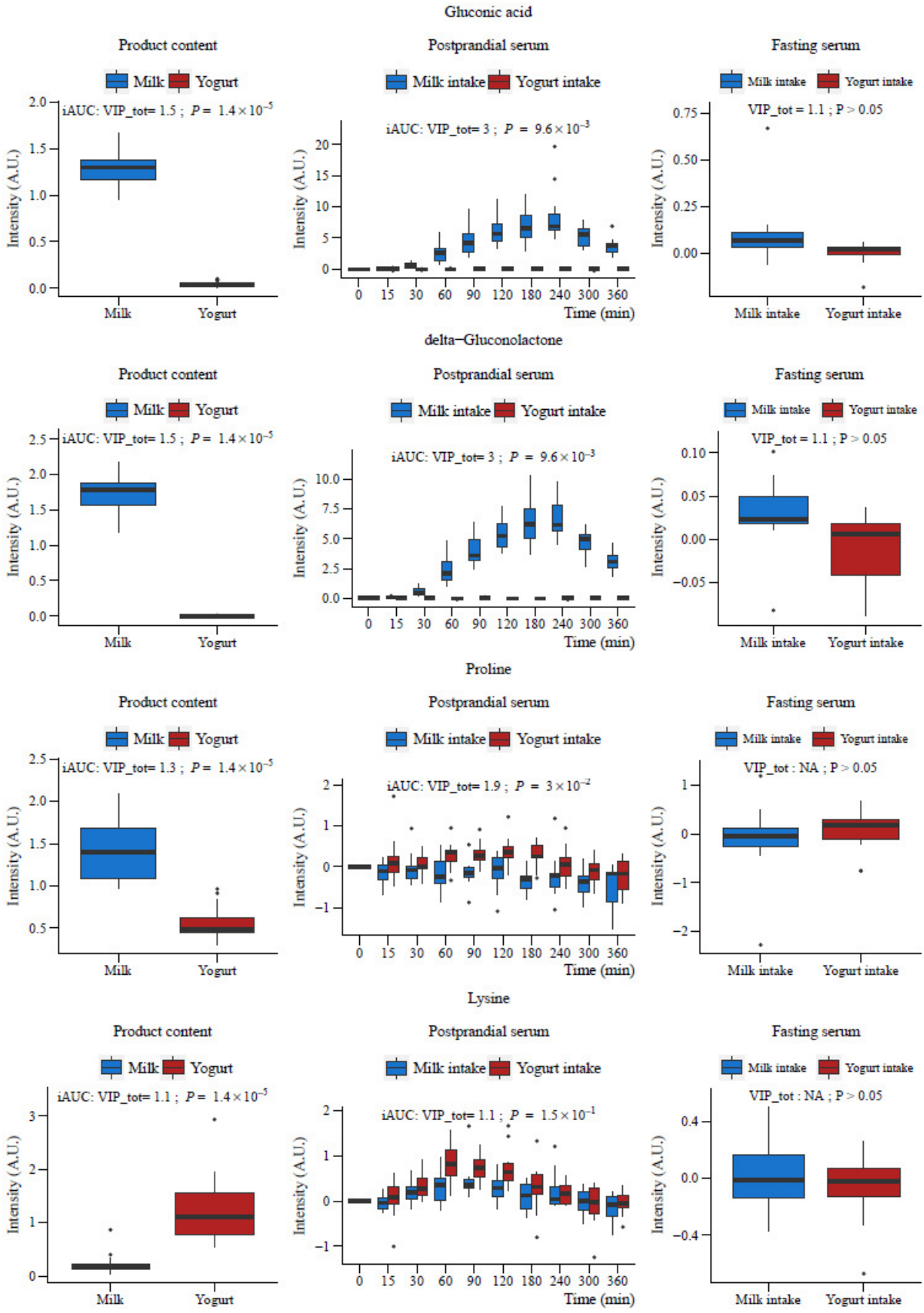


SUPPLEMENTAL FIGURE 3 Orthogonal partial least squares discriminant analysis derived from postprandial serum after milk or yogurt intake in health men: loading plot and permutation test. Number of components: 1+2+0, R2X (cum) = 0.36, R2Y (cum) = 0.97, Q2 = 0.74. (A) Loading plot of the 906 serum metabolites with a significant postprandial response after milk and/or yogurt intake and (B) permutation test with 999 permutations. The defined model was validated by higher original values of R2Y and Q2 than values obtained after permutations.

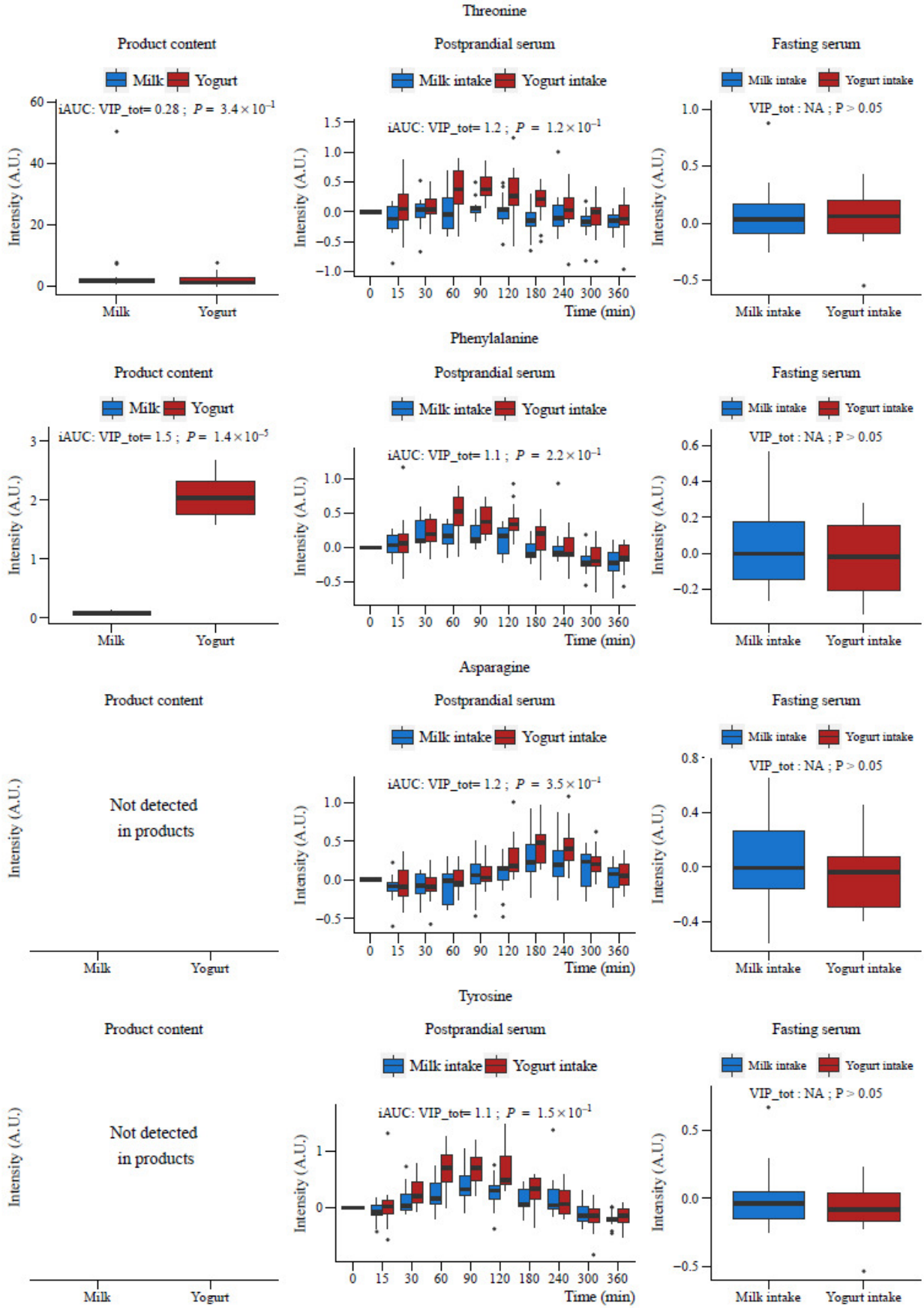


SUPPLEMENTAL FIGURE 4 Orthogonal partial least squares discriminant analysis derived from fasting serum after two weeks daily intake of milk or yogurt in healthy men: loading plot and permutation test. Number of components: 1+2+0, R2X (cum) = 0.37, R2Y (cum) = 0.97, Q2 = 0.56. (A) Loading plot of the 182 metabolites modulated by a two-week daily intake of milk and/or yogurt and (B) permutation test with 999 permutations. The defined model was validated by higher original values of R2Y and Q2 than values obtained after permutations.

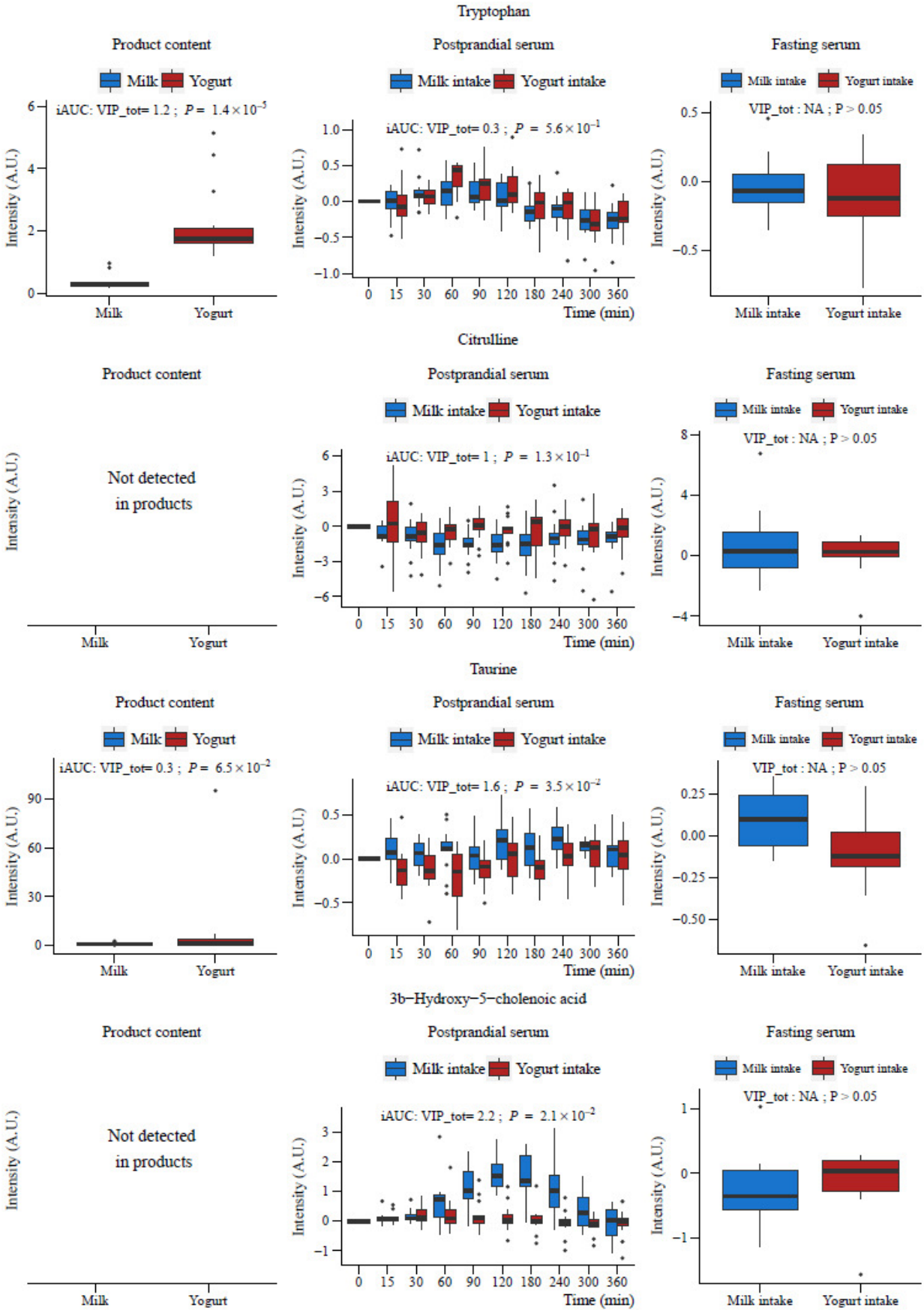
SUPPLEMENTAL FIGURE 5A



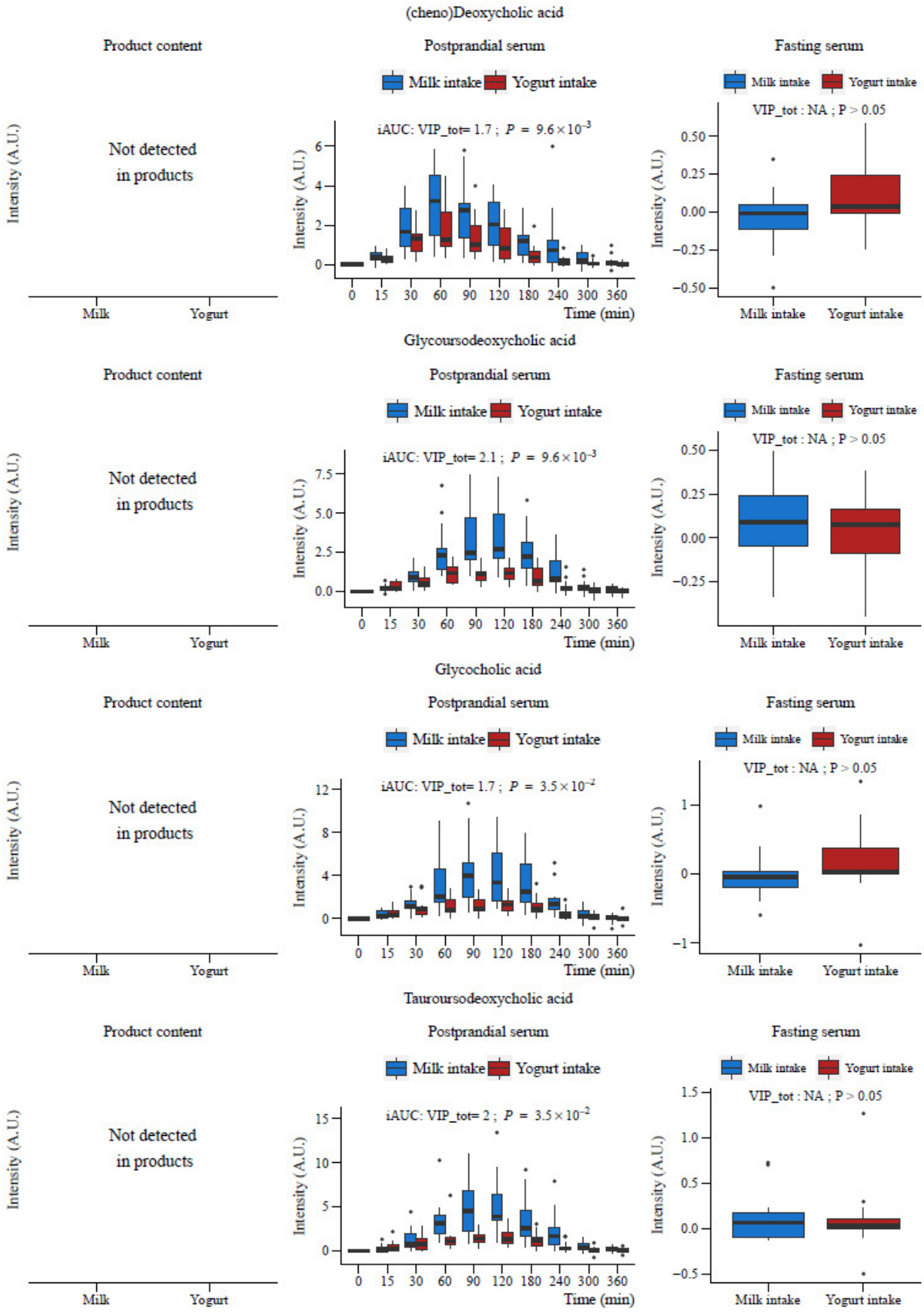
SUPPLEMENTAL FIGURE 5B



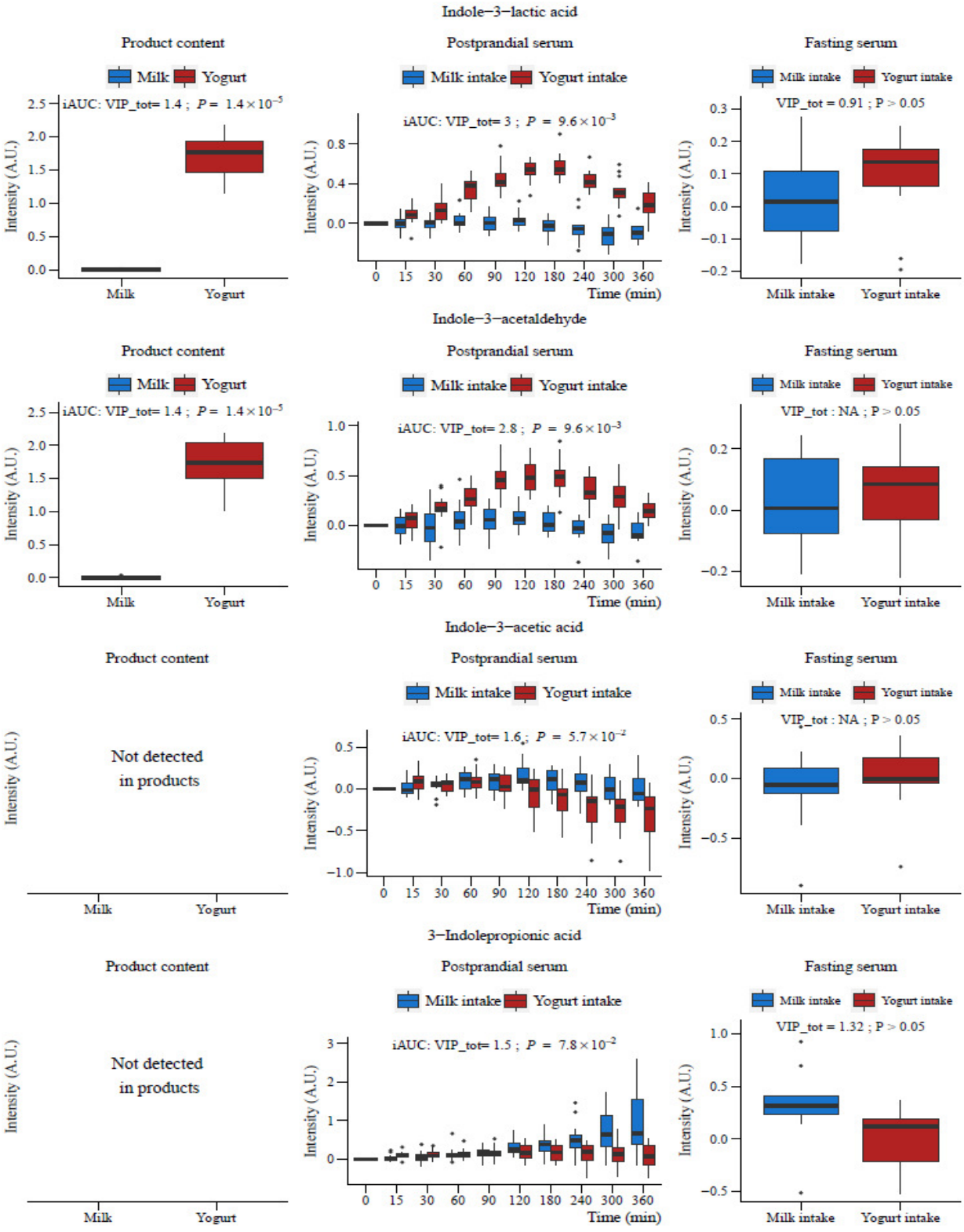
SUPPLEMENTAL FIGURE 5C



SUPPLEMENTAL FIGURE 5D



SUPPLEMENTAL FIGURE 5E



SUPPLEMENTAL FIGURE 5 Identified metabolites discriminatory for postprandial serum after milk or yogurt intake in healthy men. Levels in milk and yogurt (left side); in postprandial serum after milk and yogurt intake (center); and in fasting serum after two weeks daily intake of milk or yogurt (right side). Interquartile range plotted with median (—) and outliers (•). n = 18 for product samples, n = 13 for serum samples. OPLS-DA VIP_{tot} and paired Wilcoxon signed-rank test adjusted P are indicated. iAUC, incremental area under the curve; OPLS-DA, Orthogonal partial least squares discriminant analysis; VIP_{tot}, variable importance in projection for the predictive and orthogonal components; NA, metabolite not included in the OPLS-DA model.

SUPPLEMENTAL TABLE 1 Participant characteristics.[2]

	n	**Median (IQR)**
Age, years	13	24.0 (22.0 - 27.0)
Weight, kg	13	71.2 (67.2 - 75.7)
BMI, kg/m^2	13	22.1 (20.0 - 22.69)

[2] IQR, interquartile range. Adapted with permission from Burton *et al.* (22).

SUPPLEMENTAL TABLE 2 Nutritional composition of the milk products.[3]

	Acidified milk (mean ± SEM)	Probiotic yogurt (mean ± SEM)	UHT milk
Energy, kJ/100g	294 ± 0.80	265 ± 2.10	*270*
Carbohydrate, %	6.9 ± 0.01	2.7 ± 0.05	*4.9*
Protein, %	3.3 ± 0.02	3.4 ± 0.03	*3.2*
Low-molecular weight peptides, free amino acids and amines, mmol/100g	0.2 ± 0.00	0.5 ± 0.00	*NA*
Fat, %	3.4 ± 0.01	3.5 ± 0.01	*3.6*
Total sugars, %	6.9 ± 0.01	2.7 ± 0.05	*4.9*
Lactose monohydrate, %	4.9 ± 0.01	2.5 ± 0.04	*NA*
Galactose, %	0.0 ± 0.00	0.2 ± 0.01	*NA*
Glucose, %	0.0 ± 0.00	0.0 ± 0.00	*NA*
Total lactate, %	0.0 ± 0.00	1.1 ± 0.03	*NA*
Glucono-δ-lactone, %	2.0 ± 0.00	*NA*	*NA*

[1] UHT milk nutrients as indicated by manufacturer. *NA*, Not applicable. SEM, Standard Error of the Mean. Adapted with permission from Burton *et al.* (22).

Supplemental methods

Production of dairy products.

Both dairy products were prepared at Agroscope, Federal Research Station for Agriculture (Bern) to industrial standards in accordance with Swiss food legislation. The yogurt culture was added with LGG to milk (preheated to 39°C) and stirred for five minutes before incubation at 39°C until pH reached 4.6. The acidified milk was prepared to mimic the appearance and texture of the yogurt by the addition of 2% D-(+)-glucono-delta-lactone powder (GDL, ≥ 99.0%, Jungbunzlauer AG, Switzerland) dissolved by stirring for 5 min an kept at room temperature until the pH reached 4.6. Both products were stored immediately at 4°C upon reaching the target pH. The products were prepared in batches of 12 or 36 litres. In the yogurt, a minimum of 1.00 x 10^6 colony forming units (CFU)/g per strain was reached in the final product: *Lactobacillus delbruckeii* ssp. *bulgaricus* 9.04 x 10^{-7} (SD 3.55 x 10^7) CFU/g, *Streptococcus thermophilus* 6.50 x 10^8 (SD 1.04 x 10^8) CFU/g and LGG 2.83 x 10^6 (SD 6.53 x 10^5) CFU/g. Quality controls were carried out by the Quality Assurance Laboratory (Agroscope).

Biochemical analysis of products samples.

Protein content was assessed by Kjeldahl method with potentiometric titration.

Low-molecular peptides, amino acids, and amines were assessed with the OPA method as described in Kopf-Bolanz *et al.* (44).

Fat content was assayed by Roese-Gottlieb gravimetric.

Lactose monohydrate, galactose, glucose and total lactate were assayed by enzymatic spectrophotometric analysis.

Total sugars was calculated as the sum of assayed sugars.

Carbohydrate was calculated by sum of all assayed carbohydrates.

Protein content was calculated by assessment of total nitrogen content using Jones conversion factor for milk. Atwater factors were used to calculate total energy content.

Samples preparation for LC-MS metabolomics.

Serum samples were allowed to thaw on ice for 45min and were kept on ice along the preparation. In order to limit ion-suppression, phospholipids were removed from the samples with the use of a phospholipids removal device which consists in a 96-well plate with a filter membrane (Phree®, Phenomenex Inc., Torrance, California, USA). Protein precipitation was obtained in the well plate with the addition 1:3 (vol/vol) of acetonitrile containing 1% (vol/vol) formic acid. The Phree® plate was vortexed for 2min at 600rpm and then centrifuged for 5min at 500g and at 4°C. Test products samples were prepared using the same procedure.

LC-MS metabolomics analysis.

The chromatographic separation was performed using a high-pressure liquid chromatography system (HPLC, UltiMate™ 3000, Thermo Fisher Scientific™/Dionex™, Waltham, MA, USA) on a C18 hybrid silica column (YMC-Triart 150 x 2 mm, YMC Co., Ltd., Kyoto, Japan), reverse phase. The mobile phase consisted in water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The elution gradient that was

used (A:B, vol/vol) was as follows: 95:5 at 0 min to 5:95 at 10 min; 5:95 from 10 to 20 min; 95:5 from 20 to 30 min. The flow rate was 0.3 ml/min. The HPLC system was coupled to a quadrupole time-of-flight mass spectrometer (QTOF-MS, maXis™ 4G+, Bruker Daltonik GmbH, Bremen, Germany). The MS electrospray interface operated in positive ion mode. Electrospray voltage was set to 4.5 kV, end plate offset to 500V, capillary voltage to 3400V, nitrogen flow set to 4ml/min at 200°C. The spectra acquisition rate was set to 1Hz in profile mode, spectra were recorded from m/z 75 to m/z 1500 at a resolution of 80,000 FWHM. An internal calibration was used with 50:50 water/isopropanol containing 0.2% (vol/vol) formic acid and 1% (vol/vol) 1M sodium hydroxide. All solvents and reagents were LC-MS grade (Sigma-Aldrich GmbH, Stettlen, Switzerland). Test products samples were analyzed as described here for serum measurements.