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Functional foods acting on gut microbiota-related wellness: The multi-unit *in vitro* colon model to assess gut ecological and functional modulation

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A R T I C L E I N F O

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A B S T R A C T

The aim of this study was to investigate the effect of a functional probiotic cheese (FPC) on gut microbiota (GM), after simulated digestion performed by a multi-unit *in vitro* colon model (MICODE).

Squacquerone-like cheese was produced using the starter *Streptococcus thermophilus* (control, CTRL), and supplemented with the probiotic *Lacticaseibacillus rhamnosus*, which was either subjected to high pressure homogenization (LrH) or not (Lr). Samples were stratified by cheese type, storage time, and colonic fermentation phase. Samples were then digested with MICODE and digests were characterized for ecological and functional profiles.

The lactobacilli detected in Lr and LrH cheeses (9.0 log CFU/g) were represented by the probiotic strain *L. rhamnosus* and remained unchanged after storage at 4 °C. Lactobacilli levels in CTRLs increased from 1.5 log CFU/g to 2.0 log CFU/g after six days at 4 °C, while total coliforms remained below 1.5 log CFU/g in all samples.

Real-time qPCR indicated a positive GM response after FPC simulated digestion, highlighting an abundance of bifidobacteria, lactobacilli and *Clostridium* group IV in LrH samples.

Metataxonomy revealed higher levels of Firmicutes and Proteobacteria ($p \leq 0.05$) after simulated digestion, as well as *Megasphaera*, *Escherichia*, *Prevotella* and *Dorea*. Moreover, an increase of short and medium chain fatty acids were detected by metabolomics. Overexpression of inferred KEGG metabolic pathways showed mainly fatty acids, novobiocin and amino acid metabolism.

Understanding how functional foods can modify the GM may lead to the development of targeted microbiome-based therapies and the exploitation of these foods for the benefit of human health.

1. Introduction

Lifestyle, including dietary habits, represent one of the key elements involved in gut microbiota (GM) modulation in terms of composition and activity, which in turn has an impact on human health (Martina et al., 2019). Nowadays, a healthy diet can be considered to consist of more than sufficient energy intake, the quality of ingredients, and micronutrient content, insofar as it should include foods with additional health benefits, called functional foods (FFs) (Topolska et al., 2021). Particularly, FFs supply the organism's nutritional requirements and give a beneficial effect to human body, if consumed in moderate quantities and according to proper nutritional guidelines (European Commission. Directorate-General for Research, 2010). In this view, FFs that able to modulate the GM are foods that include nutrient-rich ingredients, fiber, and probiotics and/or prebiotics (Baker et al., 2022; Bosscher et al., 2009; The European Parliament and of the Council of the

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European union, 2006). FFs can be eaten daily to improve overall health, prevent malnutrition, and protect against chronic diseases, such as metabolic syndromes and cardiovascular disease (Hasler, 2002).

Among these are fermented products, particularly dairy foods containing prebiotics and/or probiotic bacteria, which have been studied deeply for their beneficial effects on human health (Heller, 2001; Patrignani et al., 2019). These effects depend on moving live bacteria to hypothetical interaction sites within the host, either directly, *via* microbial components (Lebeer et al., 2010; Patrignani et al., 2019; Prakash et al., 2011), or indirectly, by releasing bacterial constituents at specific locations (Taverniti & Guglielmetti, 2011), or by modifying GM activities (Flint et al., 2007).

Particularly, the search for strains able to resist gastrointestinal (GI) barriers, such as the highly acidic environment within the stomach, combined with certain technological and functional features, could result in new probiotics suitable for tailor-made FFs (Ugarte et al., 2006). Moreover, physic-chemical treatments performed on probiotic strains during food making, such as heat treatment, High pressure Homogenization (HPH), or Pulsed Electric Field (PEF), are reported to influence their viability, functionality, and even change their physiological state (Sumeri et al., 2008). Therefore, studies into the effects of probiotic strains on the GM should also take variables such as these into account.

Amongst probiotics, *Lactobacillus* and *Bifidobacterium* represent the most commonly used genera in human probiotics, being relatively easy to produce in large quantities as frozen or freeze-dried cultures (Brodmann et al., 2017), and which have been used for their ability to treat gut dysbiosis and reestablish healthy conditions in many different contexts (Gerritsen et al., 2011; Grimm et al., 2014).

Probiotics added to food are more likely to survive the journey through the stomach because the food itself provides a protective environment can significantly affects the survival of these microorganisms (Charalampopoulos et al., 2002; Morelli, 2000; Patel et al., 2004).

Moreover, cheese, having low acidity and high buffering capacity, as well as a good level of protein and high fat, represents a good choice for FF creation, as it may be an ideal candidate to protect bacteria against changes during production, ripening and ingestion (Patrignani et al., 2018, 2020; Vinderola et al., 2002) and offer health benefits to consumers, which in turn are linked to the quality and viability of the probiotic strains (Shah, 2000; Varcoe et al., 2002).

To date, several studies have reported evidence of *L. rhamnosus* remaining viable when incorporated into cheese, primarily from a biotechnological perspective (Boylston et al., 2004; Phillips et al., 2006) while the effects of *L. rhamnosus*-fortified foods on the GM still remain to be fully elucidated, making this research a novel dietary strategy for enhancing human health also in a pediatric context.

In order to provide detailed information on specific mechanisms of action of FFs, prior to an in-human assessment, an *in vitro* study to test probiotic viability and the effects of a new FF on the GM has an important role (Dupont et al., 2019; Williams et al., 2015).

In this study, we evaluate the ability of functional probiotic cheese (FPC), specifically the soft cheese Squacquerone-like, as a vector for a strain of *Lacticaseibacillus rhamnosus* with and without HPH treatment, to modulate GM ecology and activity by using an *in vitro* gut model that combines the simulation of oro-gastro-duodenal digestion by the INFOGEST® protocol (Minekus et al., 2014) to the simulation of proximal colon fermentation with human colon microbiota by the MICODE (Multi-Unit *in vitro* Colon Model) protocol (Nissen et al., 2021). Moreover, the originality of this study lies in the investigation of the potential of this probiotic cheese as a "healthy and good tasting product" to ameliorate gut wellness, and thus quality of life, in children and adults.

2. Materials and methods

2.1. Probiotics culture selection and formulation of Squacquerone-like cheeses

The probiotic strain *L. rhamnosus* FBC belongs to the Department of Agricultural and Food Sciences of Bologna University (Doron et al., 2005). Fresh *L. rhamnosus* FBC cultures were obtained by two consecutive daily transfers in De Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, UK) using a 1 % (v/v) inoculum, incubated at 37 °C in anaerobic conditions by using an Oxoid gas generating kit, for 18 h. After that, it was harvested by centrifugation (8000*g*, 20 min, 4 °C). The resultant pellet was washed twice with saline solution and re-suspended in commercial milk for the inoculums.

2.2. HPH treatment of probiotic strain

An aliquot of the probiotic strain re-suspended in commercial milk was subjected to a high-pressure homogenization (HPH) treatment at 50 MPa with a PANDA high-pressure homogenizer (Gea, Parma, Italy). The machine was supplied with a homogenizing PS-type valve. The valve assembly includes a ball type impact head made of ceramics, a stainless steel large inner diameter impact ring and a tungsten carbide passage head. The inlet temperature of samples was 20 °C and the increase rate of temperature was 2.5 °C/10 MPa (Burns et al., 2015).

2.3. Cheesemaking

Cheesemaking was carried out in a pilot scale plant at Mambelli dairy farm (Mambelli, Bertinoro, Italy). A commercial freeze-dried culture of *Streptococcus thermophilus* St 0.20 (Sacco S.R.L., Como, Italy) was used as starter at 6 Log CFU/ml. Three different formulations of Squacquerone-like cheese were produced at Mambelli dairy farm (Mambelli, Bertinoro, Italy) as follows:

(i) Squacquerone-like cheese produced only with starter cultures (*S. thermophilus*), named "CTRL";
(ii) Squacquerone-like cheese produced with starter and probiotic cultures (*S. thermophilus* plus *L. rhamnosus* FBC), named "Lr";
(iii) Squacquerone-like cheese produced with *S. thermophilus* and supplemented with HPH-treated *L. rhamnosus* FBC, named "LrH".

Briefly, three batches of pasteurized whole cow's milk (100 L) were warmed at 42 °C. The starter *S. thermophilus* St 0.20 was inoculated at 6.0 Log CFU/ml in all the batches and, 20 min later, batch (ii) was also inoculated with *L. rhamnosus* FBC at 9.0 Log CFU/mL, while batch (iii) was inoculated with HPH-treated *L. rhamnosus* FBC strain at 9.0 Log CFU/mL.

After 40 min, from the starter inoculum, NaCl (0.7 %) and rennet (12,000 international milk coagulating units (IMCU)/mL, 80 % chymosin, and 20 % pepsin, Bellucci Modena, Italy) were added to all the batches. After coagulation, the curd was cut and moved to traditional baskets. The products were allowed to rest until a pH of 5.15 was reached and then placed at 4 °C. After 24 h, the cheeses were packed under a modified atmosphere. About 40 packages of 300 g were obtain for each type of Squacquerone.

Moreover, Squacquerone-like cheeses were stored at 4 °C, in agreement with the manufacturer. The samples for the analyses were picked up immediately after production (T_0) or after being stored for 6 days at 4 °C (T_6). This storage time is considered by the manufacturer as the optimal one for achieving maximum proteolysis and, thus, this time of storage would align with the product's "sell-by" date.

2.4. Microbiological and chemical-physical analyses

At T_0 and at T_6, microbiological analyses were carried out for the enumeration of the starter (*S. thermophilus*), probiotic culture (*L. rhamnosus* FBC), yeasts, total coliforms and lactic acid bacteria. 20 gr of cheese were added to 180 ml of sterile sodium citrate solution (20 g/L) and homogenized by stomacher for 3 min (BagMixer 400P, Interscience, Saint-Nom-la-Bretèche, France). Serial dilutions of the homogenized samples were made in a physiological solution (0.9 % NaCl), and aliquots of each dilution were spread onto the surface of different selective agar media. The starter *S. thermophilus* was plated on M17 agar (Oxoid, Basingstoke, Hampshire, United Kingdom) (42 °C, 48 h), *L. rhamnosus FBC* and lactobacilli on MRS agar (Oxoid). Total coliforms and yeasts were detected on Violet Red Bile agar (VRBA, Oxoid, Basingstoke, Hampshire, UK) (37 °C, 24 h) and Yeast extract Peptone Dextrose agar (YPD, Oxoid, Basingstoke, Hampshire, UK) (25 °C, 48 h), respectively. Cheese pH was monitored by a pH-meter (PH BASIC 20, Crison, Hach Lange, Italy).

At each sampling point, 3 independent packages for each type of sample were considered for microbiological and pH analysis.

2.5. Human colon microbiota and in vitro gut model

In order to evaluate the probiotic *L. rhamnosus* FBC efficacy when supplemented in a cheese matrix, the experimental Squacquerone-like cheeses were subjected to *in vitro* digestion, using the INFOGEST protocol, and to colonic fermentation using MICODE (Multi-Unit In vitro Colon Model) (Nissen et al., 2021).

In vitro digestion. Experimental and control samples were digested *in vitro* with the INFOGEST protocol (Minekus et al., 2014). Briefly, 5 mL of each sample was processed for 242 min (2 min oral digestion, 120 min gastric digestion and 120 min intestinal digestion), at 37 °C. During *in vitro* digestion, several consecutive enzymatic treatments took place by addition of simulated saliva (containing 75 U/mL of α-amylase), simulated gastric juice (containing 2000 U/mL of pepsin) at an acidic pH, and simulated pancreatic juice (containing 10 mM of bile and 100 U/mL of pancreatin) at a neutral pH. See the details in **Supplementary Materials and Methods section**.

The digests were then thawed and gently centrifuged to separate the dense portion, to be applied then for colonic fermentation.

Preparation of Human Colon Microbiota (HCM). HCM was obtained from fecal samples of three healthy volunteers. Collection of samples was in accordance with previous protocols (Connolly et al., 2012; Nissen et al., 2021, 2024). Samples were obtained two times from three different donors, (2 female and 1 male, age ranged between 30 and 45 years), that did not undergo antibiotic treatment for at least 3 months prior and did not intentionally consume pre- or probiotic supplements before sample collection. The donors were told of the study's aims and procedures and gave their verbal consent to use their fecal matter, in agreement with the ethics procedures required at the University of Bologna. Fecal samples (Bristol chart types 2–5) were collected by the donors with a collection kit, which includes a stool collector (Sarstedt AG & Co. KG, Nümbrecht, Germany) and an anaerobic jar with a O_2 consuming catalyst (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA). Samples were rapidly transferred to the laboratory and processed within 2 h. A 10 % w/v HCM suspension was prepared just before the experiment and was obtained by homogenizing 2 g of each donation in 54 mL of pre-reduced phosphate buffered saline (PBS) (Cattivelli et al., 2023; Wang et al., 2020) and washing the pellet twice in PBS (16,000*g* for 5 min).

In vitro Colonic fermentation. Short-term batch proximal colonic fermentations were conducted for 24 h in independent vessels using an *in vitro* colon model, MICODE, as previously described (Cattivelli et al., 2023; Connolly et al., 2012; Nissen et al., 2021, 2024; Wang et al., 2020). Sampling of colonic effluates was conducted at the beginning and at the endpoint of colonic fermentations and were subsequently used to perform analytical activities. See the details in **Supplementary Materials and Methods section.**

2.6. Workflow of analytical activities

The effluates from colonic fermentation at BL (Baseline = 0 h) and EP (Endpoint = 24 h) of fermentation time (in biological duplicate and technical duplicate) were used for metataxonomic sequencing (16S-rRNA), metabolomic analysis (gascromatography/mass spectrometry coupled with solid phase microextraction, SPME-GC–MS) and enumeration of selected bacterial taxa by Real Time qPCR analyses (Fig. 1). After sterile sampling of 5 mL of bioreactor contents, samples were centrifuged at 16,000*g* for 7 min to separate the pellets and the supernatants were stored at −80 °C until further analysis.

Pellets were washed twice in PBS prior DNA extraction. Microbial DNA extraction was conducted just after sampling so as not to reduce Firmicutes content.

Quality controls for the *in vitro* model were applied as: i) a biodiversity check, ii) a stool-ubiquitary metabolomics (*i.e.,* volatile organic compounds, VOCs) check, iii) trends of fermentation parameters by the Lucullus 3.1 software (Securecells, CH), and iv) a blank control (BC).

In summary, 14 digested samples (12/14 samples were produced in replicates) (Table 1), were stratified according to the described variables: i) cheese type; ii) commercial shelf life at refrigerated temperature; iii) colonic fermentation phase.

2.6.1. Absolute enumeration of bacterial groups by real time real time qPCR

To investigate changes in the microbiota after fermentation, enumeration of bacterial groups was made by quantifications of short fragments of monocopy or multicopy genes of selected taxa by Real Time qPCR, as previously described (Modesto et al., 2011; Tamargo et al., 2022; Tanner et al., 2014; Tsitko et al., 2019; Westfall et al., 2018). Specifically, some bacterial groups were selected as generally accepted indicators of eubiosis or dysbiosis of colon microbiota, and their perturbations were considered closely correlated (positively or inversely) to the prebiotic potential of foods. Nine different bacterial taxa (**Supplementary Table S1**) were assessed by qPCR on a QuantStudio5® System with SYBR Green I chemistry (Applied Biosystem, Thermo Fisher, USA). See details in **Supplementary Materials and Methods section**

2.6.2. Omics analysis

Metataxonomy. DNA was extracted from the MICODE effluates at the BL and EP time points, using a Nucleo Spin DNA stool Kit (Macherey Nagel, Darmstadt, Germany). Nucleic acid purity was tested on a BioDrop Spectrophotometer (Biochrom Ltd., Cambridge, UK). The V3 and V4 hypervariable regions of the 16S rRNA gene (~460 bp) were amplified by PCR (Polymerase Chain Reaction) using primers specified in the 16S Metagenomic Library Preparation protocol (Illumina, San Diego, CA, USA). After PCR clean-up with magnetic beads (CleanNA, Coenecoop 75, 2741 PH Waddinxveen, The Netherlands), an index PCR step was performed to attach dual indices and Illumina sequencing adapters, using a Nextera XT Index Kit (Illumina, San Diego, CA, USA). After the second PCR clean-up, final 16S libraries were quantified by a Quant-iT™ PicoGreen® dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA), pooled, normalized to 4 nM, denatured and sequenced on an Illumina MiSeq™ platform, according to the manufacturer's specifications. Negative controls were used in all

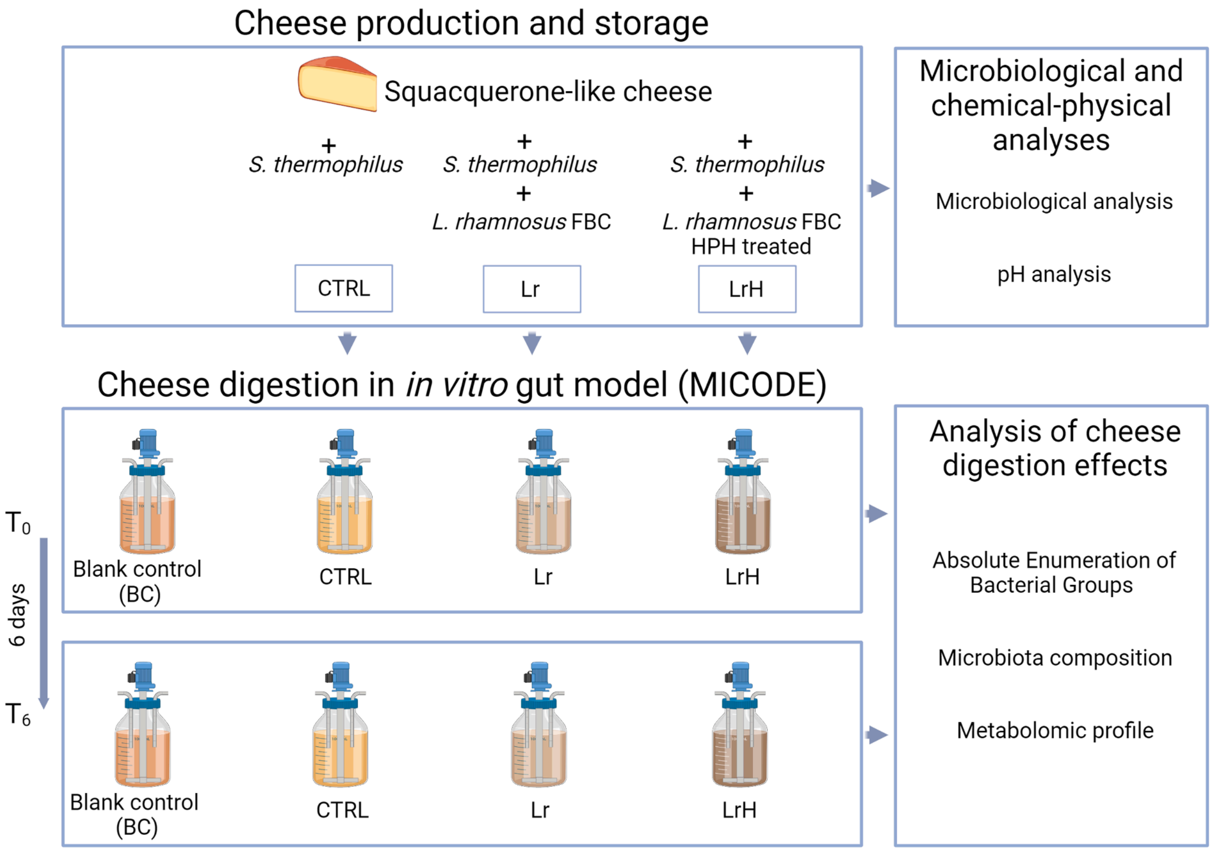

Fig. 1. Graphical representation of the experimental workflow performed.

amplification steps to exclude potential environmental contaminations.

A total of 52 paired-end fastq files were analysed with the Quantitative Insights into Microbial Ecology software (QIIME 2 v2023.2) (Bolyen et al., 2019). With the q2-DADA2 plugin, Amplicon Sequences Variants (ASVs) were detected, and through the plugin q2-features classifiers amplicon sequences were taxonomically assigned using a naïve Bayes model pre-trained on the Greengenes database (v13.8, August 2019, https://greengenes.secondgenome.com/).

Volatilome determination. The volatilome, that represents VOCs in a sample, was estimated on a Gas Chromatograph 7890A (Agilent Technologies, Santa Clara, CA, USA) coupled to a mass spectrometer 5977 (Agilent Technologies, Santa Clara, CA, USA) in electron impact mode (ionization voltage of 70 eV) equipped with a DB-HeavyWAX (60 m × 0.250 mm × 0.25 μm) capillary column Agilent Technologies. The SPME GC–MS protocol and the identification of volatile compounds were conducted as previously described (Vernocchi et al., 2023). See the details in **Supplementary Materials and Methods section.**

2.6.3. Data processing and statistical analysis

The metataxonomic data were analyzed by statistical tools with R (v4.3.0). For α- and β-diversity analyses, the rarefaction method based on the minimum sample depth was applied on absolute frequency, filtering out 724 ASVs. Statistical analyses on α-diversity indices were performed using the non-parametric Mann–Whitney and Kruskal–Wallis tests with the R phyloseq package. The permutational analysis of variance (PERMANOVA) test was applied on a matrix of dissimilarity produced with the Bray-Curtis dissimilarity algorithm.

Moreover, the metataxonomic (ASVs) and metabolomic (VOCs) data normality distributions were evaluated using the Shapiro-Wilk test and non-parametric Mann-Whitney and Kruskal-Wallis test were applied to compare taxonomic differences at the phylum (L2), family (L5), and genus (L6) levels and to identify differential VOCs profiles.

For the Phylogenetic Investigation of Communities by Reconstruction of Unobserved States of Correlation 2 (PICRUSt2) analysis and other details see the **Supplementary Materials and Methods section.**

3. Results

3.1. Microbiological and chemical-physical analysis

As shown in Table 2, the cell load of the starter *S. thermophilus* was higher in control samples compared to those supplemented with the probiotic strain throughout the storage period, ranging between 7.5 and 8.6 log CFU/g. The lactobacilli detected were represented by the probiotic strain *L. rhamnosus* FBC in Lr and LrH samples in which this strain was added. In these samples, they were found to be around 9.0 log CFU/g throughout the storage period. In control samples, lactobacilli had an initial load of 1.5 log CFU/g, which increased to 2.0 log CFU/g after 6 days of refrigerated storage. Total coliforms were below 1.5 log CFU/g in all samples throughout the shelf-life period considered. Finally, yeasts were below the detection limit at the initial time-point, but increased in cell load during refrigerated storage.

Furthermore, the control samples,showed a slight increase in pH over time, reaching 5.22 after 6 days (T_6) of commercial shelf life at refrigerated temperature. In Lr and LrH samples, on the other hand, there was a decrease in pH which, after 6 days of storage, reached 5.06 and 5.11, respectively.

3.2. Enumeration and modulation of core microbiota groups by real time qPCR

After colonic fermentation of functional probiotic cheese (FPC), the

Table 1
Samples obtained with *in vitro* colonic fermentation model.

Labels of samples	Replicates	Description
CTRL T_0 BL	2	Squacquerone-like cheese only added with starter cultures (*S. thermophilus*) CTRL) at the initial time of commercial shelf life (T_0), digested and collected at the baseline (BL) (0 h) of colonic fermentation.
CTRL T_0 EP	2	Squacquerone-like cheese only added with starter cultures (*S. thermophilus*) (CTRL) at the initial time of commercial shelf life (T_0), digested and collected at the endpoint (EP) (24 h) of colonic fermentation.
Lr T_0 BL	2	Squacquerone-like cheese added with starter and probiotic cultures (*S. thermophilus* plus *L. rhamnosus* FBC) (Lr) at the initial time of commercial shelf life (T_0), digested and collected at the baseline (BL) (0 h) of colonic fermentation.
Lr T_0 EP	2	Squacquerone-like cheese added with starter and probiotic cultures (*S. thermophilus* plus *L. rhamnosus* FBC) (Lr) at the initial time of commercial shelf life (T_0), digested and collected at the endpoint (EP) (24 h) of colonic fermentation
LrH T_0 BL	2	Squacquerone-like cheese including *S. thermophilus* and added with *L. rhamnosus* FBC treated with high pressure of homogenization (HPH) (LrH), at the initial time of commercial shelf life (T_0), digested and collected at the baseline (BL) (0 h) of colonic fermentation.
LrH T_0 EP	2	Squacquerone-like cheese including *S. thermophilus* and added with *L. rhamnosus* FBC treated with high pressure of homogenization (HPH) (LrH), at the initial time of commercial shelf life (T_0), digested and collected at the endpoint (EP) (24 h) of colonic fermentation.
BC of BL	1	Blank control (BC) made with water, digested and collected at the baseline (BL) (0 h) of colonic fermentation.
BC of EP	1	Blank control (BC) made with water, digested and collected at the endpoint (EP) (24 h) of colonic fermentation.
CTRL T_6 BL	2	Squacquerone-like cheese only added with starter cultures (*S. thermophilus*) (CTRL) on the sixth day of commercial shelf life (T_6), digested and collected at the baseline (BL) (0 h) of colonic fermentation.
CTRL T_6 EP	2	Squacquerone-like cheese only added with starter cultures (*S. thermophilus*) (CTRL) on the sixth day of commercial shelf life (T_6), digested and collected at the endpoint (EP) (24 h) of colonic fermentation.
Lr T_6 BL	2	Squacquerone-like cheese added with starter and probiotic cultures (*S. thermophilus* plus *L. rhamnosus* FBC) (Lr) on the sixth day of commercial shelf life (T_6), digested and collected at the baseline (BL) (0 h) of colonic fermentation.
Lr T_6 EP	2	Squacquerone-like cheese added with starter and probiotic cultures (*S. thermophilus* plus *L. rhamnosus* FBC) (Lr) on the sixth day of commercial shelf life (T_6), digested and collected at the endpoint (EP) (24 h) of colonic fermentation.
LrH T_6 BL	2	Squacquerone-like cheese including *S. thermophilus* and added with *L. rhamnosus* FBC treated with high pressure of homogenization (HPH) (LrH), on the sixth day of commercial shelf life (T_6), digested and collected at the baseline (BL) (0 h) of colonic fermentation.
LrH T_6 EP	2	Squacquerone-like cheese including *S. thermophilus* and added with *L. rhamnosus* FBC treated with high pressure of homogenization (HPH) (LrH), on the sixth day of commercial shelf life (T_6), digested and collected at the endpoint (EP) (24 h) of colonic fermentation.

two main bacterial phyla Bacteroidetes and Firmicutes showed trends of underrepresentation, particularly when LrH was supplemented. In contrast, the total bacterial abundance increased after colonic fermentation of FPC (**Supplementary Fig. S1**; **Supplementary Table S2**).

In general, all digested FPC samples generated a shift in microbiota composition associated with positive health outcomes, particularly in the case of LrH-fortified samples (**Supplementary Fig. S1b**; **Supplementary Table S2**). This modulation was significant for most FPC samples, and the abundances of these beneficial bacterial groups was higher in supplemented samples than in CTRLs ($p \leq 0.05$). In particular, FPC samples caused shifts in 3 out of 3 of the selected beneficial taxa, namely *Lactobacillus*, *Bifidobacterium* and *Clostridium* group IV. The highest increases were attributed to bifidobacteria and lactobacilli in LrH-fortified FPC at T_6, followed by *Clostridium* group IV (**Supplementary Fig. S1b**; **Supplementary Table S2**). Furthermore, no opportunistic pathogens were increased in any of the FPC samples during commercial shelf life (T_6) at refrigerated temperature.

3.3. Modulation of GM ecology and metabolome: Effect of FPC digestion

After data filtering, a total of 2591 sequence reads of 16S rRNA gene amplicons were obtained with an average of 3482 reads/sample and an average length of 447 bp after primer trimming. Resulting taxa were detected by interrogating the Greengenes database (v. 13.8).

Bacterial communities in BL and EP samples were analyzed by α- and β-diversity in order to see if *in vitro* colonic fermentation altered species richness. No statistically significant differences were found in α-diversity as measured by Shannon's entropy, Chao-1 or Simpson's index (**Supplementary Fig. S2**). However, β-diversity algorithms revealed statistically significant ($p \leq 0.05$) differences between samples collected at BL and EP (**Supplementary Fig. S3**).

At the phylum level (L2), four main phyla were significant altered after *in vitro* colonic fermentation. Particularly, Firmicutes and Proteobacteria were statistically significant higher ($p \leq 0.05$) in EP, while Actinobacteria and Bacteroidetes were higher in BL ($p \geq 0.05$) (Fig. 2).

At the family level, Lachnospiaceae, Ruminococcaceae, Streptococcaceae, Prevotellaceae, and Veillonellaceae (Clostridiales) were significantly more abundant in BL, while Veillonellaceae (Selemonadales) and Enterobacteriaceae were abundant in EP (Fig. 3**A**).

At the genus level, *Roseburia*, *Blautia*, *Faecalibacterium*, *Streptococcaceae bacterium* RF32 and *Ruminococcus* were significantly more abundant ($p \leq 0.05$) in BL, while *Megasphaera*, *Escherichia* and *Dorea*, were increased ($p \leq 0.05$) in EP samples (Fig. 3**B**). Furthermore, only *Dialister* was significantly altered in FPC samples during commercial storage at 4 °C, showing higher abundance at T_0 compared to T_6 days after refrigerated temperature (**Supplementary Fig. S4**).

Regarding PCA biplot executed on 20 taxa, the percentage of almost 71.4 % and 10.9 % of explained variance characterized the two groups PC1 and PC2. The over impressed loadings revealed that taxa as *Blautia*, *Roseburia*, *Bacteroides*, *Faecalibacterium*, *Ruminococcus* and *Phascolarctobacterium* were involved in the separation of BL group, while *Escherichia*, *Prevotella*, *Dorea* and *Megasphaera* define the EP group (**Supplementary Fig. S5A**).

A stable PLS-DA (**Supplementary Fig. S5B**), characterized by a predictive performance of $R^2 = 0.868$, $Q^2 = 0.755$ and RMSEE = 0.193, assessed the well separation between BL and EP along C1 and C2 component directions. On the contrary, the PCA and PLS-DA performed on variables as commercial time of exposure (T_0 and T_6) and type of cheese (CTRL, Lr and LrH) were not well separated (data not shown).

However, a clear separation was evidenced only between BL and EP condition that was revealed as two major clusters (green/brown), for OTUs (Fig. 4) while for all of others comparison many separated subclusters were produced. In particular, for ASV *Megasphaera*, *Escherichia* were more abundant in EP condition, while *Blautia*, *Roseburia* and *Bacteroides* were abundant in BL (Fig. 4). On the other hand, samples did not cluster by cheese type (CTRL, Lr and LrH) (Fig. 4), nor did they cluster by refrigerated temperature exposition (data not shown).

Next, metabolomics analysis was performed on digested samples to investigate how GM functional profiles were altered following simulated FPC digestion. The metabolomics analysis detected 112 metabolites in these samples, divided into 14 different chemical classes: terpenes (6), alkenes (8), alcanes (14), alcohols (17), aldehydes (18), ketones (13), esters (10), acids (12), organic compounds (7), indoles (2), phenols (2) pyrazines (1), pyridines (1) and amines (1).

Moreover, PCoA plots performed on 12 BL and 12 EP samples identified two distinct clusters mainly separated along the first two

Table 2

Cell load (log CFU/g) of *Streptococcus thermophilus*, lactobacilli, total coliforms and yeasts and pH values in control Squacquerone-like cheeses (CTRL), and cheeses added with probiotic *L. rhamnosus* FBC (Lr) and HPH treated *L. rhamnosus* FBC (LrH) during commercial shelf life at refrigerated temperature (T_0, and T_6). For the same microbial group and pH, the means ± SD followed by different superscript letters (a, b, c, d) are significantly different, $p \leq 0.05$.

Time	**Sample name**	***St. thermophilus* log CFU/g ± SD**	**Lactobacilli Log CFU/g ±** SD	**Total coliforms log CFU/g ±** SD	**Yeasts log CFU/g ± SD**	**pH ± SD**
T_0	CTRL	8.0 ± 0.2^{b}	1.5 ± 0.1^{d}	1.1 ± 0.2^{ab}	*	5.09 ± 0.02^{bc}
	Lr	7.5 ± 0.2^{d}	9.0 ± 0.2^{a}	1.2 ± 0.3^{ab}	*	5.12 ± 0.03^{bc}
	LrH	7.6 ± 0.2^{d}	9.0 ± 0.2^{a}	1.4 ± 0.2^{a}	*	5.14 ± 0.03^{b}
T_6	CTRL	8.6 ± 0.3^{a}	2.0 ± 0.2^{c}	0.8 ± 0.1^{bc}	2.0 ± 0.2^{c}	5.22 ± 0.02^{a}
	Lr	7.8 ± 0.1^{cd}	9.0 ± 0.2^{a}	0.5 ± 0.1^{c}	1.6 ± 0.3^{c}	5.06 ± 0.03^{c}
	LrH	7.7 ± 0.1^{d}	8.9 ± 0.3^{a}	1.1 ± 0.2^{ab}	1.7 ± 0.2^{c}	5.11 ± 0.02^{bc}

*: under the detection limit (0.5 log CFU/g).

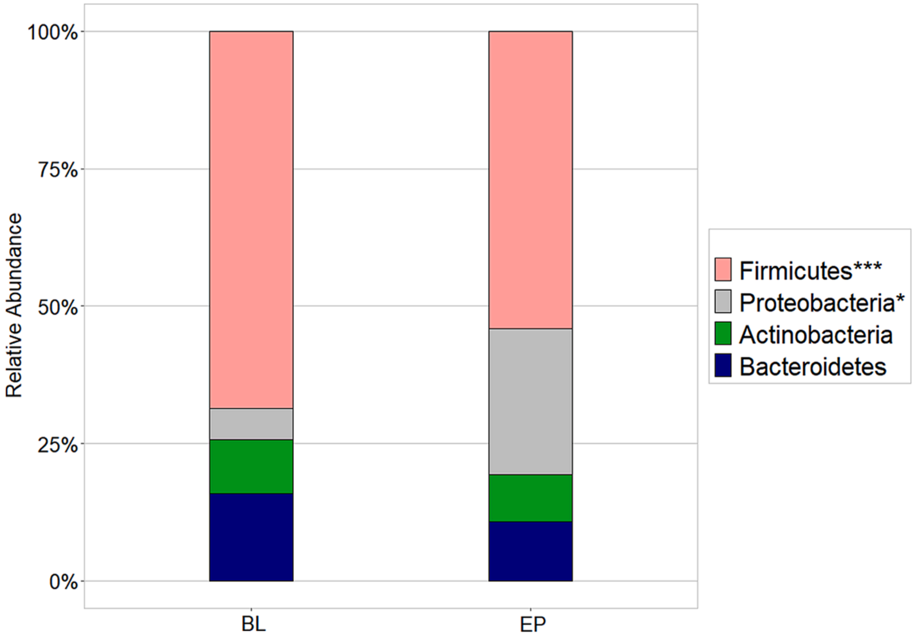

Fig. 2. Taxa distribution performed by the Mann-Whitney *U* test. At phylum level between BL versus EP of fermentation. Only statistically significant taxa (Mann-Whitney U *p* FDR ≤ 0.05) were represented. BL, baseline; EP, endpoint.

principal components, PC1 and PC2, with a percentage of explained variance of 23.5 % and 10.3 %, respectively (**Supplementary Fig. S6A**). The over impressed loadings revealed that metabolites such as butanoic, propanoic, pentanoic, hexanoic, octanoic and n-decanoic acids, pentanoic acid 3-methyl, and phenol were involved in the separation of the EP group, while caryophyllene and 1-butanol, benzothiazole and benzenamine 4-methyl defined BL samples (**Supplementary Fig. S6A**).

A stable PLS-DA (**Supplementary Fig. S6B**), characterized by a predicting performance of $R^2 = 0.972$, $Q^2 = 0.839$ and $RMSEE = 0.089$, assessed the well separation between BL and EP along C1 and C2 component directions. On the other hand, PCA and PLS-DA performed on variables as refrigerated storage (T_0 and T_6) data and on the cheese type (CTRL, Lr and LrH) were not well separated (data not shown).

In order to explore the volatilome profile more deeply, the β-diversity algorithm, based on Bray-Curtis dissimilarity, PCoA was performed, which showed a statistically significant distance (PERMANOVA $p = 0.001$) in the metabolomic profile of BL and EP datasets (Fig. 5**A**). β-diversity was also performed by considering commercial shelf life at refrigerated temperature (T_0 and T_6) and cheese type, these variables did not cause the samples to cluster separately ($p \geq 0.05$) (data not shown).

VOCs associated with BL were caryophyllene, 1-Nonanol, 1-Octanol, 1-Heptanol, and 1-Hexanol, while pentanoic acid 3-methyl, hexanoic acid, SCFAs (pentanoic and butanoic acid), phenol, benzaldehyde and p-Cresol were significantly associated with EP samples (Fig. 5**B**). On the other hand, no clusterization was detected between samples from different cheese types (CTRL, Lr and LrH) (Fig. 5**B**), nor for refrigeration time (T_0 and T_6) (data not shown).

Moreover, the 112 detected VOCs were from 14 chemical classes, which exhibited some statistically significant shifts between EP and BL samples (Fig. 6). Particularly, statistically significant differences were detected for EP samples in respect to BL for phenols (increased up to 10 %) and acids (increased up to 80 %); while terpenes (increased up to 20 %) and alkenes (increased up to 20 %) showed significant changes in BL (increased up to 20 %) (Fig. 6). Moreover, molecules belonging to aldehydes and alcohols were also increased ($p \geq 0.05$) in BL samples.

In addition, univariate analysis, was performed on fermentation time (BL and EP) and time of commercial shelf life at refrigerated temperature (T_0 and T_6 days) to identify possible biomarkers, by considering all type.

Within these classes, 20 metabolites were significantly altered ($p \leq 0.05$) between BL and EP samples, such as alcohols, aldehydes, acids, terpenes, esters, phenols, ketones and indoles (Fig. 7). The alcohols 1-Heptanol, 1-Hexanol, 1-Octanol and 1-Nonanol were significantly increased in all samples (BC, CTRL, Lr, LrH) at BL (Fig. 7**A**). Furthermore, benzyl alcohol was present in EP samples after CRTL and LrH

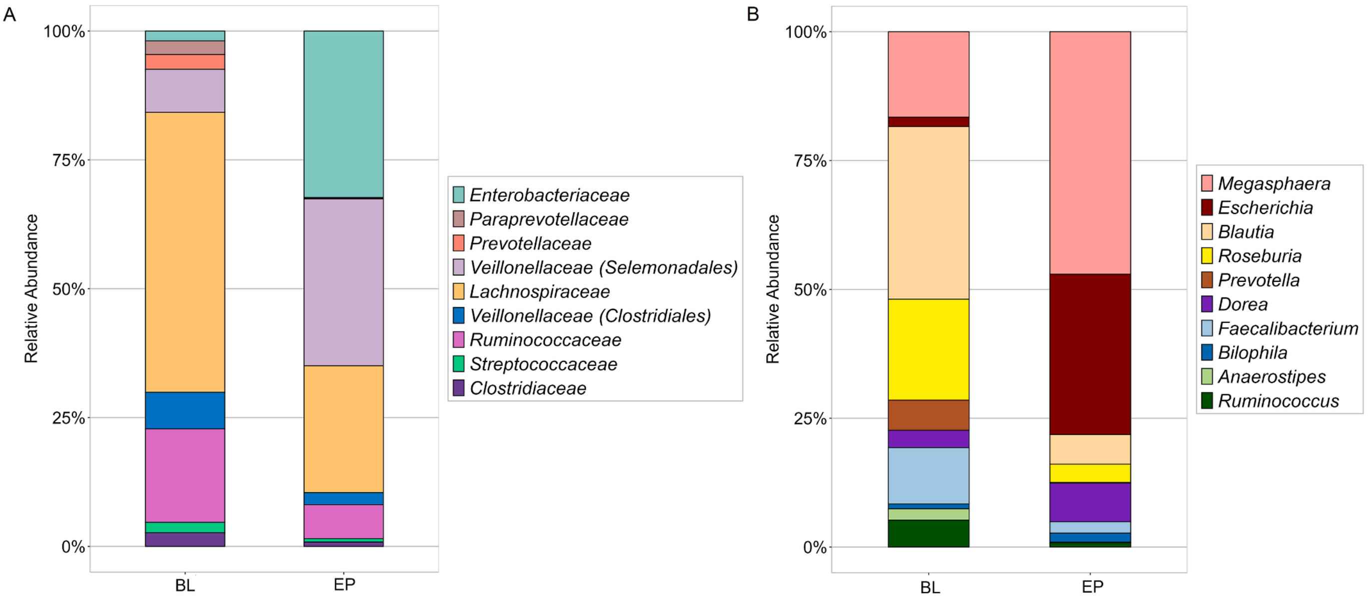

Fig. 3. Taxa distribution performed by the Mann-Whitney *U* test, at family (**panel A**) and genus (**panel B**) level between BL versus EP. Only statistically significant taxa (Mann-Whitney U *p* FDR ≤ 0.05) were represented. BL, baseline; EP, endpoint.

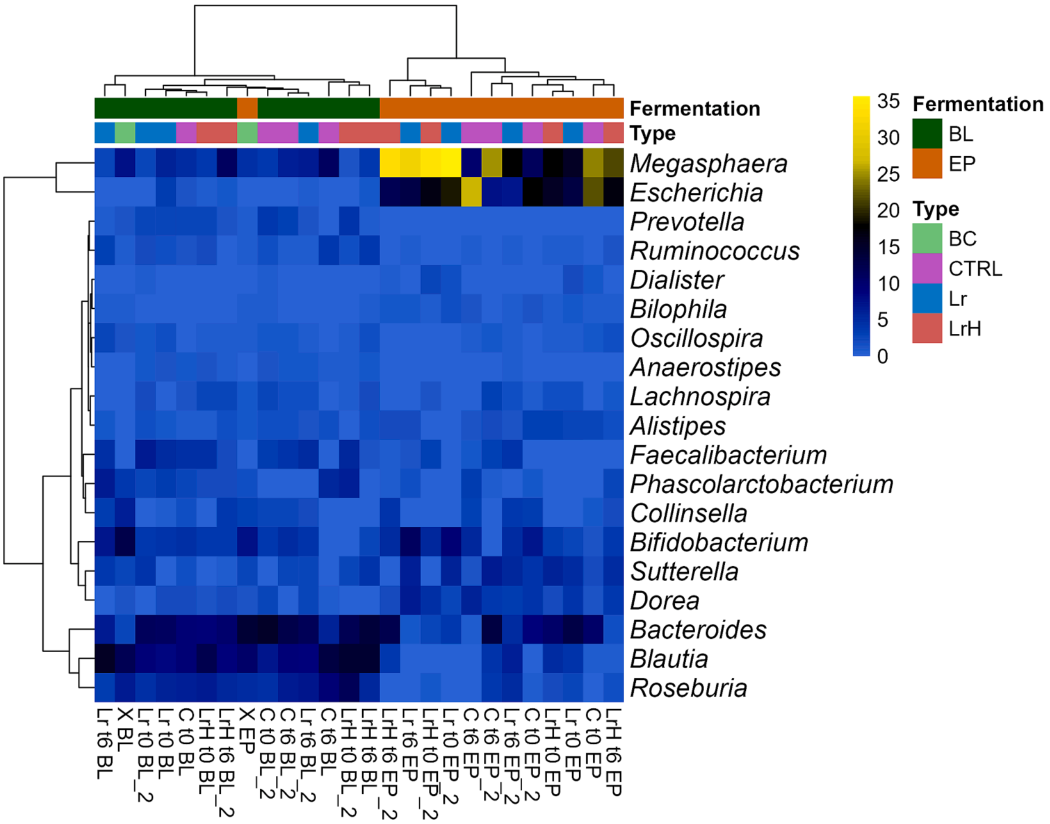

Fig. 4. Heatmap. Clustering analysis of taxa between conditions constructed using Euclidean distance and Ward's clustering method. The color scale characterizes the Z- score values for each variable: yellow, high level; blue, low level. The *p* ≤ 0.05 is corrected with FDR method. BL: baseline; EP: endpoint; BC: blank control; CTRL: control cheese with starter cultures; Lr: cheese with starter cultures and probiotic *L. rhamnosus* FBC; LrH: cheese with starter cultures and probiotic *L. rhamnosus* FBC subjected to HPH treatment.

cheese digestion while it was absent in BL samples (Fig. 7**A**). Aldehydes, on the other hand, showed a different trend, with 2-Nonenal and 2-Decenal being increased at BL, and benzaldehyde being increased in EP samples (Fig. 7**B**).

All significantly altered acids were increased in EP samples compared to BL (Fig. 7**C**). Furthermore propanoic and pentanoic acid 3-methyl, were completely absent in BL and were only detected in EP samples. On the other hand, terpenes were only present in at BL, particularly in CTRL, Lr and LrH samples (Fig. 7**D**). The only statistically significant ester, benzoic acid methyl ester, was significantly increased

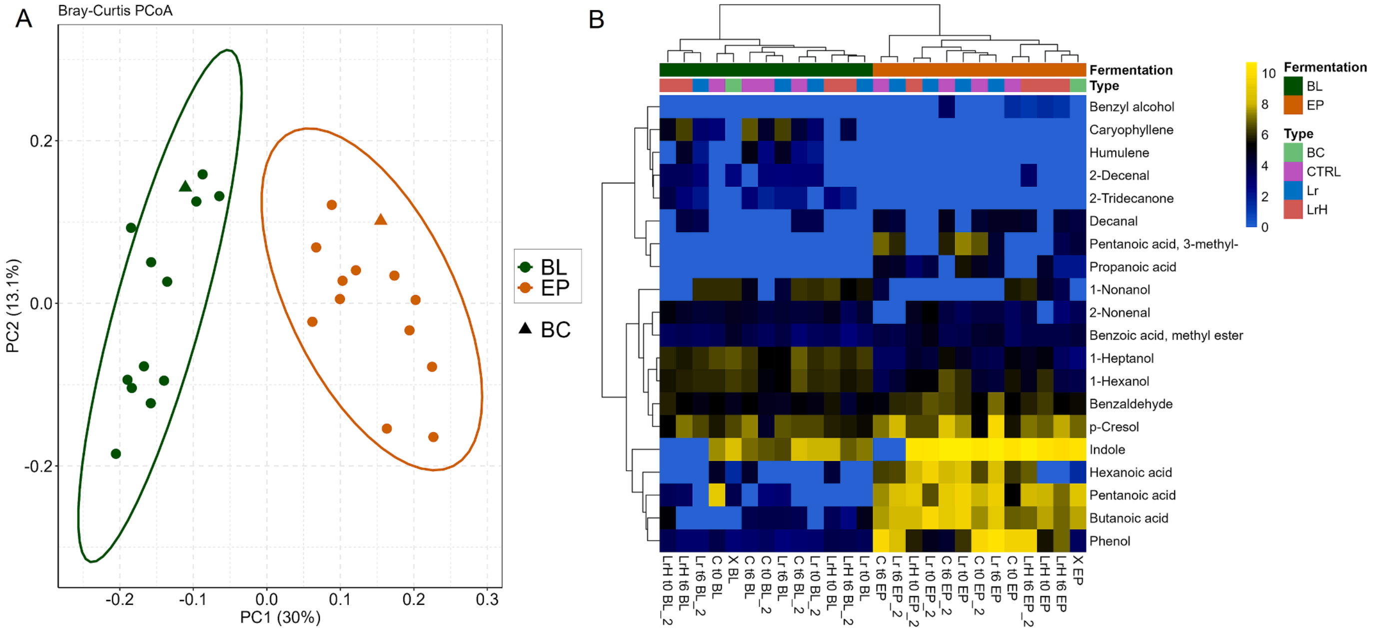

Fig. 5. Panel A. Principal Coordinate Analysis (**PCoA**) plots show Bray Curtis dissimilarity. **Panel B. Heatmap.** Clustering analysis of statistically significant VOCs between conditions constructed using Euclidean distance and Ward's clustering method. The color scale characterizes the Z- score values for each variable: yellow, high level; blue, low level. The $p \leq 0.05$ is corrected with FDR method. BL: baseline; EP: endpoint; BC: blank control; CTRL: control cheese with starter cultures; Lr: cheese with starter cultures and probiotic *L. rhamnosus* FBC; LrH: cheese with starter cultures and probiotic *L. rhamnosus* FBC subjected to HPH treatment.

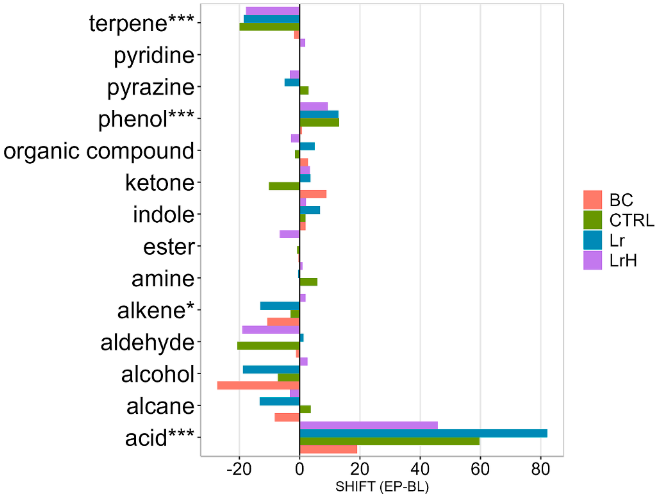

Fig. 6. Bar plot of VOCs changes expressed as relative abundances (%). Variations were recorded after fermentation respect to baseline of *in vitro* batch human colonic fermentations (MICODE). Significant differences detected by Mann-Whitney test ($p \leq 0.05$) within a chemical class group: *0.05; <0.01**; <0.001***. BL: baseline; EP: endpoint; BC: blank control; CTRL: control cheese with starter cultures; Lr: cheese with starter cultures and probiotic *L. rhamnosus* FBC; LrH: cheese with starter cultures and probiotic *L. rhamnosus* FBC subjected to HPH treatment.

in all EP samples except for the blank control (BC) (Fig. 7**E**). Phenols metabolites were also increased in EP samples (Fig. 7**F**), while ketones, such as 2-Tridecanone, was enriched at BL, particularly in CTRLs, Lr and LrH (Fig. 7**G**). Finally, indole was also significantly increased in EP samples.

When comparing the time points of commercial shelf life (T_0 and T_6 days) at refrigerated temperature samples, only 3 metabolites, belonging to 2 chemical classes (aldehydes and phenols) were significantly affected. Specifically hexanal, *p*-Cresol and decanal were significantly increased ($p \leq 0.05$) after 6 days (T_6) compared to T_0 at refrigerated temperature (Fig. 8).

3.4. Predictive functional analysis and network correlation between microbial and metabolic signatures

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) analysis was applied on metataxonomic profiles to predict abundances of KEGG orthologs (KOs) (Fig. 9). Several KOs were predicted to be significantly affected between EP and BL ($p \leq 0.05$). The twenty most abundant metabolic microbial pathways were all predicted to be enriched in EP samples (Fig. 9). These top 20 pathways include the bacterial secretion system, phosphotransferase systems (PTS), glyoxylate and dicarboxylated metabolism, ascorbate and aldarate metabolism, fatty acids biosynthesis, butanoate metabolism, nitrogen metabolism ubiquinone, glutathione metabolism and carbohydrate digestion and absorption, novobiocin biosynthesis, and amino acid metabolism (tryptophan, lysine, phenylalanine, tyrosine, valine, leucine and isoleucine), which were all over represented in EP samples (Fig. 9).

Moreover, the 20 ASVs and 112 VOCs identified in ecological and functional datasets were analyzed to select the most important correlations between GM taxonomic lineages and VOCs. Only statistically significant correlations were reported, and disconnected nodes were removed. At the end of the data processing, 10 ASVs and 10 VOCs were included in the network analysis (Fig. 10). This analysis revealed one large network, connecting most of the ASVs and VOCs included in this analysis, as well as two smaller ones (Fig. 10). The two small networks were composed of taxa and VOCs connected by both with positive and negative correlations, including *Sutterella* with hexanoic acid, and *Bacteroides* with 2-Heptanone, 4-methyl. Interestingly, butanoic acid, *Roseburia* and *Blautia* showed a central role in the network, and also correlated both positively and negatively with other metabolites and taxa (**Supplementary Table S3**; Fig. 10).

4. Discussion

In the last decade, multiple studies have shown that dietary

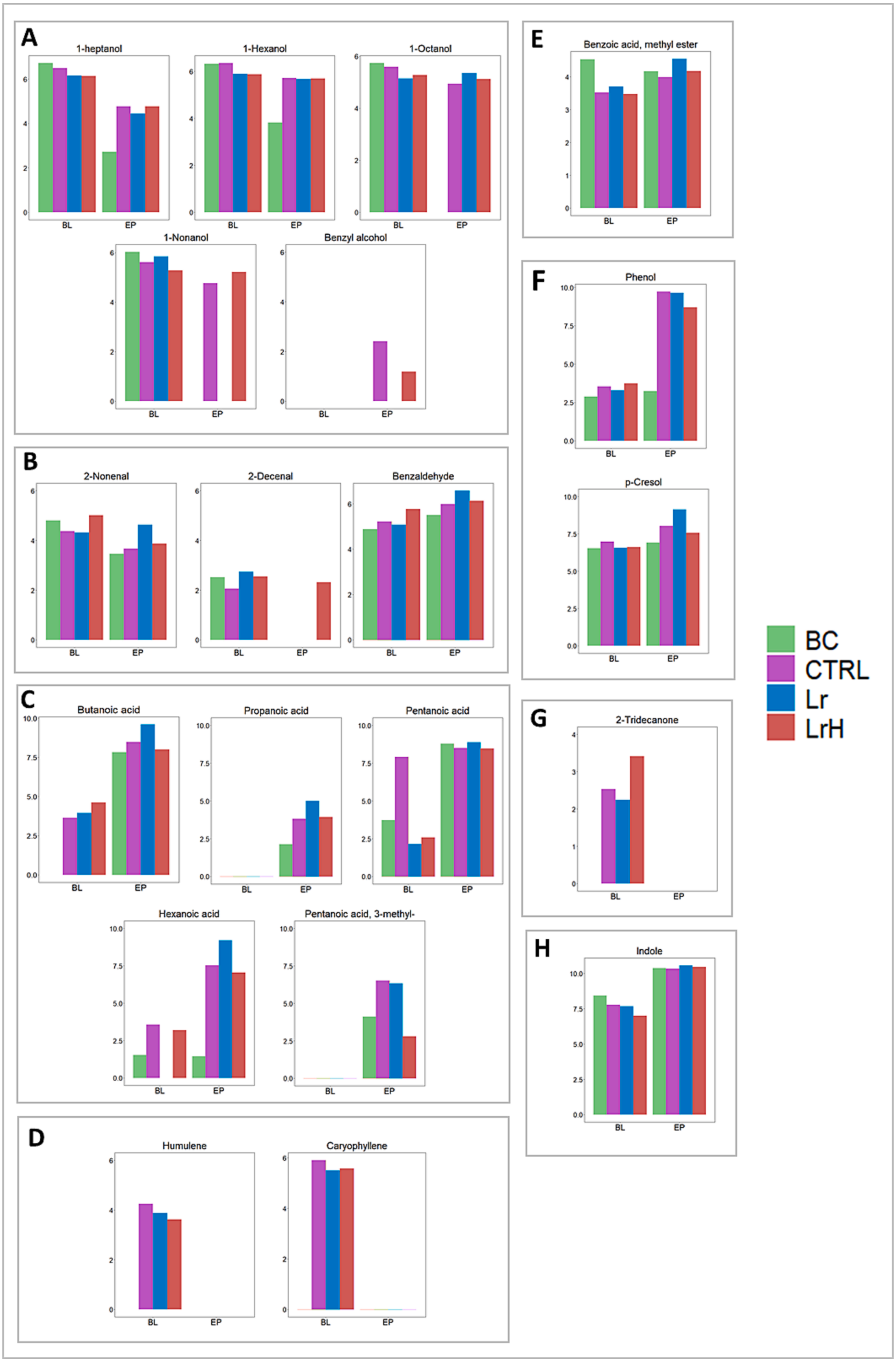

Fig. 7. Differential abundances (mg/kg) of VOCs ($p \leq 0.05$, Mann–Whitney test) for baseline and end point condition. (**A**) alcohols, (**B**) aldehydes, (**C**) acids, (**D**) terpenes, (**E**) esters, (**F**), phenols, (G) ketones and (H) indoles. BL: baseline; EP: endpoint; BC: blank control; CTRL: control cheese with starter cultures; Lr: cheese with starter cultures and probiotic *L. rhamnosus* FBC; LrH: cheese with starter cultures and probiotic *L. rhamnosus* FBC subjected to HPH treatment.

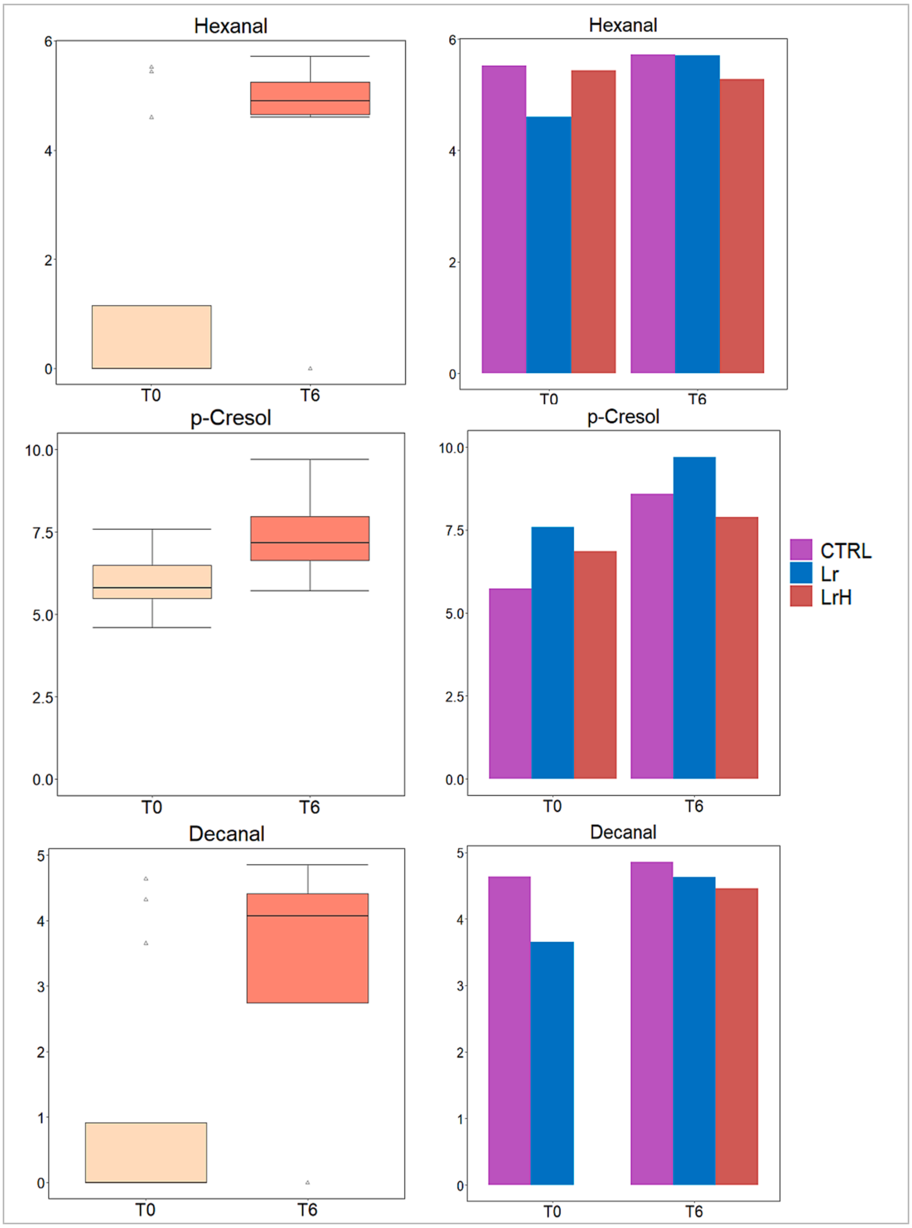

Fig. 8. Differential abundances of VOCs ($p \leq 0.05$, Mann–Whitney test) for T_0 and T_6 days of cheese commercial exposure. CTRL: control cheese with starter cultures; Lr: cheese with starter cultures and probiotic *L. rhamnosus* FBC; LrH: cheese with starter cultures and probiotic *L. rhamnosus* FBC subjected to HPH treatment. T_0: initial time; T_6: after 6 days of commercial exposure at 4 °C.

components can impact the composition of GM. Consuming foods with lactic acid bacteria, like fermented milks or cheeses, has been found to affect the GM by allowing these bacteria to thrive in the gastrointestinal tract (Putignani et al., 2014). The rationale for probiotic administration, regardless of the carrier used to deliver them, is that they can have substantial effects on human health and could change the metabolic activity of the gut's microbes (Claus et al., 2011; Larsen et al., 2010). However, the use of foods such as fermented milk or soft cheese as carriers for probiotics, in addition to protecting them from several stress conditions related to the digestive process, also strengthens the concept that food can be used as a dietary strategy to improve human wellbeing (D'Alessandro et al., 2021, 2023). In the present study, the potential probiotic properties of Squacquerone-like cheese, fortified with HPH −treated or −untreated *L. rhamnosus* FBC, were investigated on the GM after simulated digestion *via* the *in vitro* gut model MICODE. In particular, the *L. rhamnosus* FBC strain was treated at 50 MPa, since several papers have found that sub-lethal HPH treatments can increase probiotic features, such as hydrophobicity and auto-aggregation, in a strain-dependent manner (Siroli et al., 2020). For example, Braschi et al reported that 50 MPa significantly increased cell surface hydrophobicity (CSH) percentage (H%), autoaggregation and *in vitro* adhesion on mucin of *L. acidophilus 08* cells compared with untreated cells (Braschi et al., 2021). Moreover, the hyperbaric hurdle induced an upregulation of the stress response genes *groEL* and *ef-TU* (related to adhesion processes), conferring a competitive advantage to treated bacteria by increasing their resilience in the human gastrointestinal tract. The results obtained showed that the cell load reached by *S. thermophilus* in CTRL Squacquerone-like cheese was significantly higher with respect the samples in which the *L. rhamnosus* strain was added, independently of HPH treatment. These data, though inconsistent with those of Patrignani et al. (Patrignani et al., 2019) for the same type of product but with a different probiotic adjunct, showed, in any case, high starter cell loads, confirming its ability to reach desirable pH levels that, for this kind of product, need to be lower than 5.2. Furthermore, *L. rhamnosus* FBC was

Fig. 9. Error bar with relative abundance of the top 20 statistically significant pathways (p FDR < 0.05) between BL and EP obtained with the Linear models for differential abundance analysis (LINda) method. Color legend: dark green, baseline BL; dark orange, endpoint EP.

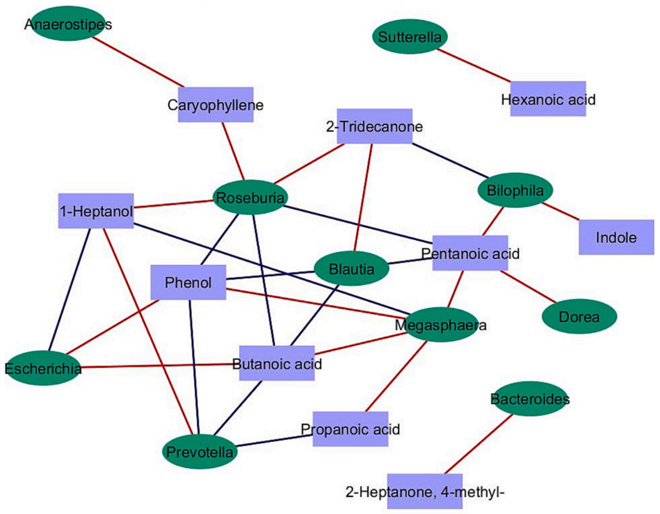

Fig. 10. Correlation network analysis between taxa and VOCs. Nodes represent the taxa (circles) and the VOCs (square), and an edge between two nodes occurs if they exhibit a statistically significant correlation ($p \leq 0.05$). The color of the network edges indicates positive (red) and negative (blue) correlations.

present at high concentrations in Lr and LrH FPCs, both at the beginning and after 6 days of commercial exposure at refrigerated temperature, corresponding to the highest point of proteolysis and to the most common conditions encountered by customers when acquiring similar products. These results fulfil two of the main criteria for selecting probiotic microorganisms to be included in food systems: i) the compatibility with eventual starter culture during cheese making and ii) the maintenance of cell loads of at least 6 log CFU/g throughout product shelf life in order to ensure its potential for some beneficial effects on the host (Burns et al., 2015).

As various dietary compounds gradually pass through the small intestine and enter the colon, they can be processed by bacterial communities, leading to the synthesis of a diverse array of microbial metabolites and producing significant changes in both microbial community composition and metabolite profiles (Liu et al., 2023).

The assays performed here revealed possible correlations between taxa such as *Megasphaera*, *Escherichia, Prevotella* and *Dorea*, and metabolites such as SCFAs, indoles, and medium chain fatty acids (MCFAs), when the cheese was fortified with *L. rhamnosus* FBC.

Our results, in terms of enumeration and modulation of core microbiota groups, are also consistent with the evidence that FPC has probiotic potential, as it is a substrate that selectively fosters the growth of microbes that confer health benefits, such as *Bifidobacterium* and *Clostridium IV* (Gibson et al., 2017).

Moreover, functional probiotic cheese (FPC) administration in all conditions resulted in an increased production of SCFAs, particularly butyric acid, which was likely linked to the increase of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus* and which, in turn, may result in reduced inflammation and gut barrier damage (Marzorati et al., 2022).

Microbial fermentation of dietary polysaccharides produces SCFAs, and hence gut bacteria, such as *Lactobacillus*, Proteobacteria, and Bacteroidetes communities (Dinu et al., 2023; Yousof et al., 2023), may help to increase nutrient absorption and metabolism, leading to improvements in health and energy balance (Portincasa et al., 2022).

The gut commensals *Bacteroides* and *Prevotella* are the main bacteria in the gut responsible for breaking down complex polysaccharides and releasing carbohydrate-active enzymes, and considered to be the main producers of propionate and other SCFAs in the intestine (Flint et al., 2012; Louis & Flint, 2017; De Filippis et al., 2016). In addition, *Megasphaera,* which was highly enriched after gut bacterial fermentation, could also contribute to the increase in butyrate production (Kamke et al., 2016).

Megasphaera, is a lactate-utilizing bacterium in the gut, which was found to be particularly increased in EP samples following fermentation of probiotic-fortified FPC. This bacterium can convert lactate to propionate through the acrylate pathway (Prabhu et al., 2012). Previous research indicated that *Megasphaera* typically converts lactate to butyrate instead of propionate under certain conditions. However, in the current study, higher levels of SCFAs were observed when FPC was administered. Therefore, it is hypothesized that the increased presence of *Megasphaera* may contribute to elevated propionate and butyrate levels in EP digests (Prabhu et al., 2012; Zhang et al., 2024).

These findings also suggest that *L. rhamnosus* FBC stimulates lactate metabolism, which could be used by *Megasphaera,* highlighting the importance of microbial interactions in the metabolism of complex carbohydrates and the production of beneficial metabolites such as butyrate (Lopez-Siles et al., 2012; Rios-Covian et al., 2015).

After FPC fermentation in the gut, MCFAs such as hexanoic, octatonic and decanoic acid were increased in EP samples. These metabolites are also known to have several benefits for the host, including improved metabolic health and immune function. MCFAs are generated by colon microbiota during chain elongation of intermediate fermentation fibers derivatives (Scarborough et al., 2020) or by straight fiber degradation by Bifidobacteriaceae (Rivière et al., 2018). Furthermore, Enterobacteriaceae and *Bacteroides* spp. can produce MCFAs from lactate (Scarborough et al., 2020).

In addition, indole was increased in EP samples after simulated digestion of with *L. rhamnosus*-fortified FBC. Indole has been shown to have anti-inflammatory properties through the activation of the aryl hydrocarbon receptor (AHR) pathway (Hou et al., 2023). Different commensal bacteria catabolize tryptophan by using tryptophanase, converting it into indoles and other derivatives (Roager & Licht, 2018). It can also modulate immune responses and promote the production of antimicrobial peptides in the gut, contributing to the maintenance of gut homeostasis (Ye et al., 2022). Additionally, indole has been found to have antioxidant and neuroprotective effects (Alexeev et al., 2018; Barresi et al., 2024). Consistently, tryptophan metabolism, was predicted to be increased in EP samples following PICRUSt2 analysis of GM metataxonomy (Agus et al., 2018).

Indole formation from tryptophan occurs in several Gram-negative and Gram-positive bacterial species, including *Escherichia coli*, *Clostridium, Bacteroides* (Lee & Lee, 2010), *Lactobacillus* (Cervantes-Barragan et al., 2017) and *Bifidobacterium* (Roager & Licht, 2018). Tryptophan metabolism in the gut is therefore an actionable actor from a therapeutic perspective, using either molecules targeting a specific pathway or by exploiting microorganisms to modulate tryptophan metabolism *via* probiotic administration. Thus, targeting tryptophan metabolism in the gut can be a viable therapeutic approach by utilizing molecules that target a specific pathway or incorporating probiotics that manipulate this metabolic pathway.

PICRUSt2 analysis was used to deepen the investigation into the functional activity of the GM, and to overlay the metaxanonomic and metabolomic datasets. Particularly, glyoxylate and dicarboxylate metabolism is able to catabolize acetate and amino acids for energy production (Maniscalco et al., 2017) and may be related to the increase of other pathways such as beta-alanine metabolism.

Ascorbate and aldarate metabolism are carbohydrate metabolic pathways, which can also aid the body in counteracting diseases. Ascorbic acid is a degradation product of vitamin C, which exhibits a protective role by counteracting free radicals (Trezzi et al., 2017).

Particularly, a diet rich in nutraceutical ingredients are catabolized in the colon by some microorganisms, which alter the composition of the GM and affect its ecological composition and functional profile (Cui et al., 2024). Gut microbiota could quicken the release and breakdown of phenolic compounds that, in turn, affect the pathogen inhibition.

Moreover, alpha linoleinc acid metabolism could be involved in a-linolenic acid (ALA) metabolism, which is known to be a metabolite that contributes to the differentiation of anti-inflammatory M2-type macrophages (Ohue-Kitano et al., 2018).

Possibly, these changes in the GM of EP samples may play a role in the metabolism of butanoate (butyrate), which could help maintain the balance of intestinal epithelial cells and regulate immune tolerance to antigens in the intestine (Chang et al., 2014).

The biosynthesis of novobiocin falls under the category of aminocoumarin antibiotics (Steffensky et al., 2000) and has been shown to promote gut health by inhibiting the growth of pathogens (Mandler et al., 2018; Wang et al., 2022). A notable increase in novobiocin biosynthesis function was observed at the end of colonic fermentation. Moreover, novobiocin is known for its effectiveness against gram-positive bacteria, with a particular affinity for *Staphylococcus aureus* ("Practical Antimicrobial Therapeutics," 2017).

On the other hand, HPH treatment does not seem to lead to significantly different ecological and metabolic activities in any of the comparisons examined. This may be due to strain-dependent responses to HPH treatment in relation to simulated gastric acidity (SGA). Indeed, some studies have indicated that HPH can enhance the SGA tolerance of certain strains, while others have reported no significant effect or even a reduction in tolerance. For instance, *Lactobacillus paracasei* and *Lactobacillus acidophilus* showed greater resistance compared to *Lactobacillus delbrueckii* subsp. *lactis* following HPH treatment (Tabanelli et al., 2013). Furthermore, the enhanced quality of cheeses made with HPH-treated *L. paracasei* cells might also be linked to changes in proteolytic, lipolytic, and volatile compound profiles (Burns et al., 2015). However, there is no evidence to suggest that this treatment has a direct effect on GM composition or function.

Finally, the small number of samples, the use of *in vitro* models missing the intricacy of the human physiology, and microbiota analysis based on the 16S rRNA metataxonomy, which has less resolution than other approaches such as shotgun metagenomics, could be considered the limitations of this study.

5. Conclusions

In conclusion, this study explored the effects of the probiotic strain *L. rhamnosus* FBC on gut microbial ecology and function when incorporated into cheese under various cell-pretreatment conditions, utilizing MICODE-based simulated digestion.

The effects were investigated immediately after cheese manufacturing and after 6 days of product refrigerated storage, coinciding with both the maximum point of proteolysis and the most common condition encountered by consumers who would buy and consume the product if it were on the market.

Our findings indicate that the most significant health benefits emerged during the colonic fermentation phase, highlighting the advantages of dietary probiotics over the considered traditional commercial exposure. This underscores the critical role of maintaining intestinal balance, rather than merely focusing on food shelf life. Understanding microbial interactions may lead to novel microbiome-based therapies that enhance gut health and address metabolic disorders through enjoyable dietary strategies, rather than relying solely on conventional probiotic supplements.

Particularly, to realize new probiotic food as a "healthy and good tasting product" to promote health starting from the gut to general well-being in adults and children.

Further investigation are needed to clarify the mechanisms and ecological relevance of these interactions within the human GM. By promoting a healthy microbiome *via* dietary intervention and lifestyle adjustments, individuals may enhance their overall health.

In alignment with the One Health approach and the 3Rs principles, as stipulated by Directive 2010/63/EU and Regulation (EU) 2019/1010, our research contributes valuable preclinical evidence to support nutritional interventions, reduce reliance on animal testing, and enhance our understanding of food functionality and safety.

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CRediT authorship contribution statement

Chiara Marangelo: Writing – original draft, Formal analysis, Data curation. **Riccardo Marsiglia:** Visualization, Formal analysis. **Lorenzo Nissen:** Writing – review & editing, Formal analysis. **Matteo Scanu:** Formal analysis. **Francesca Toto:** Writing – review & editing, Funding acquisition, Conceptualization. **Lorenzo Siroli:** Writing – review & editing, Formal analysis. **Davide Gottardi:** Formal analysis. **Giacomo Braschi:** Formal analysis. **Federica Del Chierico:** Writing – review & editing. **Alessandra Bordoni:** Writing – review & editing. **Andrea Gianotti:** Writing – review & editing. **Rosalba Lanciotti:** Supervision. **Francesca Patrignani:** Writing – review & editing, Funding acquisition, Conceptualization. **Lorenza Putignani:** Writing – review & editing, Supervision. **Pamela Vernocchi:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Conceptualization.

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.foodres.2024.115577.

Data availability

Data will be made available on request.

References

Agus, A., Planchais, J., & Sokol, H. (2018). Gut microbiota regulation of tryptophan metabolism in health and disease. *Cell Host & Microbe, 23*(6), 716–724. https://doi.org/10.1016/j.chom.2018.05.003

Alexeev, E. E., Lanis, J. M., Kao, D. J., Campbell, E. L., Kelly, C. J., Battista, K. D., Gerich, M. E., Jenkins, B. R., Walk, S. T., Kominsky, D. J., & Colgan, S. P. (2018). Microbiota-derived indole metabolites promote human and murine intestinal homeostasis through regulation of interleukin-10 receptor. *The American Journal of Pathology, 188*(5), 1183–1194. https://doi.org/10.1016/j.ajpath.2018.01.011

Baker, M. T., Lu, P., Parrella, J. A., & Leggette, H. R. (2022). Investigating the effect of consumers' knowledge on their acceptance of functional foods: a systematic review and meta-analysis. Article 8 *Foods, 11*(8). https://doi.org/10.3390/foods11081135.

Barresi, E., Baglini, E., Poggetti, V., Castagnoli, J., Giorgini, D., Salerno, S., Taliani, S., & Da Settimo, F. (2024). Indole-based compounds in the development of anti-neurodegenerative agents. *Molecules, 29*(9), 2127. https://doi.org/10.3390/molecules29092127

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C. C., Al-Ghalith, G. A., Alexander, H., Alm, E. J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J. E., Bittinger, K., Brejnrod, A., Brislawn, C. J., Brown, C. T., Callahan, B. J., Caraballo-Rodríguez, A. M., Chase, J., & Caporaso, J. G. (2019). Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nature Biotechnology, 37*(8), 852–857. https://doi.org/10.1038/s41587-019-0209-9

Bosscher, D., Breynaert, A., Pieters, L., & Hermans, N. (2009). Food-based strategies to modulate the composition of the intestinal microbiota and their associated health effects. *Journal of Physiology and Pharmacology: An Official Journal of the Polish Physiological Society, 60*(Suppl 6), 5–11.

Boylston, T. D., Vinderola, C. G., Ghoddusi, H. B., & Reinheimer, J. A. (2004). Incorporation of bifidobacteria into cheeses: Challenges and rewards. *International Dairy Journal, 14*(5), 375–387. https://doi.org/10.1016/j.idairyj.2003.08.008

Braschi, G., D'Alessandro, M., Gottardi, D., Siroli, L., Patrignani, F., & Lanciotti, R. (2021). Effects of sub-lethal high pressure homogenization treatment on the adhesion mechanisms and stress response genes in lactobacillus acidophilus 08. *Frontiers in Microbiology, 12*, Article 651711. https://doi.org/10.3389/fmicb.2021.651711

Brodmann, T., Endo, A., Gueimonde, M., Vinderola, G., Kneifel, W., De Vos, W. M., Salminen, S., & Gómez-Gallego, C. (2017). Safety of novel microbes for human consumption: practical examples of assessment in the European Union. *Frontiers in Microbiology, 8*, 1725. https://doi.org/10.3389/fmicb.2017.01725

Burns, P. G., Patrignani, F., Tabanelli, G., Vinderola, G. C., Siroli, L., Reinheimer, J. A., Gardini, F., & Lanciotti, R. (2015). Potential of high pressure homogenisation on probiotic Caciotta cheese quality and functionality. *Journal of Functional Foods, 13*, 126–136. https://doi.org/10.1016/j.jff.2014.12.037

Cattivelli, A., Nissen, L., Casciano, F., Tagliazucchi, D., & Gianotti, A. (2023). Impact of cooking methods of red-skinned onion on metabolic transformation of phenolic compounds and gut microbiota changes. *Food & Function, 14*(8), 3509–3525. https://doi.org/10.1039/D3FO00085K

Cervantes-Barragan, L., Chai, J. N., Tianero, Ma. D., Di Luccia, B., Ahern, P. P., Merriman, J., Cortez, V. S., Caparon, M. G., Donia, M. S., Gilfillan, S., Cella, M., Gordon, J. I., Hsieh, C.-S., & Colonna, M. (2017). *Lactobacillus reuteri* induces gut intraepithelial CD4$^+$ CD8αα$^+$ T cells. *Science, 357*(6353), 806–810. https://doi.org/10.1126/science.aah5825

Chang, P. V., Hao, L., Offermanns, S., & Medzhitov, R. (2014). The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. *Proceedings of the National Academy of Sciences, 111*(6), 2247–2252. https://doi.org/10.1073/pnas.1322269111

Charalampopoulos, D., Wang, R., Pandiella, S. S., & Webb, C. (2002). Application of cereals and cereal components in functional foods: A review. *International Journal of Food Microbiology, 79*(1–2), 131–141. https://doi.org/10.1016/S0168-1605(02)00187-3

Claus, S. P., Ellero, S. L., Berger, B., Krause, L., Bruttin, A., Molina, J., Paris, A., Want, E. J., De Waziers, I., Cloarec, O., Richards, S. E., Wang, Y., Dumas, M.-E., Ross, A., Rezzi, S., Kochhar, S., Van Bladeren, P., Lindon, J. C., Holmes, E., & Nicholson, J. K. (2011). Colonization-induced host-gut microbial metabolic interaction. *mBio, 2*(2), e00271–e00310. https://doi.org/10.1128/mBio.00271-10

Connolly, M. L., Tuohy, K. M., & Lovegrove, J. A. (2012). Wholegrain oat-based cereals have prebiotic potential and low glycaemic index. *British Journal of Nutrition, 108*(12), 2198–2206. https://doi.org/10.1017/S0007114512000281

Cui, W., Chen, F., Sun, Z., Cui, C., Xu, B., Shen, W., Wan, F., & Cheng, A. (2024). Catabolism of phenolics from grape peel and its effects on gut microbiota during *in vitro* colonic fermentation. *Journal of the Science of Food and Agriculture*. https://doi.org/10.1002/jsfa.13540

D'Alessandro, M., Gottardi, D., Parolin, C., Glicerina, V. T., Vitali, B., Lanciotti, R., & Patrignani, F. (2023). Development and characterization of fermented soy beverages containing encapsulated or non-encapsulated vaginal probiotics. *LWT, 180*, Article 114713. https://doi.org/10.1016/j.lwt.2023.114713

D'Alessandro, M., Pisanu, F., Baldo, D., Parolin, C., Filippini, G., Vitali, B., Lanciotti, R., & Patrignani, F. (2021). Unravelling the functional and technological potential of soy milk based microencapsulated Lactobacillus crispatus and Lactobacillus gasseri. *Journal of Functional Foods, 87*, Article 104745. https://doi.org/10.1016/j.jff.2021.104745

De Filippis, F., Pellegrini, N., Vannini, L., Jeffery, I. B., La Storia, A., Laghi, L., Serrazanetti, D. I., Di Cagno, R., Ferrocino, I., Lazzi, C., Turroni, S., Cocolin, L., Brigidi, P., Neviani, E., Gobbetti, M., O'Toole, P. W., & Ercolini, D. (2016). High-level adherence to a Mediterranean diet beneficially impacts the gut microbiota and associated metabolome. *Gut, 65*(11), 1812–1821. https://doi.org/10.1136/gutjnl-2015-309957

Dinu, L.-D., Gatea, F., Roaming Israel, F., Lakicevic, M., Dedović, N., & Vamanu, E. (2023). The modulation effect of a fermented bee pollen postbiotic on cardiovascular microbiota and therapeutic perspectives. *Biomedicines, 11*(10), 2712. https://doi.org/10.3390/biomedicines11102712

Doron, S., Snydman, D. R., & Gorbach, S. L. (2005). Lactobacillus GG: Bacteriology and clinical applications. *Gastroenterology Clinics of North America, 34*(3), 483–498. https://doi.org/10.1016/j.gtc.2005.05.011

Dupont, D., Alric, M., Blanquet-Diot, S., Bornhorst, G., Cueva, C., Deglaire, A., Denis, S., Ferrua, M., Havenaar, R., Lelieveld, J., Mackie, A. R., Marzorati, M., Menard, O., Minekus, M., Miralles, B., Recio, I., & Van Den Abbeele, P. (2019). Can dynamic *in vitro* digestion systems mimic the physiological reality? *Critical Reviews in Food Science and Nutrition, 59*(10), 1546–1562. https://doi.org/10.1080/10408398.2017.1421900

European Commission. Directorate-General for Research. (2010). *Functional foods*. Publications Office. https://data.europa.eu/doi/10.2777/82512.

Flint, H. J., Duncan, S. H., Scott, K. P., & Louis, P. (2007). Interactions and competition within the microbial community of the human colon: Links between diet and health. *Environmental Microbiology, 9*(5), 1101–1111. https://doi.org/10.1111/j.1462-2920.2007.01281.x

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P., & Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut Microbes, 3*(4), 289–306. https://doi.org/10.4161/gmic.19897

Gerritsen, J., Smidt, H., Rijkers, G. T., & De Vos, W. M. (2011). Intestinal microbiota in human health and disease: The impact of probiotics. *Genes & Nutrition, 6*(3), 209–240. https://doi.org/10.1007/s12263-011-0229-7

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K., & Reid, G. (2017). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology & Hepatology, 14*(8). https://doi.org/10.1038/nrgastro.2017.75. Article 8.

Grimm, V., Westermann, C., & Riedel, C. U. (2014). Bifidobacteria-host interactions—an update on colonisation factors. *BioMed Research International, 2014*, 1–10. https://doi.org/10.1155/2014/960826

Hasler, C. M. (2002). Functional foods: benefits, concerns and challenges—a position paper from the American council on science and health. *The Journal of Nutrition, 132* (12), 3772–3781. https://doi.org/10.1093/jn/132.12.3772

Heller, K. J. (2001). Probiotic bacteria in fermented foods: Product characteristics and starter organisms. *The American Journal of Clinical Nutrition, 73*(2), 374s–379s. https://doi.org/10.1093/ajcn/73.2.374s

Hou, J.-J., Ma, A.-H., & Qin, Y.-H. (2023). Activation of the aryl hydrocarbon receptor in inflammatory bowel disease: Insights from gut microbiota. *Frontiers in Cellular and Infection Microbiology, 13*, Article 1279172. https://doi.org/10.3389/fcimb.2023.1279172

Kamke, J., Kittelmann, S., Soni, P., Li, Y., Tavendale, M., Ganesh, S., Janssen, P. H., Shi, W., Froula, J., Rubin, E. M., & Attwood, G. T. (2016). Rumen metagenome and metatranscriptome analyses of low methane yield sheep reveals a Sharpea-enriched microbiome characterised by lactic acid formation and utilisation. *Microbiome, 4*(1), 56. https://doi.org/10.1186/s40168-016-0201-2

Larsen, N., Vogensen, F. K., Van Den Berg, F. W. J., Nielsen, D. S., Andreasen, A. S., Pedersen, B. K., Al-Soud, W. A., Sørensen, S. J., Hansen, L. H., & Jakobsen, M. (2010). Gut microbiota in human adults with type 2 diabetes differs from non-diabetic adults. *PLoS One1, 5*(2). https://doi.org/10.1371/journal.pone.0009085. e9085.

Lebeer, S., Vanderleyden, J., & De Keersmaecker, S. C. J. (2010). Host interactions of probiotic bacterial surface molecules: Comparison with commensals and pathogens. *Nature Reviews Microbiology, 8*(3), 171–184. https://doi.org/10.1038/nrmicro2297

Lee, J.-H., & Lee, J. (2010). Indole as an intercellular signal in microbial communities. *FEMS Microbiology Reviews, 34*(4), 426–444. https://doi.org/10.1111/j.1574-6976.2009.00204.x

Liu, L., Narrowe, A. B., Firrman, J. A., Mahalak, K. K., Bobokalonov, J. T., Lemons, J. M. S., Bittinger, K., Daniel, S., Tanes, C., Mattei, L., Friedman, E. S., Soares, J. W., Kobori, M., Zeng, W.-B., & Tomasula, P. M. (2023). Lacticaseibacillus rhamnosus Strain GG (LGG) regulate gut microbial metabolites, an in vitro study using three mature human gut microbial cultures in a simulator of human intestinal microbial ecosystem (SHIME). *Foods, 12*(11), 2105. https://doi.org/10.3390/foods12112105

Lopez-Siles, M., Khan, T. M., Duncan, S. H., Harmsen, H. J. M., Garcia-Gil, L. J., & Flint, H. J. (2012). Cultured representatives of two major phylogroups of human colonic faecalibacterium prausnitzii can utilize pectin, uronic acids, and host-derived substrates for growth. *Applied and Environmental Microbiology, 78*(2), 420–428. https://doi.org/10.1128/AEM.06858-11

Louis, P., & Flint, H. J. (2017). Formation of propionate and butyrate by the human colonic microbiota. *Environmental Microbiology, 19*(1), 29–41. https://doi.org/10.1111/1462-2920.13589

Mandler, M. D., Baidin, V., Lee, J., Pahil, K. S., Owens, T. W., & Kahne, D. (2018). Novobiocin enhances polymyxin activity by stimulating lipopolysaccharide transport. *Journal of the American Chemical Society, 140*(22), 6749–6753. https://doi.org/10.1021/jacs.8b02283

Maniscalco, M., Paris, D., Melck, D. J., D'Amato, M., Zedda, A., Sofia, M., Stellato, C., & Motta, A. (2017). Coexistence of obesity and asthma determines a distinct respiratory metabolic phenotype. *Journal of Allergy and Clinical Immunology, 139*(5), 1536–1547.e5. https://doi.org/10.1016/j.jaci.2016.08.038

Martina, A., Felis, G. E., Corradi, M., Maffeis, C., Torriani, S., & Venema, K. (2019). Effects of functional pasta ingredients on different gut microbiota as revealed by TIM-2 in vitro model of the proximal colon. *Beneficial Microbes, 10*(3), 301–314. https://doi.org/10.3920/BM2018.0088

Marzorati, M., Bubeck, S., Bayne, T., Krishnan, K., & Giusto, M. (2022). Effects of combined prebiotic, probiotic, IgG and amino acid supplementation on the gut microbiome of patients with inflammatory bowel disease. *Future Microbiology, 17* (16), 1307–1324. https://doi.org/10.2217/fmb-2022-0066

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., & Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food – an international consensus. *Food & Function, 5*(6), 1113–1124. https://doi.org/10.1039/C3FO60702J

Modesto, M., Stefanini, I., D'Aimmo, M. R., Nissen, L., Tabanelli, D., Mazzoni, M., Bosi, P., Strozzi, G. P., & Biavati, B. (2011). Strategies to augment non-immune system based defence mechanisms against gastrointestinal diseases in pigs. *NJAS: Wageningen Journal of Life Sciences, 58*(3–4), 149–156. https://doi.org/10.1016/j.njas.2011.04.001

Morelli, L. (2000). In vitro selection of probiotic lactobacilli: A critical appraisal. *Current Issues in Intestinal Microbiology, 1*(2), 59–67.

Nissen, L., Casciano, F., Chiarello, E., Di Nunzio, M., Bordoni, A., & Gianotti, A. (2024). Sourdough process and spirulina-enrichment can mitigate the limitations of colon fermentation performances of gluten-free breads in non-celiac gut model. *Food Chemistry, 436*, Article 137633. https://doi.org/10.1016/j.foodchem.2023.137633

Nissen, L., Valerii, M. C., Spisni, E., Casciano, F., & Gianotti, A. (2021). Multiunit in vitro colon model for the evaluation of prebiotic potential of a fiber plus D-limonene food supplement. *Foods, 10*(10), 2371. https://doi.org/10.3390/foods10102371

Ohue-Kitano, R., Yasuoka, Y., Goto, T., Kitamura, N., Park, S., Kishino, S., Kimura, I., Kasubuchi, M., Takahashi, H., Li, Y., Yeh, Y., Jheng, H., Iwase, M., Tanaka, M., Masuda, S., Inoue, T., Yamakage, H., Kusakabe, T., Tani, F., & Kawada, T. (2018). α-Linolenic acid-derived metabolites from gut lactic acid bacteria induce differentiation of anti-inflammatory M2 macrophages through G protein-coupled receptor 40. *The FASEB Journal, 32*(1), 304–318. https://doi.org/10.1096/fj.201700273R

Patel, H. M., Pandiella, S. S., Wang, R. H., & Webb, C. (2004). Influence of malt, wheat, and barley extracts on the bile tolerance of selected strains of lactobacilli. *Food Microbiology, 21*(1), 83–89. https://doi.org/10.1016/S0740-0020(03)00016-9

Patrignani, F., Parolin, C., D'Alessandro, M., Siroli, L., Vitali, B., & Lanciotti, R. (2020). Evaluation of the fate of Lactobacillus crispatus BC4, carried in Squacquerone cheese, throughout the simulator of the human intestinal microbial ecosystem (SHIME). *Food Research International, 137*, Article 109580. https://doi.org/10.1016/j.foodres.2020.109580

Patrignani, F., Siroli, L., Parolin, C., Serrazanetti, D. I., Vitali, B., & Lanciotti, R. (2019). Use of Lactobacillus crispatus to produce a probiotic cheese as potential gender food for preventing gynaecological infections. *PLoS One1, 14*(1). https://doi.org/10.1371/journal.pone.0208906. e0208906.

Patrignani, F., Siroli, L., Serrazanetti, D. I., & Lanciotti, R. (2018). Potential of high pressure homogenization and functional strains for the development of novel functional dairy foods. In N. Koca (Ed.), *Technological Approaches for Novel Applications in Dairy Processing*. InTech. https://doi.org/10.5772/intechopen.74448.

Phillips, M., Kailasapathy, K., & Tran, L. (2006). Viability of commercial probiotic cultures (L. acidophilus, Bifidobacterium sp., L. casei, L. paracasei and L. rhamnosus) in cheddar cheese. *International Journal of Food Microbiology, 108*(2), 276–280. https://doi.org/10.1016/j.ijfoodmicro.2005.12.009

Portincasa, P., Bonfrate, L., Vacca, M., De Angelis, M., Farella, I., Lanza, E., Khalil, M., Wang, D.-Q.-H., Sperandio, M., & Di Ciaula, A. (2022). Gut microbiota and short chain fatty acids: implications in glucose homeostasis. *International Journal of Molecular Sciences, 23*(3), 1105. https://doi.org/10.3390/ijms23031105

Prabhu, R., Altman, E., & Eiteman, M. A. (2012). Lactate and acrylate metabolism by Megasphaera elsdenii under batch and steady-state conditions. *Applied and Environmental Microbiology, 78*(24), 8564–8570. https://doi.org/10.1128/AEM.02443-12

Practical Antimicrobial Therapeutics. (2017). In *Veterinary Medicine* (pp. 153–174). Elsevier. https://doi.org/10.1016/B978-0-7020-5246-0.00006-1.

Prakash, S., Tomaro-Duchesneau, C., Saha, S., & Cantor, A. (2011). The gut microbiota and human health with an emphasis on the use of microencapsulated bacterial cells. *BioMed Research International, 2011*(1), Article 981214. https://doi.org/10.1155/2011/981214

Putignani, L., Del Chierico, F., Petrucca, A., Vernocchi, P., & Dallapiccola, B. (2014). The human gut microbiota: A dynamic interplay with the host from birth to senescence settled during childhood. *Pediatric Research, 76*(1), 2–10. https://doi.org/10.1038/pr.2014.49

Rios-Covian, D., Gueimonde, M., Duncan, S. H., Flint, H. J., & De Los Reyes-Gavilan, C. G. (2015). Enhanced butyrate formation by cross-feeding between *Faecalibacterium prausnitzii* and *Bifidobacterium adolescentis*. *FEMS Microbiology Letters, 362*(21), Article fnv176. https://doi.org/10.1093/femsle/fnv176

Rivière, A., Selak, M., Geirnaert, A., Van Den Abbeele, P., & De Vuyst, L. (2018). Complementary mechanisms for degradation of inulin-type fructans and arabinoxylan oligosaccharides among bifidobacterial strains suggest bacterial cooperation. *Applied and Environmental Microbiology, 84*(9), e02893–e02917. https://doi.org/10.1128/AEM.02893-17

Roager, H. M., & Licht, T. R. (2018). Microbial tryptophan catabolites in health and disease. *Nature Communications, 9*(1), 3294. https://doi.org/10.1038/s41467-018-05470-4

Scarborough, M. J., Myers, K. S., Donohue, T. J., & Noguera, D. R. (2020). Medium-chain fatty acid synthesis by "*Candidatus* Weimeria bifida" gen. Nov., sp. Nov., and "*Candidatus* Pseudoramibacter fermentans" sp. Nov. *Applied and Environmental Microbiology, 86*(3), e02242–e02319. https://doi.org/10.1128/AEM.02242-19

Shah, N. P. (2000). Probiotic bacteria: selective enumeration and survival in dairy foods. *Journal of Dairy Science, 83*(4), 894–907. https://doi.org/10.3168/jds.S0022-0302(00)74953-8

Siroli, L., Braschi, G., Rossi, S., Gottardi, D., Patrignani, F., & Lanciotti, R. (2020). Lactobacillus paracasei A13 and high-pressure homogenization stress response. *Microorganisms, 8*(3), 439. https://doi.org/10.3390/microorganisms8030439

Steffensky, M., Mühlenweg, A., Wang, Z.-X., Li, S.-M., & Heide, L. (2000). Identification of the novobiocin biosynthetic gene cluster of *Streptomyces spheroides* NCIB 11891. *Antimicrobial Agents and Chemotherapy, 44*(5), 1214–1222. https://doi.org/10.1128/AAC.44.5.1214-1222.2000

Sumeri, I., Arike, L., Adamberg, K., & Paalme, T. (2008). Single bioreactor gastrointestinal tract simulator for study of survival of probiotic bacteria. *Applied Microbiology and Biotechnology, 80*(2), 317–324. https://doi.org/10.1007/s00253-008-1553-8

Tabanelli, G., Patrignani, F., Vinderola, G., Reinheimer, J. A., Gardini, F., & Lanciotti, R. (2013). Effect of sub-lethal high pressure homogenization treatments on the in vitro functional and biological properties of lactic acid bacteria. *LWT - Food Science and Technology, 53*(2), 580–586. https://doi.org/10.1016/j.lwt.2013.03.013

Tamargo, A., Cueva, C., Silva, M., Molinero, N., Miralles, B., Bartolomé, B., & Moreno-Arribas, M. V. (2022). Gastrointestinal co-digestion of wine polyphenols with glucose/whey proteins affects their bioaccessibility and impact on colonic microbiota. *Food Research International, 155*, Article 111010. https://doi.org/10.1016/j.foodres.2022.111010

Tanner, S. A., Zihler Berner, A., Rigozzi, E., Grattepanche, F., Chassard, C., & Lacroix, C. (2014). In vitro continuous fermentation model (PolyFermS) of the swine proximal colon for simultaneous testing on the same gut microbiota. *PLoS One1, 9*(4). https://doi.org/10.1371/journal.pone.0094123. e94123.

Taverniti, V., & Guglielmetti, S. (2011). The immunomodulatory properties of probiotic microorganisms beyond their viability (ghost probiotics: Proposal of paraprobiotic concept). *Genes & Nutrition, 6*(3), 261–274. https://doi.org/10.1007/s12263-011-0218-x

The European Parliament and of the Council of the European Union. (2006, December 20). *Regulation (EC) No 1924/2006 of the European parliament and of the council*. Official Journal of the European Union. https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:404:0009:0025:En:PDF.

Topolska, K., Florkiewicz, A., & Filipiak-Florkiewicz, A. (2021). Functional food—consumer motivations and expectations. *International Journal of Environmental Research and Public Health, 18*(10), 5327. https://doi.org/10.3390/ijerph18105327

Trezzi, J., Galozzi, S., Jaeger, C., Barkovits, K., Brockmann, K., Maetzler, W., Berg, D., Marcus, K., Betsou, F., Hiller, K., & Mollenhauer, B. (2017). Distinct metabolomic signature in cerebrospinal fluid in early parkinson's disease. *Movement Disorders, 32* (10), 1401–1408. https://doi.org/10.1002/mds.27132

Tsitko, I., Wiik-Miettinen, F., Mattila, O., Rosa-Sibakov, N., Seppänen-Laakso, T., Maukonen, J., Nordlund, E., & Saarela, M. (2019). A small in vitro fermentation model for screening the gut microbiota effects of different fiber preparations. *International Journal of Molecular Sciences, 20*(8), 1925. https://doi.org/10.3390/ijms20081925

Ugarte, M. B., Guglielmotti, D., Giraffa, G., Reinheimer, J., & Hynes, E. (2006). Nonstarter lactobacilli isolated from soft and semihard argentinean cheeses: genetic characterization and resistance to biological barriers. *Journal of Food Protection, 69* (12), 2983–2991. https://doi.org/10.4315/0362-028X-69.12.2983

Varcoe, J., Zook, C., Sui, J., Leighton, S., Busta, F., & Brady, L. (2002). Variable response to exogenous Lactobacillus acidophilus NCFMR consumed in different delivery vehicles. *Journal of Applied Microbiology, 93*(5), 900–906. https://doi.org/10.1046/j.1365-2672.2002.01764.x

Vernocchi, P., Marangelo, C., Guerrera, S., Del Chierico, F., Guarrasi, V., Gardini, S., Conte, F., Paci, P., Ianiro, G., Gasbarrini, A., Vicari, S., & Putignani, L. (2023). Gut microbiota functional profiling in autism spectrum disorders: Bacterial VOCs and related metabolic pathways acting as disease biomarkers and predictors. *Frontiers in Microbiology, 14*, Article 1287350. https://doi.org/10.3389/fmicb.2023.1287350

Vinderola, C. G., Costa, G. A., Regenhardt, S., & Reinheimer, J. A. (2002). Influence of compounds associated with fermented dairy products on the growth of lactic acid starter and probiotic bacteria. *International Dairy Journal, 12*(7), 579–589. https://doi.org/10.1016/S0958-6946(02)00046-8

Wang, X., Gibson, G. R., Sailer, M., Theis, S., & Rastall, R. A. (2020). Prebiotics inhibit proteolysis by gut bacteria in a host diet-dependent manner: a three-stage continuous *in vitro* gut model experiment. *Applied and Environmental Microbiology, 86* (10), e02730–e02819. https://doi.org/10.1128/AEM.02730-19

Wang, Y., An, M., Zhang, Z., Zhang, W., Kulyar, M.-F.-A., Iqbal, M., He, Y., Li, F., An, T., Li, H., Luo, X., Yang, S., & Li, J. (2022). Effects of milk replacer-based *lactobacillus* on growth and gut development of yaks' calves: a gut microbiome and metabolic study. *Microbiology Spectrum, 10*(4), e01155–e01222. https://doi.org/10.1128/spectrum.01155-22

Westfall, S., Lomis, N., & Prakash, S. (2018). A novel polyphenolic prebiotic and probiotic formulation have synergistic effects on the gut microbiota influencing *Drosophila melanogaster* physiology. *Artificial Cells, Nanomedicine, and Biotechnology, 46*(sup2), 441–455. https://doi.org/10.1080/21691401.2018.1458731

Williams, C. F., Walton, G. E., Jiang, L., Plummer, S., Garaiova, I., & Gibson, G. R. (2015). Comparative analysis of intestinal tract models. *Annual Review of Food Science and Technology, 6*(1), 329–350. https://doi.org/10.1146/annurev-food-022814-015429

Ye, X., Li, H., Anjum, K., Zhong, X., Miao, S., Zheng, G., Liu, W., & Li, L. (2022). Dual role of indoles derived from intestinal microbiota on human health. *Frontiers in Immunology, 13*, Article 903526. https://doi.org/10.3389/fimmu.2022.903526

Yousof, S. M., Alghamdi, B. S., Alqurashi, T., Alam, M. Z., Tash, R., Tanvir, I., & Kaddam, L. A. (2023). Modulation of gut microbiome community mitigates multiple sclerosis in a mouse model: the promising role of palmaria palmata alga as a prebiotic. *Pharmaceuticals, 16*(10), 1355. https://doi.org/10.3390/ph16101355

Zhang, Y., Ye, Y., Guo, J., Wang, M., Li, X., Ren, Y., Zhu, W., & Yu, K. (2024). Effects of 2'-fucosyllactose on the composition and metabolic activity of intestinal microbiota from piglets after *in vitro* fermentation. *Journal of the Science of Food and Agriculture, 104*(3), 1553–1563. https://doi.org/10.1002/jsfa.13037