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Metabolic profiling reveals enrichment of health-related metabolites in yoghurt by variation of strain consortium

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ABSTRACT

Enrichment of yoghurts with additional bacterial strains holds the potential to improve the nutritional value of the product. By incorporating an adjunct strain in combination with the starter culture, 183 enriched yogurts were produced, covering 19 species from 9 different genera. Interestingly, this enrichment substantially increased the total number of metabolites produced. Moreover, the set of newly produced metabolites alone was sufficient for a clear cluster-separation of yoghurts enriched with adjunct strains from the same genus. Furthermore, 35 metabolites known to be aroma compounds or associated with beneficial health effects were identified by targeted metabolomics. Determination of the relative concentration of these metabolites revealed that a set of enriched yoghurts produced high concentrations of DL-indole-3-lactic acid, 3-phenyllactic acid, short-chain fatty acids, amino acids and the neurotransmitter gamma-aminobutyric acid, particularly in yoghurts enriched with *Lactobacillus, Propionibacterium*, or *Acidipropionibacterium* strains, having the potential to further improve the health benefits associated with consumption of yoghurt. This study is the first in which a comprehensive series of enriched yoghurts were produced by adding an adjunct strain, followed by a metabolic profiling using three different platforms (LC-MS, GC-MS, and GC-MS volatiles), allowing the identification of a wide variety of different compounds.

1. Introduction

A pivotal determinant of human health is nutrition. The correlation between diet quality and the risk of non-communicable chronic diseases (NCCD) is well documented. It is estimated that in 2017, approximately 11 million deaths were attributed to dietary factors (Afshin et al., 2019). An umbrella review found that healthy dietary patterns—including low-fat dairy products, such as certain yoghurt varieties—may be associated with a reduced risk for metabolic diseases (Jayedi et al., 2020). At the same time, the human gut microbiota is increasingly being recognized as a key factor in health and disease (Wastyk et al., 2021). Although deeper insights of factors shaping its composition and function are required, diet in particular has been identified as a major modifier of the gut microbiota (García-Vega et al., 2020; Lama Tamang et al., 2023; Milani et al., 2025; Ross et al., 2024; Sanz et al., 2025). A food group that is generally associated with beneficial health effects is fermented foods such as yoghurt (Caffrey et al., 2024; Leeuwendaal et al., 2022; Marco et al., 2017; Mukherjee et al., 2024; Wastyk et al., 2021). Fermented foods are defined as foods made through desired microbial growth and enzymatic conversions of food components (Marco, 2021).

Many health-related effects of fermented foods are attributed to the microbial strains contained, some of which may have probiotic

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properties – defined as viable microorganisms which, when administered in adequate amounts, confer a health benefit on the host (Salminen et al., 2021). The ability of probiotics to robustly colonize the mucosa of humans after oral ingestion is highly species-specific and varies greatly between individuals, with about 60 % of successful expansion after supplementation and 40 % of a near-total colonization resistance (Zmora et al., 2018). However, beneficial effects may also arise from metabolites produced by lactic acid bacteria (LAB) during fermentation, which can act on the microbiota and the host, independent of mucosal colonization (Vera-Santander et al., 2023). Therefore, this study focused on developing yoghurts with increased abundance of health-related metabolites.

2. Methods

2.1. Strain selection

The strains for yoghurt production were selected as described in (Roder et al., 2022). In short, from the 869 entries in our sequencing database, we removed legally restricted strains, strain mixtures, strains that could not be clearly assigned taxonomically, duplicates and assemblies with low reliability. Of the 31 species, 7 were excluded as they are not relevant for the development of dairy products (*Brevibacterium linens, Corynebacterium variabilis, Desemzia incerta, Glutamicibacter arilaitensis*, *Anaerosphaera aminiphila, Listeria monocytogenes* and *Listeria innocua*). After removal, 626 strains remained, which were analyzed with the pathway tool of the *OpenGenomeBrowser* (Roder et al., 2022) for their genetic potential to produce bioactive substances relevant to the human organism, such as for example indole compounds. Based on this analysis, 183 strains covering 19 different species were selected for yoghurt production (Table 1).

2.2. Yoghurt production

All 183 yoghurts, including the 9 yoghurt remakes, were produced at Agroscope (Bern) to industrial standards in accordance with Swiss food legislation. After the strains (Table 1) had been pre-cultured for 16–24 h at 30 °C or 37 °C in their respective growth media, they were added to lactose-free, homogenized, pasteurized whole milk (3.5 %) (Aha! IP Suisse, Migros, Switzerland) in combination with a classic yoghurt starter culture consisting of a mixture of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* (Yoflex® YC-381, Chr. Hansen A/S, Denmark). As control, a yoghurt containing only the starter culture without adjunct strain was used. Fermentation took place at 37 °C for 16 h, after which the sample was cooled to 4 °C and stored at – 20 °C prior to analysis for a maximum of 6 month.

Table 1
Strains used for production of enriched yoghurts. Binomial name is specified, where appropriate, with subspecies classification. Number of adjunct strains used for generation of enriched yoghurts is shown for LC-MS, GC-MS and GC-MS volatile.

Binomial name (where appropriate with subspecies)	LC-MS and GC-MS	GC-MS volatile
	# Strains	# Strains
Acidipropionibacterium acidipropionici	1	1
Acidipropionibacterium thoenii	2	2
Lactobacillus delbrueckii subsp. *bulgaricus*	3	3
Lactobacillus delbrueckii subsp. *lactis*	2	2
Lactobacillus helveticus	2	2
Lentilactobacillus parabuchneri	9	9
Lacticaseibacillus paracasei	17	17
Lentilactobacillus parafarraginis	1	1
Lactiplantibacillus plantarum	2	2
Lacticaseibacillus rhamnosus	3	3
Lactococcus cremoris	20	20
Lactococcus lactis subsp. *lactis*	31	30
Lactococcus raffinolactis	1	1
Leuconostoc mesenteroides	4	4
Pediococcus acidilactici	4	4
Pediococcus stilesii	1	1
Propionibacterium freudenreichii	45	33
Staphylococcus xylosus	1	1
Streptococcus thermophilus	34	31

2.3. Liquid chromatography-mass spectrometry (LC-MS)

2.3.1. Sample preparation

100 mg (±10 %) of yoghurt was mixed with 1:3 (vol/vol) of pre-chilled acetonitrile containing 1 % (vol/vol) formic acid. The samples were then centrifuged for 15 min at 12′000 g and 4 °C. The supernatant was filtered through a 0.22 μm regenerated cellulose filter (WhatmanTM UnifloTM 13/0.2 RC) and the resulting filtrate was loaded on a Phree® phospholipids removal plate. The Phree® plate was centrifuged for 5 min at 500 g and 4 °C. The final filtrate was kept at 4 °C during the analysis.

2.3.2. Sample analysis

Each yoghurt in the first experiment with 183 yoghurts was analyzed in technical triplicates and for the remake experiment in biological replicates as described in (Roder et al., 2024). Quality control samples (QC), consisting of a pool of all yoghurt samples, were injected every five samples for signal drift correction. Blanks (ultrafiltered water) were regularly injected as well to account for contaminants. Solvents and reagents were obtained from Sigma-Aldrich GmbH, Switzerland in LC-MS grade quality.

2.3.3. Data processing

Progenesis QI (v.March 2, 6198.24128; NonLinear Dynamics Ltd.) was used for retention time correction, peak-picking, deconvolution, adducts annotation, and normalization (default automatic sensitivity and without minimum peak width) and database search for identification. The Human Metabolome Database (Wishart et al., 2018) was used with 10 ppm as mass accuracy threshold. No outliers were excluded from the dataset. Standardization was applied using StandardScaler from scikit-learn (v.0.24.2) before performing UMAP (v.0.5-dev) dimensionality reduction. To assess the statistical significance of group separation, Permutational Multivariate Analysis of Variance (PERMANOVA) was performed using the permanova function from scikit-bio (v.0.6.3) (Jai Ram Rideout). The test was conducted on standardized data using Euclidean distances and genus labels as the grouping variable, with 9999 permutations.

2.4. Gas chromatography-mass spectrometry (GC-MS)

2.4.1. Sample preparation

Samples were prepared as described in the LC-MS section, except that 25.0 mg/ml isotopically labelled D-xylose was added as an internal standard (IStd) in prechilled acetonitrile.

2.4.2. Sample analysis

Sample analysis was performed in analogy to (Münger et al., 2017). After derivatization, the samples were analyzed in randomized order on a GC-MS-Quadrupole Time-of-Flight (QTOF) system from Agilent (8890/7250).

2.4.3. Data processing

Peak-picking, deconvolution, and normalization of the untargeted metabolomics data was carried out with Agilent MassHunter Workstation. Unknown Analysis (v.10.1) and Agilent MassHunter Workstation MassProfiler Professional (v.15.1) were used for data processing. Reevaluation of specific compounds was performed using Agilent MassHunter Workstation Quantitative Analysis for QTOF (v.10.1).

2.5. Gas chromatography-mass spectrometry for volatile compounds (GC-MS volatile)

2.5.1. Sample preparation

Each yoghurt was analyzed in duplicates (technical replicates). 25 μm of IStd solution containing 0.5 ppm paraldehyde, 0.25 ppm tetradecane and 0.5 ppm d4-δ-decalactone was added to 250 mg of yoghurt.

2.5.2. Sample analysis

Sample analysis was performed in randomized order in analogy to (Fuchsmann et al., 2020), using an Agilent 7890B gas chromatography (GC) system coupled with an Agilent 5977B mass selective detector (MSD).

2.5.3. Data processing

Agilent MassHunter Profinder (v.10.0) was used for peak picking, deconvolution, and normalization. Reevaluation of specific compounds was done using Agilent MassHunter Quantitative Analysis (v.10.1), Agilent MassHunter Qualitative Analysis (v.10.0), and Unknown Analysis (v.10.1).

2.6. Statistical analysis

Data sets were analyzed with R Studio (Version 2023.6.2.561, R Version 4.3.3), using the R packages ggplot2, readxl, tidyverse, writexl, stringi, utils, RcolorBrewer, purr, ggsignif, ggpubr, readr and scales.

2.7. Pre-analysis of LC-MS, GC-MS and GC-MS volatile dataset

An analysis focusing on presence/absence of the metabolites in yoghurts to reveal general trends across all three datasets was conducted. The LC-MS dataset originally comprised 17,310 metabolites. Metabolites with a mean concentration of < 3x blank mean were excluded from the analysis, leaving 5607 compounds. In GC-MS, 10,936 metabolites were analyzed without blank filtering. In the GC-MS Volatile dataset, 6305 compounds were analyzed without blank filtering.

2.8. Selection criteria for identification of 35 metabolites produced in enriched yoghurts

For metabolite identification, the coefficient of variation (CV) of each measured compound was calculated (equation (1)), by dividing the standard deviation of the relative concentrations from all yoghurts (σ) by the mean of all yoghurts (μ):

$$CV = \frac{\sigma}{\mu} \quad (1)$$

The identification strategy followed a stepwise logic: first, screening of compounds that were present both in starter and enriched yoghurts with high CV difference between the yoghurts, followed by a screening for new metabolites present in the enriched yoghurts. After that, it was checked that preselected metabolites had high correlation between the technical replicates of a yoghurt and whether annotation compounds from Progenesis suggested ≤ 5 potential metabolites. Identified metabolites were then evaluated for scientific evidence supporting their potential health benefits in the host, based on a non-systematic literature search. Following this strategy, 48 compounds of interest were identified in the LC-MS dataset. Another 8 compounds were included that did not fulfill the criteria of the identification strategy, but were reported to have potential health benefits for the host. To these 56 compounds of interest, further criteria for selection were applied such as: i) plausibility of abundance in yoghurt, ii) plausibility of production during fermentation, and iii) relevance of the biological effect. By applying these second set of criteria, 17 of 56 compounds were selected for further analysis. Similar criteria were applied to the GC-MS dataset, resulting in the selection of 16 compounds. For the compound screening in the GC-MS volatile dataset, a more complex selection was performed. In the GC-MS volatile dataset, not all compounds correlated well between the two replicates. To reduce the likelihood of detecting random contaminants, correlation between the two replicates was used as an important criterion for further investigation. Spearman's rank correlation coefficient and the CV was calculated for every compound. Compounds with a correlation coefficient >0.6 were kept, resulting in 61 compounds. Second, the CVs between the two replicates were calculated for each yoghurt and for each compound. The remaining compounds ($CV < 1$) were all visualized and kept or opt-out based on manual inspection. This selection resulted in a total of 18 compounds. The second approach was to select the 200 compounds with the highest CV (indicating high variation between yoghurts) and good correlation between sample 1 and 2 for manual inspection. Applying this strategy, 6 compounds were selected for identification. An additional 17 health-related compounds were selected based on automatically suggestion by Profinder with high math factor (>85). Thus, a total of 41 compounds were analyzed using Unknown Analysis (v.10.1), MassHunter Agilent Qualitative (v.10.0) and MassHunter Agilent Quantitative (v.10.1). For each compound, the Kovats' retention index (RI) was calculated. RI and compound spectra from the chromatograms were compared with the NIST library for identification of the compound. Twenty-one different compounds could be identified at level 2 (match factor >80 %, deviation from RI < 10 units; Rochat, 2017; Sumner et al., 2007). For these 21 compounds the iAUC of a characteristic ion of the compound spectrum was calculated, using MassHunter Agilent Quantitative Analysis, to obtain their relative concentration. Three compounds were excluded due to ambiguous/overlapping peaks, resulting in 18 compounds total.

3. Results

3.1. The metabolomic repertoire of a yoghurt is increased by adding an adjunct strain to the starter yoghurt

The aim of this study was to create yoghurts with additional health benefits by enriching the metabolite repertoire of the yoghurts with substances that are particularly beneficial to human health. For that, 183 strains from the strain collection of the Swiss Confederation's center of excellence for agricultural research (Agroscope) were selected based on their genomic potential to produce metabolites relevant to human health, such as for example indole compounds (Table 1). The 183 strains comprise 19 different species ranging over 9 genera (Table 1). Three starter yoghurts were produced as controls, consisting of milk fermented with commercially available starter cultures. Next, 183 enriched yoghurts were generated by simultaneously adding an adjunct strain to the starter yoghurt (Table 1). Coupling untargeted LC-MS, GC-MS and GC-MS volatile measurements allowed to capture the metabolic repertoire in the enriched yoghurts.

In starter yoghurts, a median of total metabolites of 3311 ± 127 (standard error of median) was measured by LC-MS (Fig. 1A). In the GC-MS and GC-MS volatile datasets, a median of total metabolites of 241 ± 14.2 and 5073 ± 61.2 was measured in starter yoghurts (Supplementary Fig. 1C). Since the taxonomy of the genus *Lactobacillus* underwent major reclassification (Zheng et al., 2020), it is noted that throughout this manuscript, the species *Lacticaseibacillus paracasei, Lentilactobacillus parabuchneri*, *Lactobacillus delbrueckii* subsp *lactis, Lactobacillus delbrueckii* subsp *bulgaricus, Lentilactobacillus parafarraginis*, *Lacticaseibacillus rhamnosus*, *Lactiplantibacillus plantarum* and *Lactobacillus helveticus* were all assigned to *Lactobacillus*.

In the LC-MS dataset, yoghurts enriched with a *Lactobacillus, Lactococcus* or *S. thermophilus* strain (Table 1) contained more metabolites than the starter yoghurts, with a median of total metabolites measured of 3555 ± 17.4 for *Lactobacillus,* 3495 ± 11.9 for *Lactococcus* and 3556 ± 26.9 for *Streptococcus* (Fig. 1A). In yoghurts enriched with a *Propionibacterium* strain (Table 1), the median total metabolites measured was 3316 ± 27.7 (Fig. 1A). In the GC-MS dataset, the median of total

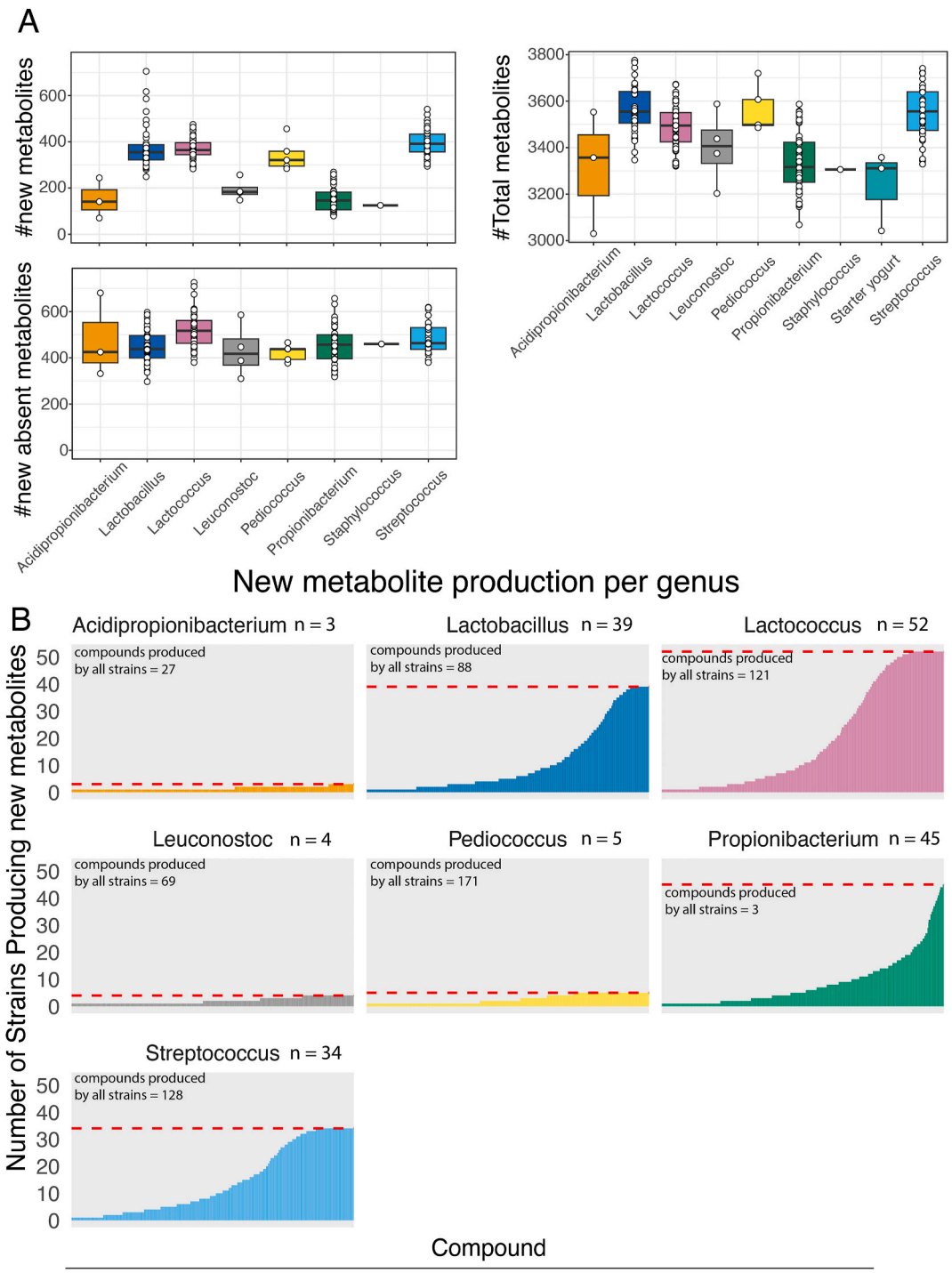

Fig. 1. Metabolic repertoire assessment of 183 enriched yoghurts spanning nine genera. (A) Boxplot showing number of new metabolites, new absent metabolites and total metabolites abundant in the new yoghurts. Central line in the boxplot represent median, lower whisker shows ≥ Q1 - 1.5 * IQR and upper whisker shows ≤ Q3 + 1.5 * IQR. (B) Barplot showing all new metabolites produced by any enriched yoghurt that has a strain of the respective genus as the adjunct strain. Red dash line intercepts with y-axis at the total of strains per genus. LC-MS, liquid chromatography–mass spectrometry; Q, quartile; IQR, interquartile range.

metabolites in enriched yoghurts was more evenly distributed, with a median of 239 ± 3.69 for *Lactobacillus,* 239 ± 3.3 for *Lactococcus,* 242 ± 3.2 for *Streptococcus* and 246 ± 3.97 for *Propionibacterium* (Supplementary Fig. 1A). Contrary to the LC-MS dataset, in the GC-MS volatile dataset, highest median of total metabolites was measured in yoghurts enriched with an *Acidipropionibacterium* strain (5228 ± 71.7), followed by yoghurts enriched with a *Propionibacterium* strain (5117 ± 9.97) (Table 1, Supplementary Fig. 1C). In yoghurts enriched with a *Lactobacillus, Lactococcus* or *Streptococcus* strain (Table 1), the lowest median for total metabolites was measured, with 4957 ± 16.5 for *Lactobacillus,* 4878 ± 28.9 for *Lactococcus* and 4944 ± 23.1 for *Streptococcus* (Supplementary Fig. 1C). Thus, the detection of a specific set of metabolites depends on the method used (Wang et al., 2024). This is valuable information that needs to be taken into account when targeting metabolites in yoghurts produced with bacteria from different genera.

Thereafter, it was investigated how many additional metabolites were detected in the enriched yoghurts that were absent in the starter yoghurts. Similar results as for total metabolites were observed: in

yoghurts enriched with a *Lactobacillus, Lactococcus*, *Streptococcus* or *Pediococcus* strain, more new metabolites were measured by LC-MS (Fig. 1 A). In yoghurts enriched with a *Propionibacterium* strain, only 146 ± 13.8 new metabolites were detected (Fig. 1A). In the GC-MS dataset, detection of new metabolites was evenly distributed among the genera, with a median of 40 ± 2.64 for *Lactobacillus*, 35.5 ± 1.68 for *Lactococcus*, 34 ± 1.54 for *Streptococcus*, 37 ± 3.31 for *Pediococcus* and 34.5 ± 1.2 for *Propionibacterium* (Supplementary Fig. 1A). In the GC-MS volatile dataset, most new metabolites were detected in yoghurts enriched with an *Acidipropionibacterium* or *Propionibacterium* strain, with a median of 117 ± 22.8 for *Acidipropionibacterium* and 101 ± 6.1 for *Propionibacterium.* For *Lactobacillus, Lactococcus* and *Streptococcus,* the median of new metabolites detected was 20 ± 3.98, 24 ± 2.56 and 18.5 ± 2.79 (Supplementary Fig. 1C). In sum, LC-MS captures more new metabolites in yoghurts enriched with an adjunct strain from the genera *Lactobacillus, Lactococcus* and *Streptococcus,* whereas GC-MS volatile captures more new metabolites in yoghurts enriched with an adjunct strain from the genera *Acidipropionibacterium* and *Propionibacterium*. Efficiency of capturing new metabolites with GC-MS is the same for all investigated genera.

In a next step, numbers of absent metabolites were assessed, consisting of metabolites that were detected in the starter yoghurts but not in enriched yoghurts. Interestingly, the number of absent metabolites were similar in all enriched yoghurts measured by LC-MS (Fig. 1A), ranging between a median of 418 ± 63.2 (*Leuconostoc*) and 517 ± 17.1 (*Lactococcus*) (Fig. 1A). In the GC-MS dataset, absent metabolites were evenly distributed among the genera, with a median of absent metabolites of 160 ± 2.98 for *Lactobacillus*, 156 ± 2.1 for *Lactococcus*, 151 ± 3.08 for *Streptococcus*, 142 ± 19.9 for *Pediococcus* and 149 ± 2.57 for *Propionibacterium* (Supplementary Fig. 1A). In the GC-MS volatile dataset, yoghurts enriched with an *Acidipropionibacterium* and *Propionibacterium* strain, although showing the highest number of new metabolites as previously mentioned, had the lowest level of absent metabolites. The median of absent metabolites was 226 ± 47.8 for *Acidipropionibacterium* and 310 ± 11.6 for *Propionibacterium.* For *Lactobacillus, Lactococcus* and *Streptococcus,* the median of absent metabolites was 391 ± 11.5, 483 ± 24.7 and 409 ± 24.6 (Supplementary Fig. 1C). In sum, the same findings were obtained for the detection of total and new metabolites as for the detection of absent metabolites.

To better understand appearance of new metabolites in enriched yoghurts, coverage of new metabolites in yoghurts enriched with strains from the same genus was investigated. In the LC-MS dataset, a higher median of total metabolites in enriched yoghurts correlated with a higher metabolite coverage within a genus (Fig. 1A and B). For example, 4 enriched yoghurts were produced with a *Leuconostoc* strain (median total metabolites = 3406 ± 91.1), and 5 with a *Pediococcus* strain (median total metabolites = 3498 ± 68.3), but the coverage of new metabolites by all *Pediococcus* strains was 171, whereas for *Leuconostoc,* it was only 69 new metabolites (Fig. 1B). In the GC-MS dataset, coverage of new metabolites in any of the yoghurts enriched with strains from a certain genus were not detected (Supplementary Fig. 1B). In the GC-MS volatile dataset, highest coverage of new metabolites by any strain of a certain genus was found in *Acidipropionibacterium* and *Propionibacterium*, with a coverage of 38 and 16 new metabolites respectively. Coverage of new metabolites in yoghurts enriched with a *Lactobacillus, Lactococcus* and *Streptococcus* strain was zero, one and zero (Supplementary Fig. 1D). In sum, the metabolic repertoire of a yoghurt increases when an adjunct strain is added, and this is not an artefact introduced by the choice of total strains per genera. Furthermore, a clear correlation was observed between total metabolites in yoghurts enriched with an adjunct strain within a genus and abundance and coverage of new metabolites. However, this is at least partially dependent on the method used, since LC-MS is the most comprehensive method in metabolomics research for the analysis of non-volatile compounds and large or thermally unstable compounds, while GC-MS mainly concentrates on volatile organic compounds, lipids and derivatisable molecules, highlighting the importance to carefully consider which mass spectrometry-based method to use for the detection of metabolites in yoghurts (Wang et al., 2024). Production of new metabolites could be at the expense of metabolites already present in the yoghurt matrix such as amino acids like tryptophan or metabolites produced by the starter strains. Emergence of new metabolites could be paralleled by the loss of such metabolites, resulting in absent metabolites in the enriched yoghurts. Yet, no positive correlation was found between new and absent metabolites in any of the genera (Fig. 2A and B). Although abundant metabolites in the yoghurt, either available from the matrix or produced by the present starter strains, are further metabolized by the adjunct strain and results in the abundance of new metabolites, emergence of new metabolites does not result in total consumption of a metabolite.

3.2. The repertoire of new metabolites is sufficient to cluster yoghurts enriched with adjunct strains from the same genus

Further, it was examined whether the set of new metabolites produced by a strain serves as a feature that enables clustering of enriched yoghurts with adjunct strains from the same genus. To explore this possibility, a uniform manifold approximation and projection (UMAP) analysis was performed. We have recently demonstrated that the total set of metabolites from enriched yoghurts measured by LC-MS and GC-MS volatile allows for clear cluster separation of strains from the same genus (Roder et al., 2024). In this study, only the set of new metabolites measured in the LC-MS dataset was used as input data. Surprisingly, this was sufficient for clear, unbiased clustering of enriched yoghurts with adjunct strains from the same genus. Statistical significance was confirmed by Permutational Multivariate Analysis of Variance (PERMANOVA, scikit-bio 0.6.3) with a pseudo-F statistic of 3.827843 and a corresponding p-value of 0.0175. The clusters of yoghurts enriched with a *Streptococcus*, *Lactococcus*, *Lactobacillus* and *Pediococcus* strain clearly separated from the clusters of yoghurts enriched with a *Acidipropionibacterium*, *Propionibacterium* and *Leuconostoc* strain (Fig. 2C). Whereas clear cluster separation was further found for most *Streptococcus*, *Lactococcus* and *Lactobacillus* enriched yoghurts, the yoghurts enriched with a *Pediococcus* strain clustered together with yoghurts enriched with a *Lactobacillus* strain. Hence, yoghurts enriched with a *Pediococcus* strain are not distinguishable from yoghurts enriched with a *Lactobacillus* strain based on the set of new metabolites in these yoghurts. The same is true for the yoghurts enriched with an *Acidipropionibacterium*, *Propionibacterium* and *Leuconostoc* strain, which clustered together and thus were not distinguishable based on their set of new metabolites. In sum, these data indicate that the repertoire of new metabolites in yoghurts is a feature that allows to distinguish yoghurts enriched with adjunct strains from different genera.

3.3. A set of enriched yoghurts is distinct in its capacity to produce unique metabolites

In some yoghurts, the production of unique metabolites was detected, defined as new metabolites that are only found in one of all assessed yoghurts. In the LC-MS dataset, 60 out of 183 yoghurts had at least one compound that was unique to them. With 88 abundant unique metabolites *Lactobacillus helveticus* (FAM22081) was ranked at the top (Fig. 3A). For this strain, a total of 3771 metabolites was measured, 705 new metabolites and 575 absent metabolites. In a yoghurt enriched with a *Pediococcus* strain (FAM18987), 14 unique metabolites were measured and in two yoghurts enriched with a *L. rhamnosus* strain (FAM20440 and FAM17361), 11 unique metabolites were measured (Fig. 3A). Also, yoghurts enriched with an adjunct *Propionibacterium strain* were top unique metabolite producers. In a yoghurt enriched with a *Propionibacterium freudenreichii* strain (FAM19022), 6 unique metabolites were measured. In yoghurts enriched with three other strains from the genus *Propionibacterium,* 3 unique metabolites were measured. In GC-MS and GC-MS volatile, unique metabolites were detected in 95 and 42 of all

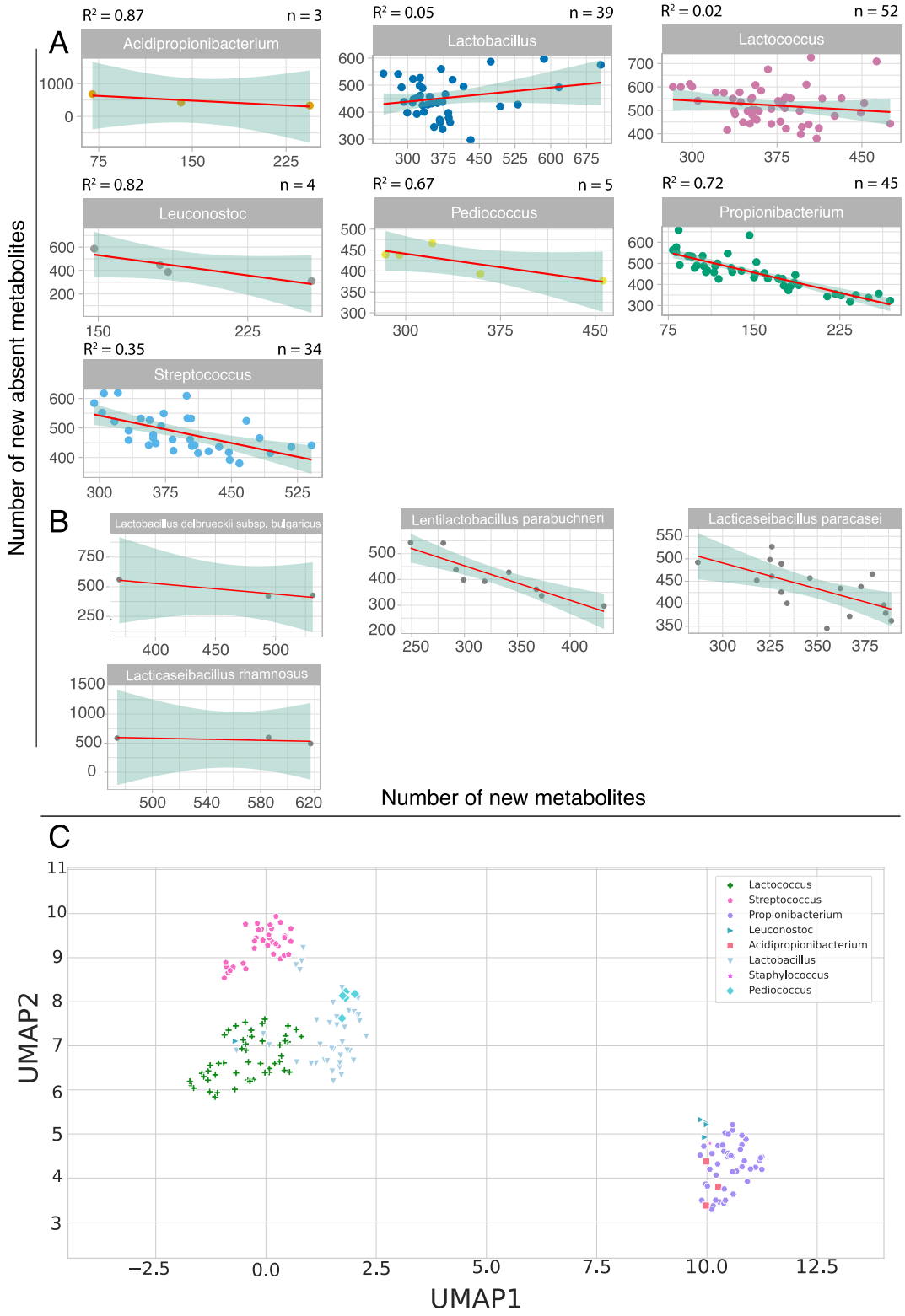

Fig. 2. Set of new metabolites is sufficient for clustering of enriched yoghurts on the genus-level. (A) Correlation plot comparing number of new metabolites versus new absent metabolites in enriched yoghurts from all genera. (B) Correlation plot comparing number of new metabolites versus new absent metabolites in enriched yoghurts and adjunct *Lactobacillus* strain. Red line in A and B represents regression line for linear regression, green area represents the standard error. (C) UMAP shows clustering of enriched yoghurts based on the detection of new metabolites. Detection of metabolites was performed by LC-MS. Data are shown from one experiment with technical triplicates for each yoghurt. UMAP, Uniform Manifold Approximation and Projection.

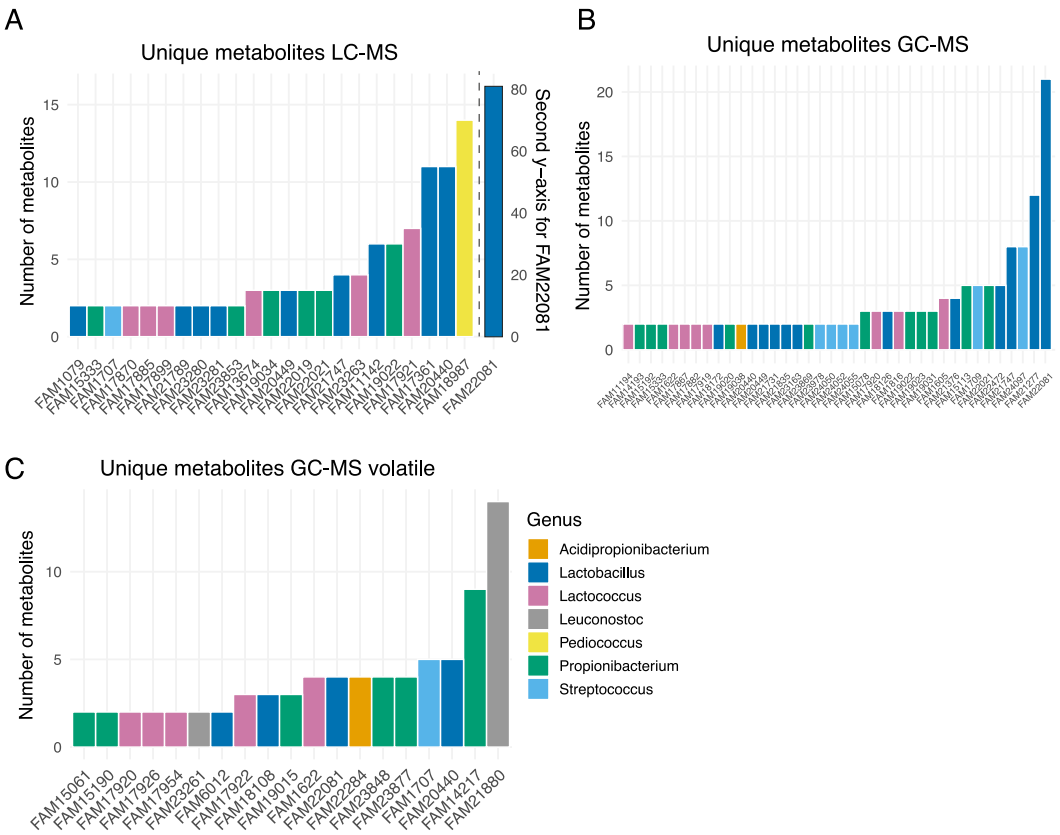

Fig. 3. Yoghurts enriched with an adjunct strain from several genus produce unique metabolites. Strains are depicted that at least produce two unique metabolites, measured by LC-MS (A), GC-MS (B) or GC-MS volatile (C). LC-MS, liquid chromatography–mass spectrometry, GC-MS, gas chromatography–mass spectrometry.

yoghurts (Fig. 3B and C).

In the LC-MS dataset, many identified unique metabolites were assigned to be gangliosides. In the yoghurt enriched with *Lactobacillus helveticus* FAM22081, 7 of the 88 unique metabolites were identified as gangliosides. However, although some studies exist that demonstrate the ability of bacteria to modify and/or degrade gangliosides present in bovine or human milk, in general, bacteria do not possess the enzymatic sets for ganglioside metabolization. Mapping genes encoding for ganglioside modification in the *OpenGenomeBrowser* (Roder et al., 2022) across all the 183 strains revealed the presence of three enzymes hypothetically associated with ganglioside metabolism. These three enzymes are β-galactosidase (EC 3.2.1.23), α1,2/α1,3-L-fucosidase (EC 3.2.1.51) and endo-β-1,3-*N*-acetylglucosaminidases (EC 3.2.1.52). Although β-galactosidase can metabolize gangliosides, it is more likely that in yoghurt, its main activity is to metabolize lactose into glucose and galactose. α1,2/α1,3-L-fucosidase can metabolize fucosyl-GM1 (Sugiyama et al., 2017). However, in the dataset, fucosyl-GM1 was not detected in the starter yoghurts, thus it is unlikely that in the yoghurts, fucose is detached from fucosyl-GM1 via enzymatic action of α1,2/α1,3-L-fucosidase. β-*N*-acetylhexosaminidase is an enzyme with the main function to produce peptidoglycans for bacterial cell walls and it is more plausible that this is the main activity in yoghurt. Gangliosides are important components of human breast milk and are thought to be important for the infants' gut health. Degradation of bovine milk gangliosides by infant-gut associated bifidobacteria has been demonstrated (Lee et al., 2014) and it is theoretically possible, that other strains have this capacity too, but studies investigating this are scarce. Although indications exist that some strains might have the capacity to metabolize or degrade gangliosides present in the yoghurt, more in-depth investigations would be needed to draw any conclusion.

3.4. Identification of 35 health-related metabolites enriched in yoghurts with adjunct strains by targeted metabolomics

Investigations were conducted to identify health-related metabolites abundant in enriched yoghurts. By applying several selection criteria (see methods), 17 candidates in the LC-MS dataset, 16 in the GC-MS dataset and 41 in the GC-MS volatile dataset were identified. Next, measurements of pure standards were performed and were able to identify 5 compounds in the LC-MS dataset, 12 compounds in the GC-MS dataset and 11 compounds in the GC-MS volatile dataset. Seven more compounds in the GC-MS volatile dataset were identified by using CV and correlation between the two batches. The relative concentration of these metabolites in the yoghurts was calculated and it was determined, in which yoghurt the highest relative concentrations was detected (Supplementary Table 1). For several species, high production of short-chain fatty acids (SCFAs) was found. For example, significantly different concentrations of acetic acid compared to starter yoghurts were found for *L. parabuchneri* ($p = 0.034$), *L. paracasei* ($p = 0.004$), *Leuconostoc mesenteroides* ($p = 0.029$), and *P. freudenreichii* ($p = 3 \times 10^{-5}$) (Fig. 4A). High concentrations of acetic acid in *L. helveticus* were observed, although significance was not reached, presumably due to low sample size (Fig. 4A). Additionally, significant differences in the relative concentration of butyric acid in *L. paracasei* ($p = 0.009$) and *P. freudenreichii* ($p = 3 \times 10^{-5}$) were observed, with high concentrations in other propionic acid producing bacteria (PAB) (Fig. 4B). Propionic acid levels were highest in yoghurts enriched with a *P. freudenreichii* strain ($p = 3 \times 10^{-5}$), followed by other PAB, such as *A. acidipropionici* and *A. thoenii,* although in these two latter species, significance was not reached (Fig. 4C). Relative concentrations of aromatic compounds were determined by GC-MS volatile and some of the most extreme differences between enriched yoghurts and starter yoghurt were observed in this

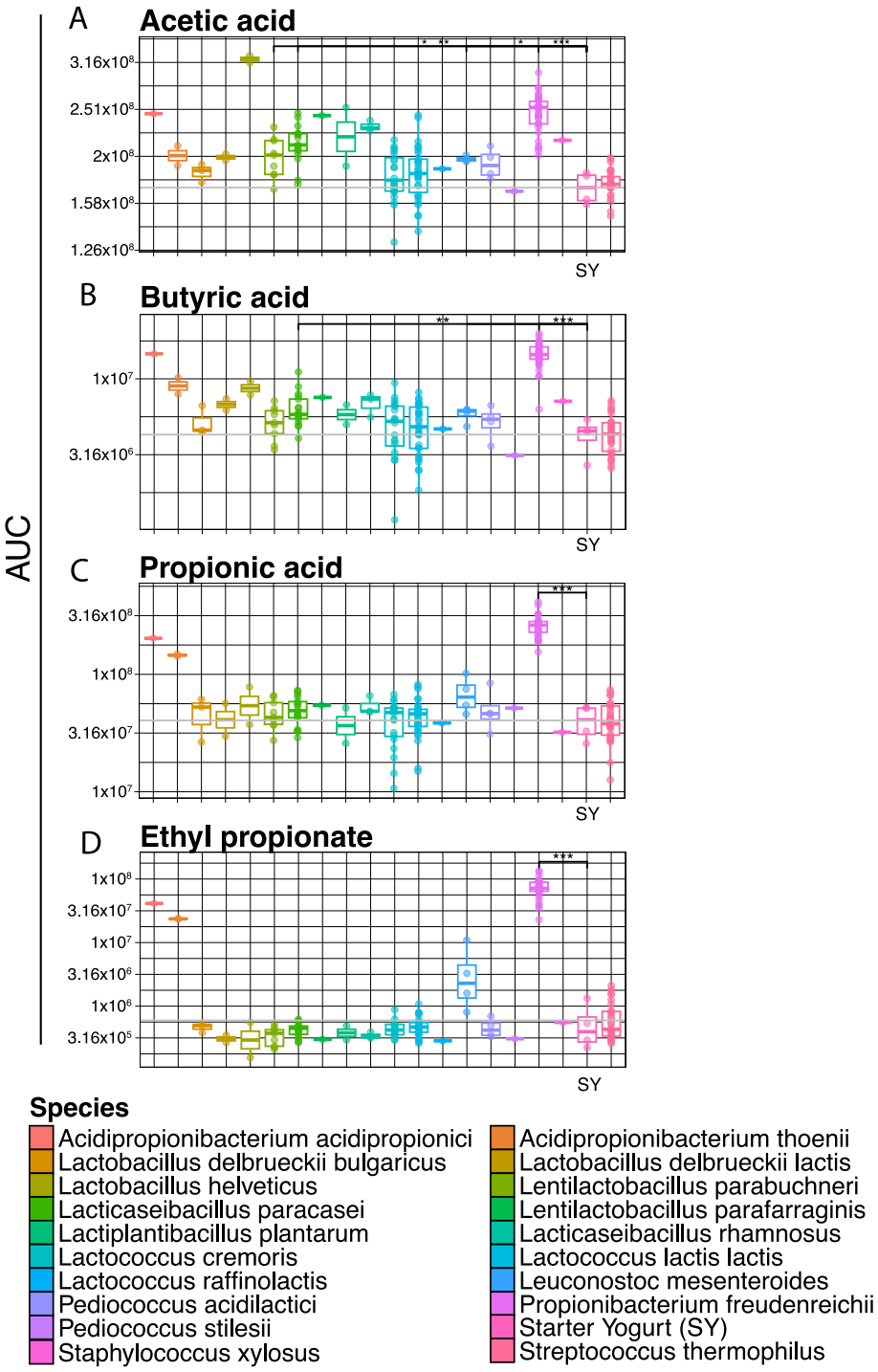

Fig. 4. Enrichment of SCFAs in a set of yoghurts. AUC measured for each yoghurt, grouped by species for acetic acid (A), butyric acid (B), propionic acid (C) and ethyl propionate (D). The gray line represents the mean value of the starter yoghurts. Each point represents the mean value of all analytical replicates from one yoghurt. Mann-Whitney-Wilcoxon test was performed to test for significant differences between each species and starter yoghurts. The level of significance is indicated by the following symbols: ***p < 0.001; **p < 0.01; *p < 0.05; N.S., p > 0.05. AUC, area under the curve; BCAAs, branched-chain amino acids.

compound class. For example, a 224-fold increase of the aromatic compound ethyl propionate was detected in the yoghurt enriched with *P. freudenreichii* (FAM14221), whereas the lowest concentration was measured in the yoghurt enriched with *L. helveticus* (FAM22081) (Fig. 4D). Furthermore, yoghurts enriched with *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, and *L. helveticus* were found to have high relative concentrations of the branched-chain amino acids (BCAAs) valine, leucine and isoleucin (Fig. 5A–C). Additionally, in yoghurts enriched with *P. freudenreichii* strains, higher valine ($p = 0.0047$) and isoleucine ($p = 0.0142$) levels were found compared to starter yoghurts (Fig. 5A and C). Valine levels were also increased in yoghurts enriched with *S. thermophilus* ($p = 0.0018$) and *L. parabuchneri* ($p = 0.0091$) strains (Fig. 5A). Furthermore, higher levels of 3-phenyllactic acid (PLA) were found in yoghurts enriched with *L. helveticus* strains compared to starter yoghurts, although without reaching statistical significance (Fig. 5D). Interestingly, higher gamma-aminobutyric acid (GABA) levels were detected in yoghurts with adjunct *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, and *L. helveticus* strains compared to starter

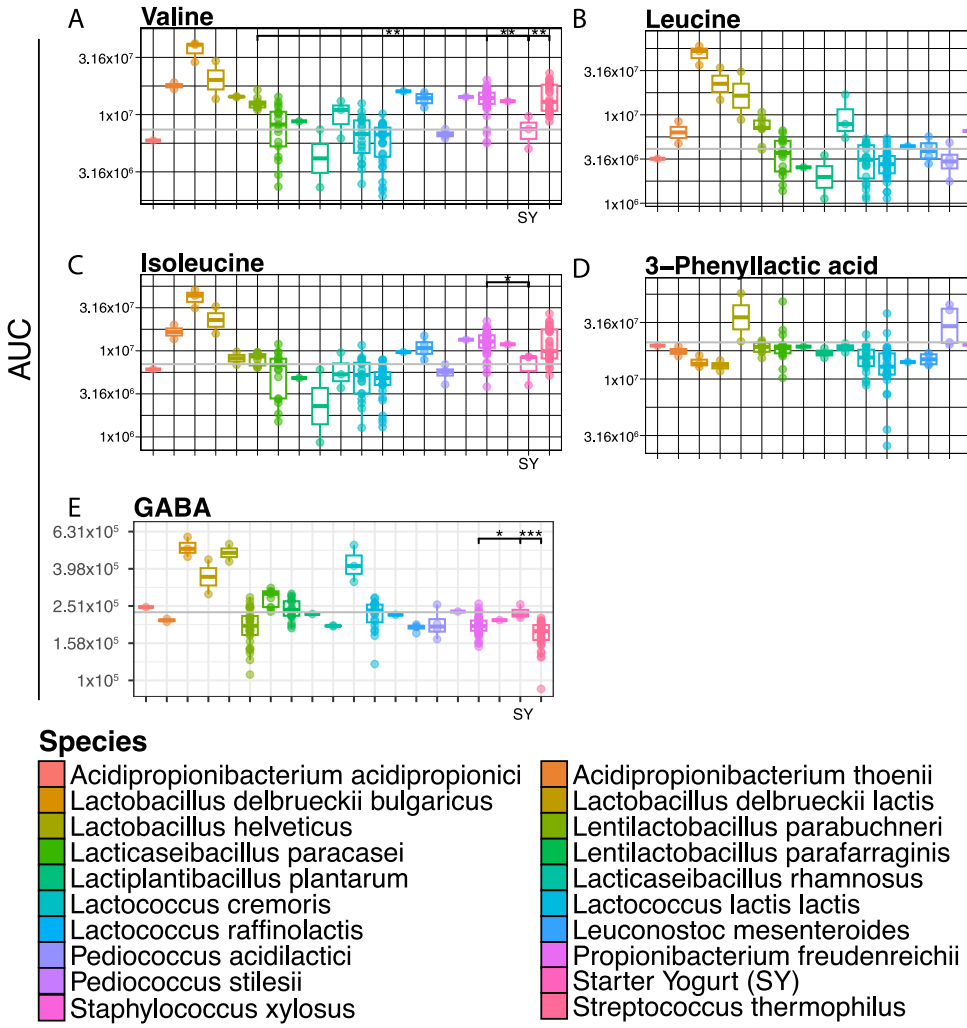

Fig. 5. Enrichment of BCAAs and 3-phenyllactic in a set of yoghurts. AUC measured for each yoghurt, grouped by species for valine (A), leucine (B), isoleucine (C) and 3-phenyllactic acid (D). The gray line represents the mean value of the starter yoghurts. Each point represents the mean value of all analytical replicates from one yoghurt. Mann-Whitney-Wilcoxon test was performed to test for significant differences between each species and starter yoghurts. The level of significance is indicated by the following symbols: ***p < 0.001; **p < 0.01; *p < 0.05; N.S., p > 0.05. AUC, area under the curve; BCAAs, branched-chain amino acids.

yoghurts, although without reaching statistical significance (Fig. 5E). Compared to starter yoghurts, significant lower GABA levels were detected in yoghurts enriched with an adjunct *Propionibacterium freudenreichii* or *S. thermophilus* strain (Fig. 5 E), indicating that those strains might consume GABA. The results of higher GABA levels for two *Lactobacillus delbrueckii* subsp. *bulgaricus* strains were reproduced and lower GABA levels were detected in yoghurts enriched with *Lactococcus cremoris* strains (Fig. 10). These results together demonstrate that yoghurts enriched with strains from different genera have distinct SCFAs, BCAAs and PLA production profiles and have the potential to increase concentrations of the neurotransmitter GABA.

To expand the analyses, unsupervised clustering was performed based on the concentration of 33 health-related metabolites in the enriched yoghurts. One cluster was detected with yoghurts enriched with a *P. freudenreichii* strain with much higher mean concentrations of propionic acid, butyric acid and ethyl propionate (Fig. 6, cluster 1). In this cluster, high levels of the SCFA pentanoic acid were found and the medium-chain fatty acids (MCFAs) decanoic and hexanoic acid. Another cluster with yoghurts enriched with *P. freudenreichii* and *S. thermophilus* produced low levels of SCFAs and MCFAs (Fig. 6, cluster 2). A further yoghurt cluster was detected with low abundance of aromatic acids such as 2-undecanone, 2-nonanone and 2-heptanone (Fig. 6, cluster 3).

To reproduce these findings, the production of 9 yoghurts was repeated and relative concentrations of 12 compounds identified by GC-MS was determined (Fig. 7). For the remake, yoghurts were chosen that had the highest concentrations of these compounds in the first experiment (Supplementary Fig. 2). Furthermore, it was investigated whether the presence of lactose in the milk used for yoghurt preparation influenced the levels of health-related compounds. To test this, enriched yoghurts both in lactose-free (LF) milk and lactose-containing (L) milk were produced. Additionally, non-fermented LF and L milk was included. As expected, higher relative concentrations of PLA, lactic acid and DL-indole-3-lactic acid (ILA) were detected in LF and L yoghurt with starter strains compared to LF and L milk only (Fig. 7). Interestingly, none of the BCAAs were detected in any of the milk samples (Fig. 7). In general, the findings of higher relative concentrations for most amino acids were reproduced, namely for leucine, isoleucine, valine, glutamic acid, tryptophan, phenylalanine, and glycine (Fig. 7). It is noteworthy to mention that tryptophan was measured in much lower concentrations in the L-compared to LF-based yoghurts (Figs. 8 and 9). This explains the higher ILA levels in LF- compared to L-based yoghurts (Figs. 8 and 9), considering that this compound is metabolized by bacteria from tryptophan. The highest relative concentration of ILA was detected in the LF-based yoghurt with the *P. acidilactici* (FAM20325) strain (Fig. 9). Another interesting finding is the high relative concentration of PLA in *L. helveticus* (FAM22081), compared to all other yoghurt remakes

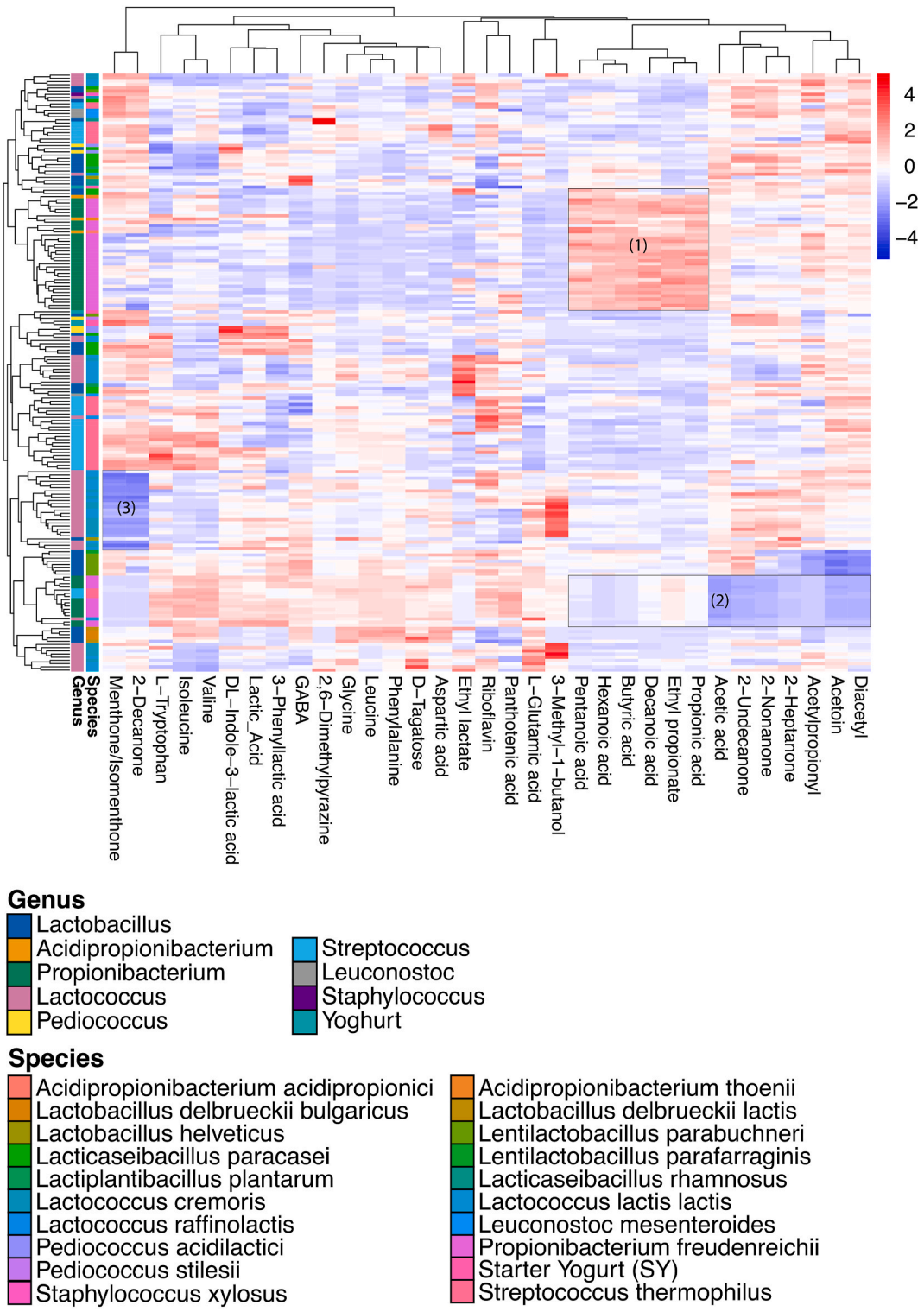

Fig. 6. Enriched yoghurts cluster based on the detection of specific health-related metabolites. Heatmap representation of metabolites identified by targeted metabolomics showing normalized (z-score) values for each yoghurt produced.

(Fig. 8). High proteolytic activity in *L. helveticus* FAM22081 may increase the phenylalanine level in the yoghurt matrix, enabling the starter cultures to increase the formation of PLA. In a future study, it would be interesting to investigate the interaction of these strains leading to the increased PLA level.

4. Discussion

Commercial yoghurt production exhibits a limited range of microbial diversity. However, increasing microbial diversity in yoghurt could improve the beneficial effects that yoghurt has on human health. Therefore, the possibilities of enriching yoghurts with the addition of adjunct strains to the starter culture was explored. A total of 183 enriched yoghurts were produced and metabolomic screening was

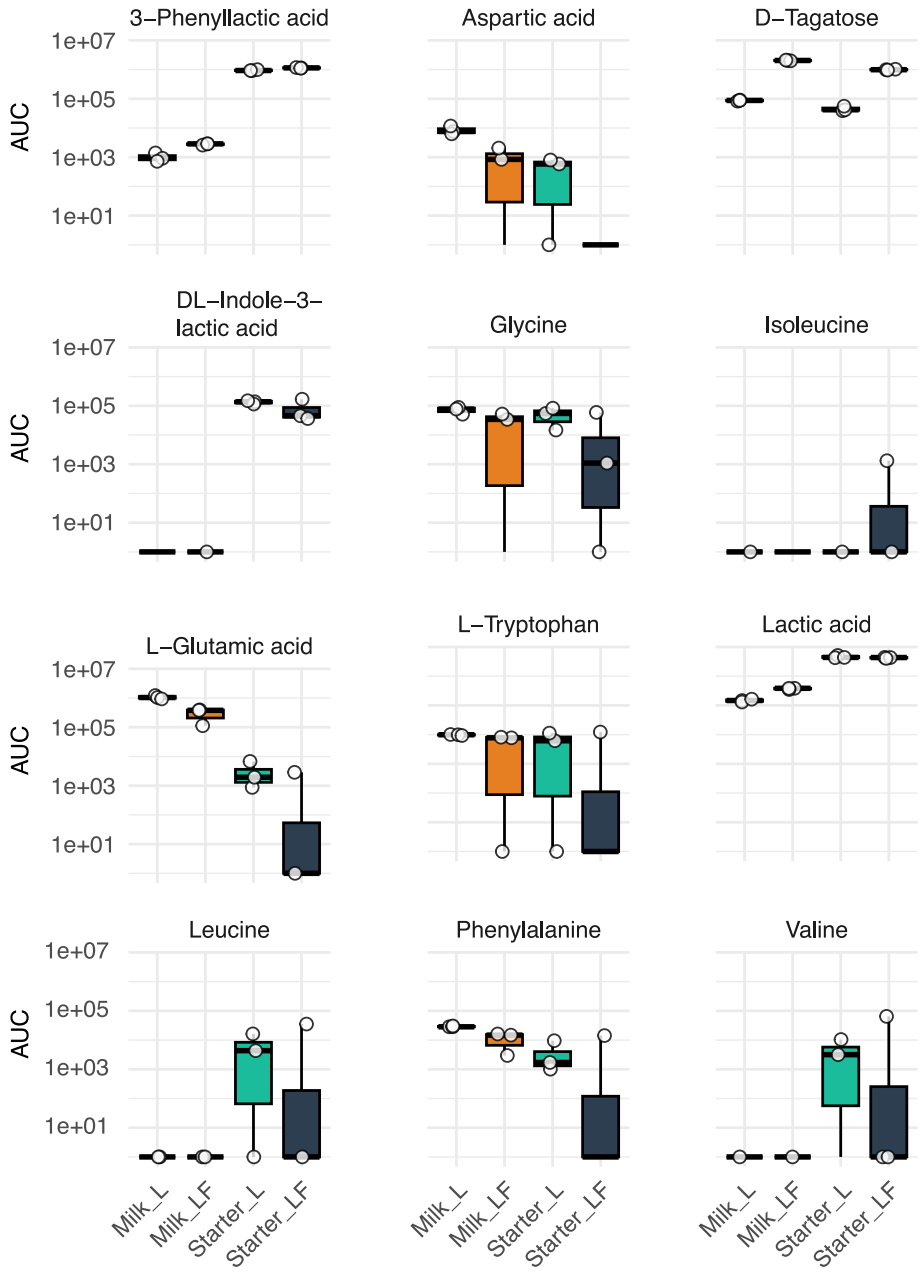

Fig. 7. Relative fold-changes of identified metabolites with potential health benefits in starter yoghurt remakes with lactose-containing yoghurt. (A) Relative concentration of 12 health-associated metabolites in lactose-containing milk, lactose-free milk, lactose-containing starter yoghurts and lactose-free starter yoghurts. AUC, Area under the curve; L, lactose; LF, lactose-free.

performed using LC-MS, GC-MS and GC-MS volatile, which allowed detecting a wide spectrum of compounds. 35 health-related or aroma compounds were identified, and their relative concentrations were assessed in the yoghurts. Significant differences in the relative concentrations of metabolites were observed between yoghurts made with different strain combinations, particularly those involving strains from different genera. Similarly, increased SCFA levels were detected in yoghurts enriched with strains from the genus *Propionibacterium* and *Acidipropionibacterium.* Beneficial effects of SCFAs are well documented and include regulation of mucosal and systemic immunity, increased intestinal barrier integrity, stimulation of mucin synthesis and quality, production of antimicrobial peptides and beneficial effects on metabolic health (Blaak et al., 2020; Mann et al., 2024). These findings are in line with a study that reported increased production of propionic acid in starter yoghurts enriched with *Propionibacterium* strains (Ekinci & Gurel, 2008). Several studies investigated the beneficial effects on the host health upon feeding with *P. freudenreichii*. *In vitro* exposure of the *P. freudenreichii* strain BIA129 to peripheral blood mononuclear cells (PBMCs) induced secretion of IL-10 in the latter (Foligné et al., 2010). In the same study, administration of *P. freudenreichii* BIA129 prevented acute colitis after administration of Trinitrobenzenesulfonic acid (TNBS) in BALB/c mice. In another study, colonic mucosa explants from piglets that received *P. freudenreichii* BIA129 secreted less IL-8 and TNF-α upon lipopolysaccharide (LPS) challenge and piglets receiving *P. freudenreichii* BIA129 consumed more feed and gained more weight (Cousin et al., 2012). These studies highlight the probiotic potential of *P. freudenreichii* in different situations, such as supporting the growth of an organism or dampening exaggerated responses in colitogenic situations. Therefore, this data could be used to screen and identify *P. freudenreichii* strains with distinct properties that could further be investigated for their beneficial effects on the host in hypothesis driven experiments.

Elevated BCAAs levels such as valine and leucine were found in

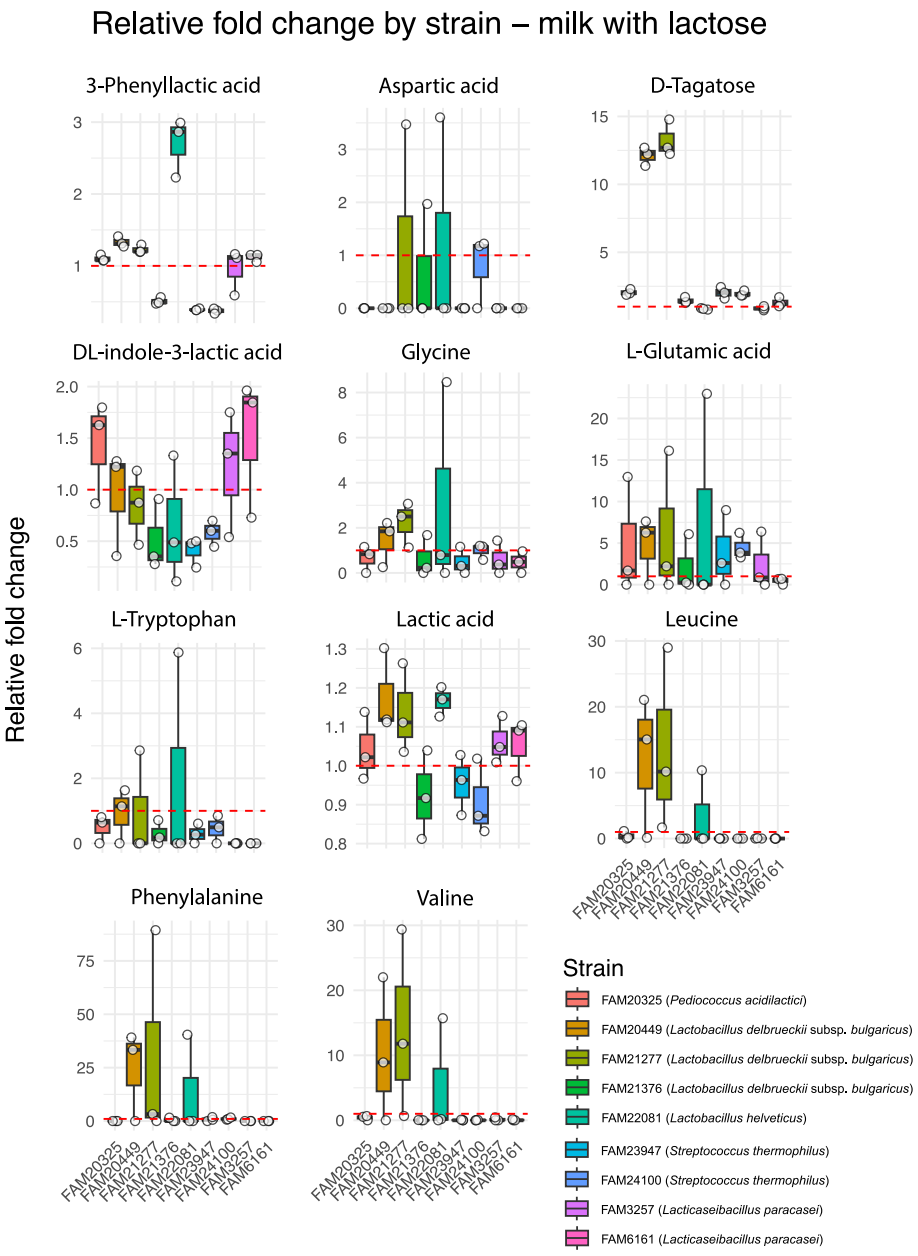

Fig. 8. Relative fold-changes of identified metabolites with potential health benefits in yoghurt remakes with lactose-containing milk, measured by GC-MS. (A) Relative concentration of 12 health-associated metabolites by targeted metabolomics in different by lactose-containing enriched yoghurts with a *Pediococcus*, *Streptococcus* or *Lactobacillus* strain characterized by exceptionally high relative concentrations of amino acids. The red line represents the starter yoghurts concentration, which was normalized to 1.

yoghurts enriched with *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis* strains, and to a lesser extent in some strains of *L. helveticus* and *L. rhamnosus*. These higher concentrations of free amino acids might be explained by the proteolytic activity of those adjunct strains. Increased uptake of BCAAs can improve metabolic health, and support protein synthesis for muscle growth, especially in the context of healthy aging (D'Antona et al., 2010; Kirk et al., 2021). Thus, these yoghurts with increased BCAA levels could be harnessed as potential supplementations in the elderly population to support healthy aging and prevent age-related muscle loss.

High levels of PLA were detected in the yoghurt enriched with *L. helveticus* (FAM22081). Recently, PLA has been identified as a metabolite that mitigates age-associated declines and extends health span in a longevity model with *C. elegans* (Kim et al., 2024). PLA is found in fermented foods, such as yoghurt (Pimentel et al., 2018), cheese (Trimigno et al., 2018) and others (Peters et al., 2019). We have shown that serum levels of PLA increases upon ingestion of cheese but not milk or soy drink (Trimigno et al., 2018). PLA was detected in high levels in the serum, sufficient to activate the hydroxycarboxylic acid receptor 3 (HCA3) on monocytes, triggering a chemotactic response (Peters et al., 2019). Furthermore, PLA alleviates colitis in a *Salmonella Typhimurium* infection model by mediating an anti-inflammatory response assisting mucosal healing (Zhou et al., 2021). Thus, a highly promising enriched yoghurt with elevated PLA was identified that could be further investigated as a potential nutraceutical in future studies.

Intriguingly, high relative concentrations of GABA were detected in enriched yoghurts with an adjunct *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, or *L. helveticus* strain and significantly lower relative concentrations of GABA in yoghurts enriched with an adjunct *Propionibacterium freudenreichii* or *S. thermophilus* strain. This could be of

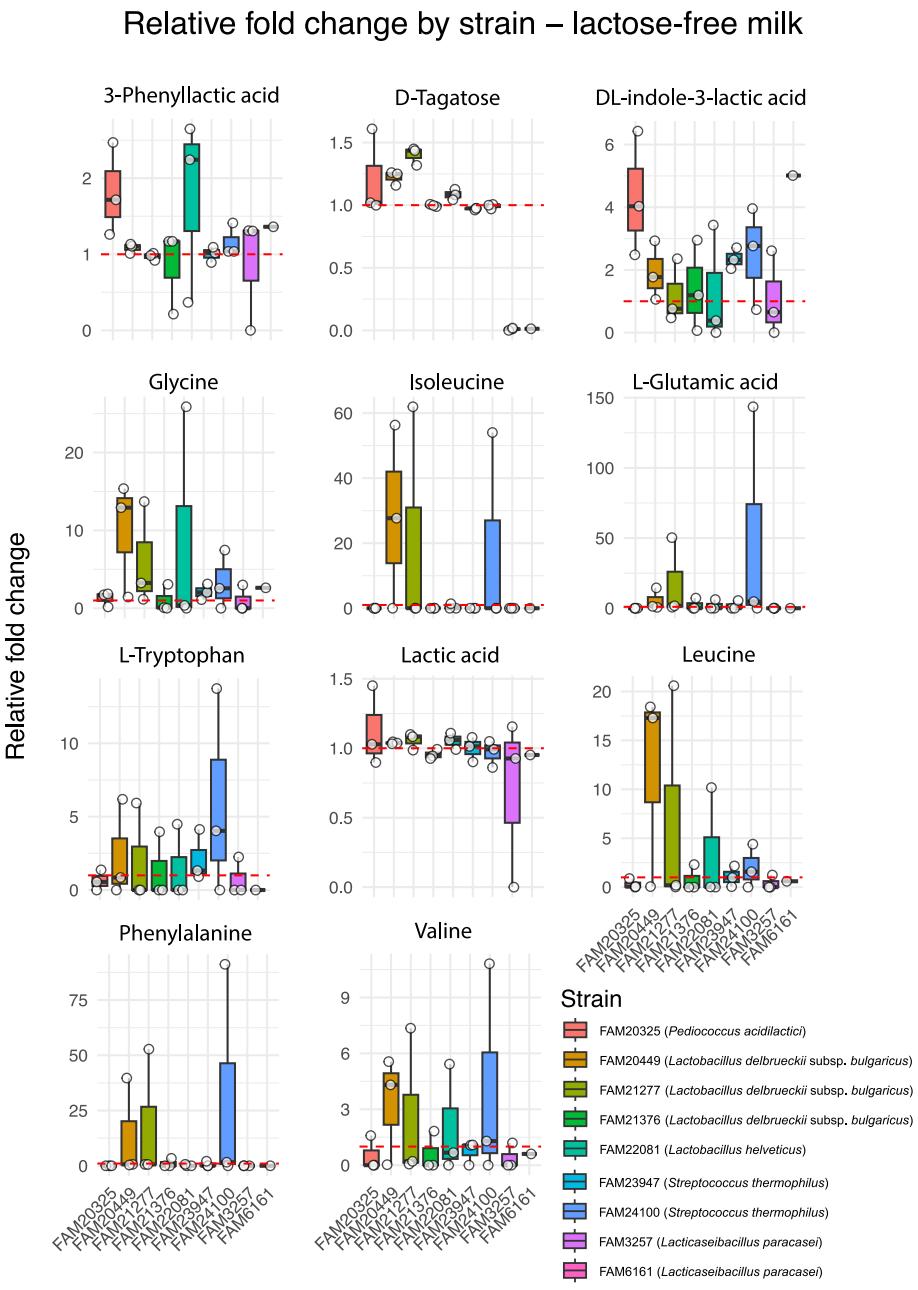

Fig. 9. Relative fold-changes of identified metabolites with potential health benefits in yoghurt remakes with lactose-free milk, measured by GC-MS. (A) Relative concentration of 12 health-associated metabolites identified by targeted metabolomics in different lactose-free enriched yoghurts with a *Pediococcus*, *Streptococcus* or *Lactobacillus* strain characterized by exceptionally high relative concentrations of amino acids. The red line represents the starter yoghurts concentration, which was normalized to 1.

interest in perspective to the possibility of GABA being a postbiotic mediator (Braga et al., 2024). Relative abundance of *Bacteroides* were found to negatively correlate with signatures in the brain that are associated with depression (Strandwitz et al., 2019). Future experiments investigating whether feeding of enriched yoghurts could increase GABA serum levels are warranted.

Furthermore, fermented foods are rich in aryl hydrocarbon receptor (Ahr) ligands, such as ILA. Ahr ligands are indispensable for proper immune responses in the mucosa upon infection but also during homeostasis (Stockinger et al., 2014, 2021) and ensure proper transition from tissue regeneration to differentiation in the intestine (Shah et al., 2022). In some enriched yoghurts, elevated levels of ILA, an Ahr ligand, was measured. In a feeding experiment with gnotobiotic dams, we have previously shown the beneficial effects of Ahr ligand enriched yoghurts on the offspring's mucosal immune system (Pimentel et al., 2024). Feeding of Ahr ligand enriched yoghurt to gnotobiotic dams during pregnancy induced elevated numbers of innate lymphoid cell type 3 cells (ILC3s) in the offspring's small intestinal lamina propria. Future investigations will include testing whether this confers protection from early life infections.

In this study, significant differences in metabolite profiles were detected in enriched yoghurts. Future investigations will specify whether this is driven by interactions of the strains present in the milk, the fermentation conditions, or the inherent functionality of the adjunct strains. Another possibility could be synergistic or competitional phenomena between present strains, which warrants further investigations.

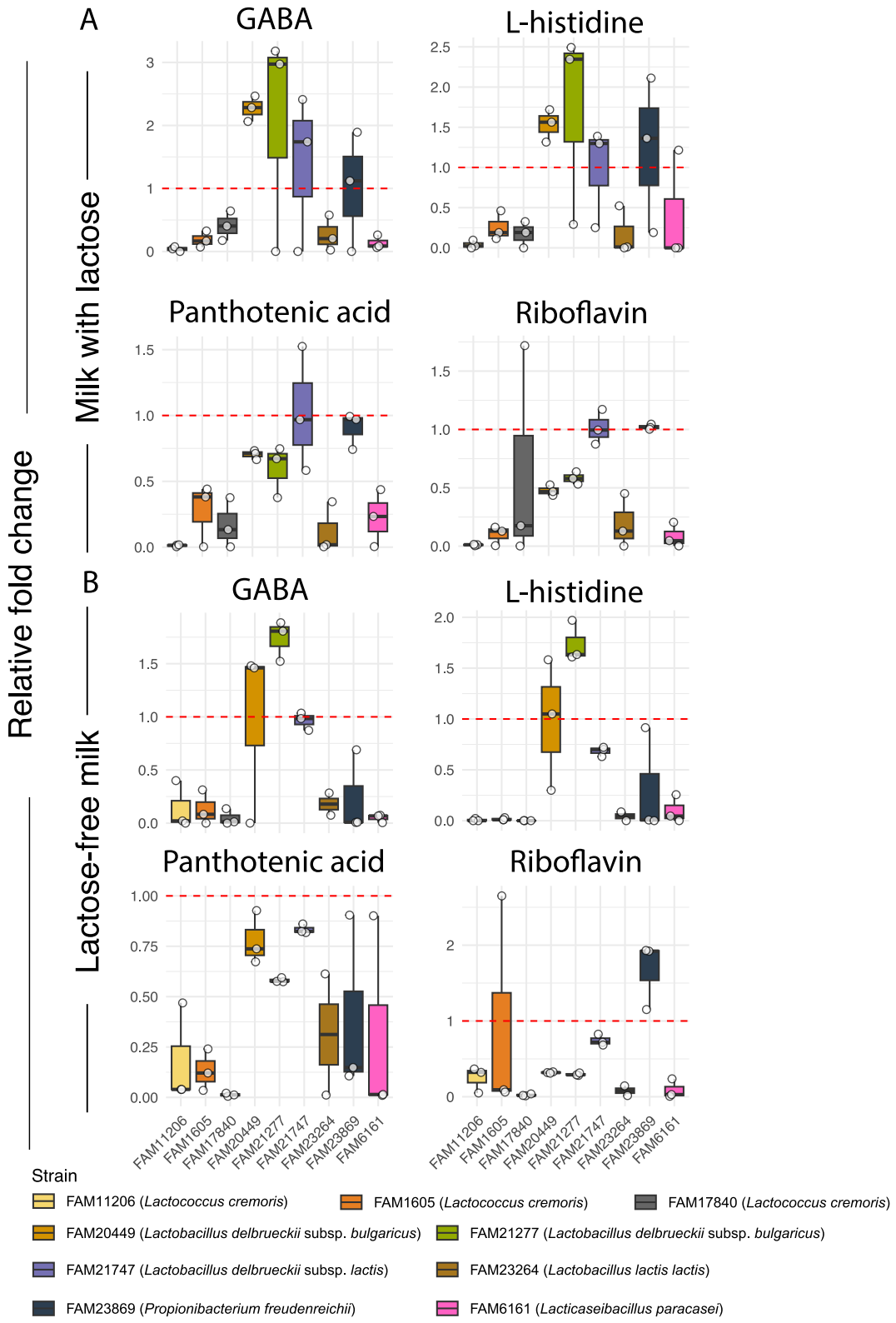

Fig. 10. Relative fold-changes of identified metabolites with potential health benefits in yoghurt remakes with lactose-free or lactose containing milk, measured by LC-MS. (A) Relative concentration of 4 health-associated metabolites identified by targeted metabolomics in different lactose-free or lactose-containing enriched yoghurts with a *Lactococcus*, *Lactobacillus* or *Propionibacterium* strain. The red line represents the starter yoghurts concentration, which was normalized to 1.

5. Conclusions

Nowadays, yoghurt is produced in many countries using commercial starter cultures consisting solely of the two species *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus*. However, this study demonstrates that expanding the microbial consortium of yoghurt results in the enrichment of yoghurt with health-related metabolites, such as SCFAs, GABA, BCAAs, PLA and ILA,

potentially influencing the integrity of the intestinal barrier, modulating the gut-brain axis or the immune system, or promoting metabolic health.

Nevertheless, the results presented in this study have some limitations, such as the fact that all yoghurts were produced under the same fermentation conditions without considering specific growth curves or needs of the individual adjunct strains, which may have resulted in different growth rates and thus might have influenced the presence or absence of metabolites in the enriched yoghurts. Furthermore, the experiments are limited to milk fermentation and subsequent metabolomic analysis without further *in vitro* or *in vivo* experiments, investigating the possible health effects. However, as a positive health effect has already been demonstrated for a yoghurt enriched in indole compounds (Pimentel et al., 2024) future *in vitro* and *in vivo* studies may reveal additional potential health benefits of promising fortified yoghurt candidates.

In conclusion, the results shown in this study open up a multitude of possibilities that may have a significant impact on the future of modulating and improving the overall nutritional value of yoghurt through natural fermentation with the ultimate goal of producing customized functional yoghurt products that no longer require the addition of vitamins or other health-promoting substances.

CRediT authorship contribution statement

Sandro Christensen: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation, Conceptualization. **David Biedermann:** Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Zahra Sattari:** Investigation. **Thomas Roder:** Visualization, Software, Resources, Methodology, Formal analysis, Conceptualization. **Mireille Tena-Stern:** Investigation. **Carola Blaser:** Methodology, Investigation. **Pascal Fuchsmann:** Resources, Methodology, Investigation, Data curation. **Ueli von Ah:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Barbara Walther:** Writing – review & editing, Conceptualization. **Rémy Bruggmann:** Software, Resources, Methodology, Conceptualization. **Stephanie C. Ganal-Vonarburg:** Writing – review & editing, Methodology, Conceptualization. **Guy Vergères:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Grégory Pimentel:** Writing – review & editing, Visualization, Supervision, Software, Methodology, Investigation, Data curation. **Cornelia Bär:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2025.107047.

Abbreviations

Ahr	Aryl hydrocarbon receptor
BAT	Brown adipose tissue
BCAAs	Branched-chain amino acids
CV	Coefficient of variation
ESI	Electrospray ionization
GABA	Gamma-aminobutyric acid
GC-MS	Gas chromatography-mass spectrometry
HCA	Hydroxycarboxylic acid receptor 3
HMDB	Human metabolome database
HPLC	High-performance liquid chromatography
ILA	DL-indole-3-lactic acid
ILC3s	Innate lymphoid cell type 3 cells
IStd	Internal standard
L	Lactose
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography-mass spectrometry
LF	Lactose-free
LPS	Lipopolysaccharide
MCFAs	Medium-chain fatty acids
MSTFA	Trimethylsilyl)trifluoroacetamide
NCCD	Non-communicable chronic diseases
OMA	O-Methylhydroxylamine hydrochloride
PAB	Propionic acid producing bacteria
PBMCs	Peripheral blood mononuclear cells
PLA	3-phenyllactic acid
SCFA	Short-chain fatty acids
TNBS	Trinitrobenzenesulfonic acid
UMAP	Uniform manifold approximation and projection
QC	Quality control
QTOF-MS	Quadrupole time-of-flight mass spectrometry

Data availability

Data will be made available on request.

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