

Strain-dependent effects of the probiotic *Lactobacillus crispatus* on the physicochemical, technological, nutritional, and antimicrobial characteristics of fermented milk formulated for women

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ABSTRACT

In this study, a fermented milk enriched with *Lactobacillus crispatus* strains (BC1, BC4, and BC5) was developed with the aim of supporting female well-being, while ensuring technological robustness. Each strain was combined with an exopolysaccharide-producing *Streptococcus thermophilus* starter culture and its impact on physicochemical, technological, nutritional, and antimicrobial properties of the final product was assessed during 28 days of refrigerated storage (4°C). All formulations exhibited optimal pH values (4.0–4.4), consistent with commercial yogurts, and the incorporation of *L. crispatus* strains influenced syneresis behavior during storage, likely as a consequence of the acidification observed in probiotic-containing samples. Rheological analysis confirmed pseudoplastic behavior in all samples, with BC4 and BC5 showing the highest consistency and more pronounced pseudoplastic behavior. The addition of the probiotic strains markedly increased total free amino acids, particularly lysine, leucine, glutamic acid, and proline, suggesting enhanced proteolytic activity and potential nutritional relevance for women's health. Volatile compound analysis revealed a shift toward higher levels of short-chain fatty acids, especially acetic and butanoic acids, which may contribute to both flavor and antimicrobial activity. All probiotic-enriched samples inhibited *Escherichia coli* H10407 and *Salmonella enterica*, with BC1 and BC5 showing the strongest antagonistic effects. Among tested strains, BC1 displayed the best survival ($\geq 7 \log$ CFU/g after 28 days), indicating suitability for industrial application, while BC5 was the most effective in improving technological and rheological properties. Overall, this study demonstrates the feasibility of producing fermented milk with promising technological and sensory characteristics, supporting its future scale-up and further validation of its health-related potential.

1. Introduction

In recent years, consumer interest in nutrition and health has steadily increased, with particular attention given to the potential disease-preventive properties of healthy foods. Fermented milk is widely recognized as a healthy food and is a popular choice for producing functional foods. It is consumed globally and offers excellent sensory appeal and an extended shelf-life, which enhances its distribution, consumption, and marketability (Sakandar & Zhang, 2021). Functional

foods are one of the fastest growing sectors of the food industry, as they are considered a dietary strategy to reduce the incidence of human illness due to their active components (Banwo et al., 2021). Probiotics are defined as living microorganisms that confer a health benefit to the host when administered in adequate amounts according to the International Scientific Association for Probiotics and Prebiotics (Hill et al., 2014) and are widely used in food and pharmaceutical industries. Their therapeutic potential against a wide range of disorders is well recognized, and their consumption is strongly supported by clinicians,

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particularly gastroenterologists worldwide (Bodke & Jogdand, 2022). Certain conventional fermented dairy products, including yogurt, kefir, and koumiss, have been reported to contain substantial levels of probiotics. Among dairy products, yogurt accounts for the largest market share, approximately 37% (Naik, 2023). A large body of literature indicates that these types of products represent excellent carriers for probiotic bacteria, because they are characterized by a high buffering capacity and a protective lipoprotein matrix (Khorshidian et al., 2020). These properties protect microbial cells during gastrointestinal transit, allowing probiotics to reach their targets in adequate amounts (Castro et al., 2015). In this context, both matrix composition and strain-specific phenotypic traits play a key role in determining probiotic viability under gastrointestinal stress conditions (Battistini et al., 2023; da Silva et al., 2024). Since at least 1×10^9 CFU are required daily to exert positive outcomes in hosts, probiotic viability should be ensured during formulation, storage, as well as throughout gastrointestinal transit after administration (D'Amico et al., 2025; Terpou et al., 2019). In addition, microbial viability and metabolic activity during fermentation can vary across food matrices due to differences in nutrient availability, physicochemical conditions, and microbial interactions (Suzuki et al., 2024; Herkenhoff et al., 2023a). Multi-omics approaches have shown that interactions between starter cultures and probiotic strains result in distinct proteomic and metabolomic profiles, highlighting strain-specific fermentation performance (Herkenhoff et al., 2023b). These factors are particularly relevant, as they can influence the organoleptic properties of the final product and, consequently, consumer acceptance.

Therefore, microbial selection is based on specific characteristics that the final product must possess. For instance, microorganisms with enhanced exopolysaccharides (EPS) production can not only affect the rheological and sensory characteristics of the product but also provide additional health-related properties, as EPS can act as prebiotics (Ale et al., 2020; Korcz et al., 2018). Other selected bacteria may enhance the intestinal barrier function, promote the production of vitamins, improve mineral bioavailability, increase short chain fatty acids (SCFAs) production, lower intestinal pH, modulate the immune system, and more broadly contribute to the prevention and management of gastrointestinal disorders (Aponte et al., 2020). In general, the technological and functional properties of microorganisms are strain dependent (Chatsirisakul et al., 2025; Tomita et al., 2017).

Beyond their well-established role in gut health (Chandrasekaran et al., 2024), probiotics have also been increasingly investigated for their impact on extra-intestinal microbiota, particularly the vaginal microbiota, owing to the close anatomical and functional connection between the gut and the urogenital tract. Indeed, in women, these microbial ecosystems form a highly interconnected network whose cross-talk plays a key role in physiological, immunological, and metabolic balance, thereby contributing to the maintenance of vaginal microbiota homeostasis (Amabebe & Anumba, 2020). In this context, functional food containing selected probiotics can be used as a daily tool to prevent and ameliorate women's dysbiosis. Although gender differences are present in all dimensions of health (Vieira et al., 2017), research on women has been largely neglected in recent decades and findings derived from men have often been directly applied to women in both medicine and nutrition (Marino et al., 2011). Consumption of probiotics can prevent/reduce the recurrence or improve the recovery rates of dysbiosis in women (Wu et al., 2024). In the last decade, several species and strains of Lactobacilli have been isolated from the vaginal niche and extensively characterized for their anti-*Candida* and anti-*Chlamydia trachomatis* activity, as well as for their inhibitory effects against food-borne pathogens (*Listeria monocytogenes*, *Listeria innocua*, *Enterococcus faecalis* and *Escherichia coli*) and pathogens associated with vaginal and urinary tract infections (for instance *Staphylococcus aureus*, *Enterococcus faecium*, *Gardnerella vaginalis*, and *Proteus mirabilis*). Their probiotic properties (cell surface hydrophobicity, deconjugation of bile salts, adhesion to Caco-2 cells) and protechnological characteristics

(metabolic characterization, acidification capacity and survival in a milk environment and at low temperature) were also assessed (D'Alessandro et al., 2021; Nardini et al., 2016; Parolin et al., 2015; Siroli et al., 2017). Among the tested strains, *Lactobacillus crispatus* BC1, BC4, and BC5, as well as *Lactobacillus gasseri* BC9 and BC12, emerged as the most promising candidates against all the pathogens and particularly *Candida* spp. In particular, *Lactobacillus crispatus* is already commercially available as a probiotic for women with recurrent urinary tract infections and for maintaining a balanced gut microbiota (Bertuccioli et al., 2022). The strains selected in this study have previously been employed in the production of fermented foods such as Squacquerone cheese and soy-based fermented drinks (D'Alessandro et al., 2021, 2023a, 2023b, 2025; Patrignani et al., 2019, 2020). Although numerous probiotic strains are available, their application in food systems requires the evaluation of additional properties, including the ability to maintain viability and probiotic functionality during storage, as well as technological traits such as acidification kinetics and the capacity to impart desirable sensory characteristics to the final product.

The aim of this study was to develop a fermented milk specifically designed to support women's well-being that is technologically robust, sensorially appealing, industrially scalable, and characterized by potential nutritional and microbiota-related benefits. To achieve this, a starter culture consisting of EPS-producing lactic acid bacteria was combined with three *L. crispatus* strains BC1, BC4, and BC5. The final product was characterized in terms of pH evolution, bacterial viability, amino acid profile, water-holding capacity, rheological behavior, antimicrobial activity, and volatilome over 28 days of refrigerated storage at 4°C.

2. Material and methods

2.1. Microorganisms

The probiotic strains used in this experimental work were *L. crispatus* BC1, BC4 and BC5, belonging to the collection of the Department of Pharmacy and Biotechnology (FABIT, University of Bologna, Italy), isolated according to Parolin et al. (2015). As starter culture, a mix of strains of *Streptococcus thermophilus* producing EPS (Lyofast ST440) was provided by Sacco srl (Italy). Fresh cultures of each strain were obtained from frozen stocks by two consecutive refreshments in De Man-Rogosa-Sharpe (MRS) broth (Oxoid, Basingstoke, United Kingdom) using a 1% (v/v) inoculum and incubated at 37°C in anaerobic conditions overnight.

2.2. Fermented milk production

The production of fermented milk was carried out under laboratory conditions. Commercial UHT skimmed milk (Centrale del latte di Cesena, Italy) was divided in 50 mL falcon tubes and each tube was inoculated with Lyofast ST440, at a ratio of 1 UC/100L, while the probiotic strains were inoculated at the final cell density of about 8 log CFU/mL. The samples were then incubated at 43°C until reaching a pH value of 4.9. At the end of fermentation, the samples were stored at 4°C for up to 28 days. Analyses were performed after 1, 14, and 28 days of refrigerated storage, corresponding to early, intermediate, and late shelf-life stages. These time points were selected to capture the main changes occurring during storage, in line with common experimental designs adopted for fermented dairy products (D'Alessandro et al., 2025). Four types of experimental conditions were obtained and defined as follows:

- Control: fermented milk with only Lyofast ST440;
- BC1: fermented milk with Lyofast ST440 and *L. crispatus* BC1;
- BC4: fermented milk with Lyofast ST440 and *L. crispatus* BC4;
- BC5: fermented milk with Lyofast ST440 and *L. crispatus* BC5.

Each condition was prepared in triplicate (n=3) for each time point considered.

2.3. Starter culture and probiotic strain viability in fermented milk

Cell viability of starter culture and vaginal strains was assessed after 1, 14 and 28 days of storage. Five grams of fermented milk were placed into 45 mL of PBS and homogenized in a stomacher (Labblender 80, Pbi International, Milan, Italy) for 3 min. Decimal dilutions of the homogenates were made in 0.9% (w/v) NaCl and 0.1 mL of appropriate dilutions were spread onto the surface of different agar media. *S. thermophilus* was counted on M17 agar (Oxoid, Basingstoke, Hampshire, UK) incubated at 45°C, while *L. crispatus* was enumerated on MRS supplemented with 0.05% (w/v) l-cysteine and incubated at 37°C, as previously described by D'Alessandro et al. (2023a).

2.4. pH and Syneresis

The decrease in pH during fermentation and its evolution during 28 days of storage at 4°C was monitored using the Basic 20 pH-meter (Crison Instruments, Modena, Italy). The syneresis of the samples was determined using a refrigerated centrifuge (Avanti J-26 XP with Ja A-10 rotor, Beckman Coulter). More specifically, fermented milk (5 g) was centrifuged at 4500 × g for 15 min at 4°C. After centrifugation, the clear supernatant formed was collected and weighed. Triplicates were measured for each sample and the means were calculated. The extent of whey separation of the fermented samples was calculated from the weight of the supernatant and the fermented milk using the following equation:

$$\text{Syneresis (\%)} = \left[1 - \left(\frac{\text{weight of supernatant (g)}}{\text{weight of fermented milk (g)}} \right) \right] \times 100$$

2.5. Antagonistic activity against intestinal pathogens

All the investigated fermented milks were tested for their antagonistic activity towards the intestinal pathogens enterotoxigenic *Escherichia coli* H10407, *Salmonella enterica* serovar Typhimurium, *Yersinia enterocolitica* (Department of Pharmacy and Biotechnology, University of Bologna, Italy) by overlay assay, as described in (D'Alessandro et al., 2023b) with minor modifications. Pathogenic strains were routinely grown in Brain Heart Infusion (BHI) broth, at 37°C with gentle agitation, and subcultures twice before being used in the experiments. Five microliters (µL) of each fermented milk were spotted over the surface of MRS plates (containing 0.05% (w/v) l-cysteine and 1.2% (w/v) agar) and incubated in anaerobic conditions at 37°C for 24 h. Then 100 µL (corresponding to approximately 7–8 log CFU) of overnight subcultures of the pathogenic strains were inoculated into 10 mL of BHI 0.7% (w/v) agar and poured over the spots. The plates were further incubated at 37°C for additional 24 h, then checked to evaluate the presence of a growth inhibition zone. The inhibition halos were measured from the outer perimeter of the spots in four directions and the average was considered. The antagonistic activity was expressed in relation to the observed zone of inhibition: -: no inhibition; +, Diameter between 1 and 3 mm; ++, Diameter between 3 and 6 mm; +++, Diameter between 6 and 10 mm; +++++, Diameter > 10 mm.

2.6. Aroma profiles

Volatile compounds of fermented milks were monitored after 1, 14 and 28 days of refrigerated storage using gas chromatography-mass spectrometry (GC-MS) coupled with solid phase micro extraction (SPME) using the method described by Patrignani et al. (2017).

2.7. Rheological measurements

Rheological measurements on fermented samples were carried out at 4°C with a controlled stress-strain rheometer mod. MCR 102 (Physica/Anton Paar, Ostfildern, Germany) equipped with a parallel-plate ge-

ometry (Ø 25 mm) and a fixed gap of 1 mm. Prior to measurement, samples were subjected to a pre-shearing of 60 s at 0.1 s⁻¹. Viscosity was then measured by gradually increasing shear rate from 0.1 to 100 s⁻¹, applying a logarithmic ramp with a data acquisition density of 20 points per decade. The flow curve data were fitted to the Herschel-Bulkey model according to previous studies (Felix da Silva et al., 2017; Santillán-Urquiza et al., 2017; Terzioğlu & Bakirci, 2024):

$$\sigma = \sigma_0 + \kappa \cdot \dot{\gamma}^\eta$$

where σ is the shear stress (Pa), σ_0 is the yield stress (Pa), κ is the consistency index (Pa*s^η), $\dot{\gamma}$ is the shear rate (s⁻¹) and η is the flow behavior index (dimensionless and $\eta = 1$ for Newtonian, $\eta < 1$ for pseudoplastic and $\eta > 1$ for dilatant fluids). All measurements were carried out in triplicate.

2.8. Free amino acids

Free amino acids (FAAs) were quantified on fermented milks following precipitation with 5% (v/v) cold sulfosalicylic acid. After 1 h incubation at 4°C, samples were centrifuged (15,000 × g, 15 min, 4°C) and filtered through a 0.22 µm membrane. FAA analysis was performed using a Biochrom 30+ Automatic Amino Acid Analyzer (Biochrom Ltd., Cambridge Science Park, Waterbeach Cambridge, UK) equipped with a sodium cation-exchange column (20 by 0.46 cm, inner diameter) and post-column ninhydrin derivatization, as described by De Pasquale et al. (2021). Total FAA were calculated as the sum of all individual amino acids quantified in each sample. Essential amino acids (EAA) were calculated as the sum of essential and conditionally essential amino acids (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, arginine, cysteine, glycine, proline). Amino acids were also classified according to their sensory properties. Bitter amino acids were calculated as the sum of valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, tryptophan, and arginine. Sweet amino acids were calculated as the sum of threonine, serine, glycine, alanine, lysine, and proline. The bitter-to-sweet ratio was calculated accordingly. These values were derived from calculated sums and were not directly measured.

2.9. Statistical analysis

Microbiological and physicochemical data are expressed as the mean of at least three independent replicates (n = 3). Statistical analyses were performed using Statistica software (version 8.0; StatSoft, Tulsa, OK, USA). For microbiological data, pH, and syneresis, a 2-way repeated measures ANOVA was performed to evaluate the effects of treatment, storage time, and their interaction. When a significant interaction was detected, simple effects were analyzed using Tukey's multiple comparisons test to compare treatments within each time point and storage times within each treatment. For rheological parameters and free amino acids, a one-way ANOVA was applied at each time point to evaluate differences among treatments, followed by Tukey's multiple comparisons test when significant differences were observed. Prior to ANOVA, data were checked for normality and homogeneity of variances using standard procedures. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Viability of the bacteria in fermented milks during the refrigerated storage

Microbial viability during refrigerated storage was evaluated by 2-way repeated measures ANOVA, which revealed significant effects of the treatment, storage time, and their interaction ($p < 0.05$) for both the starter culture and the probiotic strains. Regarding starter viability (Fig. 1), *S. thermophilus* cell load was 8.8 log CFU/g of product in all the

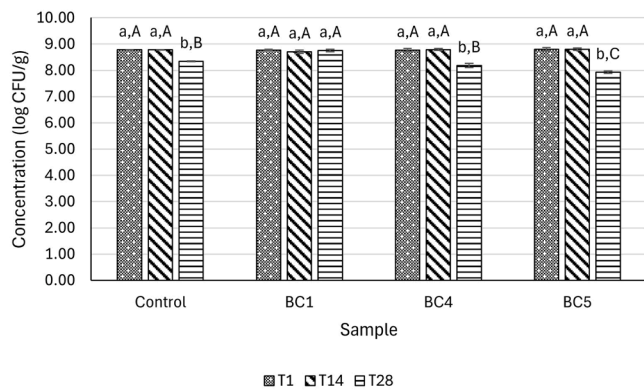


Fig. 1. Cell load viability (log CFU/g) of the starter culture (Lyofast ST440) in fermented milk after 1, 14 and 28 days (T1, T14, T28) of storage in refrigerated conditions. Control: fermented milk with only Lyofast ST440; BC1: fermented milk with Lyofast ST440 and *L. crispatus* BC1; BC4: fermented milk with Lyofast ST440 and *L. crispatus* BC4; BC5: fermented milk with Lyofast ST440 and *L. crispatus* BC5. Data are expressed as mean ± standard deviation (n = 3). Different uppercase superscript letters indicate significant differences among treatments within the same storage time, whereas different lowercase superscript letters indicate significant differences over storage time within the same treatment (p < 0.05).

samples after 1 and 14 days of storage at 4°C. After 28 days, the concentration of the starter culture decreased in all the samples except for the one containing BC1. The highest reduction (around 1 log) was observed in the sample containing BC5. This reduction may be due to faster acidification in samples containing BC4 and BC5. An increase in *S. thermophilus* concentration, or its better survival, has been previously observed when probiotic lactobacilli are added in soy drink or whole milk when the yogurt starter is applied (Cui et al., 2021; D'Alessandro et al., 2023b; Patrignani et al., 2017). In the present work only the samples with BC1 preserved the viability of *S. thermophilus* up to 28 days probably due to the positive interaction between the vaginal strain and the starter. Regarding the survival of the vaginal strains (Fig. 2), BC1 was the least affected by the fermentation process, remaining at 8.15 log CFU/g on day 1 and reducing to 7.48 log CFU/g only on day 28. On the contrary, BC4 was the strain most affected by the process, as well as by the storage condition, passing from 6.67 log CFU/g on day 1 to 5.62 log CFU/g on day 28. Strain BC5 presented an intermediate behavior,

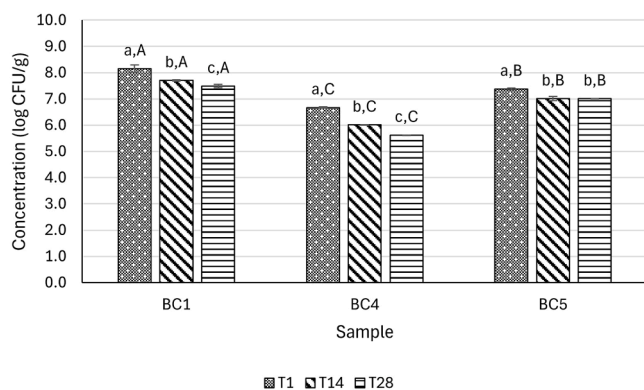


Fig. 2. Cell load viability (log CFU/g) of the vaginal strains (BC1, BC4 and BC5) in fermented milk after 1, 14 and 28 days (T1, T14, T28) of storage in refrigerated conditions. BC1: fermented milk with Lyofast ST440 and *L. crispatus* BC1; BC4: fermented milk with Lyofast ST440 and *L. crispatus* BC4; BC5: fermented milk with Lyofast ST440 and *L. crispatus* BC5. Data are expressed as mean ± standard deviation (n = 3). Different uppercase superscript letters indicate significant differences among treatments within the same storage time, whereas different lowercase superscript letters indicate significant differences over storage time within the same treatment (p < 0.05).

resulting more affected by the fermentation process and subsequent storage at 4°C (reduction of 0.63 log CFU/g) more than the 28 days of storage (further reduction of 0.37 log CFU/g, final concentration: 7.00 log CFU/g). The higher survival of BC1, compared to BC4, may be related to strain-specific tolerance to acid stress and storage conditions, as well as to a more favorable interaction with the starter culture (Papadimitriou et al., 2016).

In general, all the vaginal strains considered were previously reported to exhibit limited resistance to refrigerated storage conditions. However, compared to that study, the physicochemical and storage conditions of the fermented milk investigated in the present work negatively affected the survival of BC4, while the survival of BC5 was better preserved. Despite the reduction observed, both BC1 and BC5 remained above 7 log CFU/g during the whole storage period, which would guarantee the proper amount of living probiotics that need to be consumed per day (9 log CFU), considering product daily intake of about 120 g (Hill et al., 2014). This aspect is of great importance since *L. crispatus* strains can provide a biologically relevant basis for targeting women’s health, as these microorganisms are dominant members of the healthy vaginal microbiota and are directly involved in maintaining urogenital homeostasis (Bertuccioli et al., 2022).

3.2. pH and syneresis

Since pH is an important parameter affecting microbial viability and survival, this parameter was monitored during storage at 4°C for 28 days (Table 1).

All the fermentations were stopped when the pH of 4.9 was reached and stored at 4°C (this pH was selected in preliminary lab trial, data not shown). Two-way repeated measures ANOVA indicated that pH was significantly affected by treatment, storage time, and their interaction (p < 0.05). After one day under refrigerated conditions, all samples containing vaginal strains exhibited a lower pH (around 4.6) compared to the control (pH 4.8). This difference persisted throughout the 28-day storage period, with samples prepared using BC1, BC4, and BC5 having approximately a pH of 4.3, while the control remained at pH 4.4. However, in all cases, pHs were in the range of 4.4-4.0, corresponding to the optimal acidic conditions typically reported for commercial yogurts or similar fermented dairy products (Herkenhoff et al., 2023a; Deshwal et al., 2021). Siroli et al. (2017) previously assessed the ability of vaginal strains to be used as stand-alone starter cultures in dairy products. However, their findings highlighted the limited technological suitability of these lactobacilli when tested in whole milk. Conversely, in the present study, their use as adjunct cultures enhanced product acidification,

Table 1

pH values and syneresis (expressed in %) recorded during the refrigerated storage (1, 14 and 28 days, T1, T14, T28, respectively) for all the investigated fermented milks. Control: fermented milk with only Lyofast ST440; BC1: fermented milk with Lyofast ST440 and *L. crispatus* BC1; BC4: fermented milk with Lyofast ST440 and *L. crispatus* BC4; BC5: fermented milk with Lyofast ST440 and *L. crispatus* BC5. Data are expressed as mean ± standard deviation (n = 3). Different uppercase superscript letters indicate significant differences among treatments within the same storage time, whereas different lowercase superscript letters indicate significant differences over storage time within the same treatment (p < 0.05).

	pH			Syneresis (%)		
	T1	T14	T28	T1	T14	T28
Control	4.88 ^{a,A} ± 0.01	4.45 ^{b,B} ± 0.01	4.40 ^{c,A} ± 0.01	25.1 ^{a,A} ± 1.44	18.2 ^{b,B} ± 0.43	16.7 ^{b,B} ± 0.98
BC1	4.67 ^{a,B} ± 0.01	4.50 ^{b,A} ± 0.00	4.34 ^{c,B} ± 0.00	23.2 ^{a,A} ± 1.26	24.6 ^{a,A} ± 1.09	19.3 ^{b,AB} ± 0.43
BC4	4.62 ^{a,C} ± 0.00	4.37 ^{b,C} ± 0.01	4.31 ^{c,C} ± 0.00	23.6 ^{a,A} ± 2.68	25.1 ^{a,A} ± 2.64	21.0 ^{a,AB} ± 0.15
BC5	4.62 ^{a,C} ± 0.01	4.35 ^{b,D} ± 0.00	4.29 ^{c,CD} ± 0.00	24.1 ^{a,A} ± 2.05	23.2 ^{a,A} ± 2.19	23.3 ^{a,A} ± 0.15

which may in turn influence the physicochemical and technological properties of the final product.

Syneresis, defined as the spontaneous expulsion of whey from the gel matrix, is considered one of the most undesirable defects in fermented milk products because it negatively affects texture and consumer acceptability (Torricco et al., 2019). In the present study, syneresis was significantly influenced by treatment, storage time, and their interaction ($p < 0.05$). No significant differences among samples were observed at day 1 (Table 1). However, during refrigerated storage, the control sample showed a progressive decrease in syneresis, from 25.1% at T1 to 16.7% at T28, whereas probiotic-containing samples maintained higher values throughout storage. In particular, BC5 exhibited almost constant syneresis values (~23%) over the entire 28-day storage period. The higher syneresis observed in samples containing vaginal probiotic strains may be associated with their greater acidification activity, as demonstrated by the lower pH values recorded during storage. Increased acidification can promote contraction of the casein network, thereby reducing its water-retention ability and favoring whey separation. This behavior appears to be strain dependent and may also be related to differences in EPS production and interactions with the protein matrix, which can modulate the microstructure and physicochemical properties of fermented dairy products (Nagaoka, 2018; Han et al., 2016).

3.3. Rheological properties

Rheological properties provide valuable insights into the gel structure of fermented milks, offering important information on flow behavior and textural characteristics (Denktaş et al., 2025; Kumari et al., 2022; Terzioğlu & Bakirci, 2024). This is because factors such as processing conditions, milk composition (e.g., fat content), starter cultures, fermentation process, packaging and storage time-temperature conditions markedly influence the rheological and textural attributes of fermented milk products (Denktaş et al., 2025; Terzioğlu & Bakirci, 2024). Figure 3 shows the relationship between shear stress (σ , Pa) and shear rate ($\dot{\gamma}$, s^{-1}) of the samples at day 1 and after 28 days of storage at 4°C. All curves exhibited a non-Newtonian, pseudoplastic (shear-thinning) behavior, characterized by a non-linear increase in shear stress with increasing shear rate and the presence of an apparent yield stress. This trend is typical of fermented milk systems, where the protein network resists deformation until a critical stress threshold is reached (Felix da Silva et al., 2017; Jurado-Guerra et al., 2023; Pacheco et al., 2023). As expected, the apparent viscosity decreased with increasing shear rate, which can be attributed to the progressive breakdown of the protein network and the alignment of macromolecular chains in the direction of the shear field (Glicerina et al., 2013; Pacheco et al., 2023). Comparing day 1 (Fig. 3a) and day 28 (Fig. 3b), shear-stress values were slightly lower after storage for all samples, indicating a modest reduction in flow

resistance under shear. This agrees with Velez-Ruiz et al. (2013), who observed a similar decrease in shear stress and consistency coefficient during storage of stirred yogurts, attributed to microstructural rearrangements and a partial weakening of the gel network. The experimental data were well fitted to the Herschel-Bulkley model, yielding high correlation coefficients ($R^2 = 0.96-0.99$). The rheological parameters obtained from the model, such as flow index (η), yield stress (σ_0) and consistency index (κ), for each sample are summarised in Table 2. The flow index (η) confirms the degree of non-Newtonian behavior of all fermented milk samples, indeed values lower than 1 denote shear-thinning (pseudoplastic) fluids. Similar η values (<1) have been reported in previous studies on experimental fermented milks and yogurts (Dönmez et al., 2017; Santillán-Urquiza et al., 2017; Terzioğlu & Bakirci, 2024). In this study, at both T1 and T28, η values were significantly lower in BC1, BC4 and BC5 (approximately 0.35-0.54) than in the control (approximately 0.81-0.85). Lower η values indicate more pronounced pseudoplastic behavior, suggesting a more interconnected and shear-sensitive gel structure. Conversely, the control, which exhibited the highest η , behaved more like a Newtonian fluid and was therefore less structured.

The σ_0 , a characteristic property of pseudoplastic fluids such as fermented milk, is associated with the presence of a cross-linked or interacting structure (Costa et al., 2019; Terzioğlu & Bakirci, 2024). At day 1 (T1), all samples exhibited low σ_0 values, typical of freshly fermented milk. Among them, BC4 and BC5 showed higher σ_0 values than the control sample, indicating greater consistency (cross-linked structure). In contrast, sample BC1 did not differ significantly from the control,

Table 2

Flow index η , yield stress σ_0 and consistency index κ of the fermented milks after 1 and 28 days (T1, T28) of storage in refrigerated conditions. Control: fermented milk with only Lyofast ST440; BC1: fermented milk with Lyofast ST440 and *L. crispatus* BC1; BC4: fermented milk with Lyofast ST440 and *L. crispatus* BC4; BC5: fermented milk with Lyofast ST440 and *L. crispatus* BC5. Data are expressed as mean \pm standard deviation ($n = 3$). Within the same time point, data with different superscript letters were statistically different ($p < 0.05$).

	Flow index η		Yield stress σ_0 (Pa)		Consistency index κ (Pa·s ¹)	
	T1	T28	T1	T28	T1	T28
Control	0.808 ^a ± 0.012	0.848 ^a ± 0.089	0.178 ^b ± 0.017	0.169 ^d ± 0.064	0.670 ^c \pm 0.058	0.165 ^c ± 0.001
BC1	0.438 ^b ± 0.025	0.532 ^b ± 0.062	0.139 ^{a,b} ± 0.093	0.610 ^c ± 0.082	3.014 ^b ± 0.108	1.482 ^b ± 0.640
BC4	0.418 ^{b,c} ± 0.028	0.540 ^b ± 0.042	0.326 ^a ± 0.169	1.007 ^b ± 0.071	3.973 ^{a,b} ± 0.127	1.796 ^b ± 0.351
BC5	0.346 ^c \pm 0.027	0.426 ^b ± 0.063	0.209 ^a ± 0.177	1.163 ^a ± 0.088	5.108 ^a ± 0.374	3.301 ^a ± 1.130

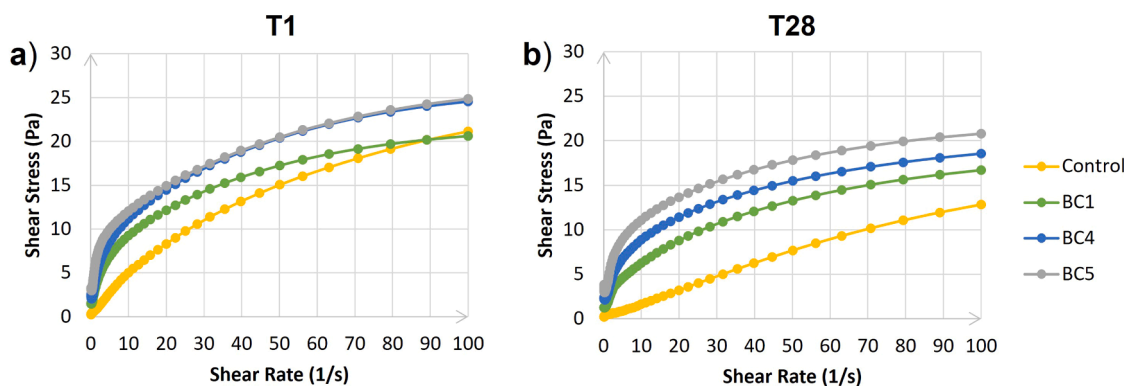


Fig. 3. Flow curves (shear stress versus shear rate) of fermented milk samples after 1 (a) and 28 (b) days (T1 and T28) of storage in refrigerated conditions: Control: fermented milk with only Lyofast ST440; BC1: fermented milk with Lyofast ST440 and *L. crispatus* BC1; BC4: fermented milk with Lyofast ST440 and *L. crispatus* BC4; BC5: fermented milk with Lyofast ST440 and *L. crispatus* BC5. Results represent the means of three independent replicates.

suggesting a minor effect of this strain on matrix firmness immediately after fermentation. After 28 days (T28), σ_0 increased markedly for BC4 and BC5 (approximately 1.0-1.16 Pa), while it remained almost unchanged in the control and only moderately increased in BC1. These results are consistent with recent evidence showing that specific strains can modulate the microstructure of fermented milk through variations in EPS production and acidification kinetics (Denktaş et al., 2025). The consistency index (κ), which represents the flow resistance once the yield stress has been exceeded, followed a similar trend. At T1, samples BC1, BC4 and BC5 showed κ values several times higher than the control (3-5 Pa·s⁻¹ vs 0.67 Pa·s⁻¹), indicating a thicker and creamier consistency even at the early stage of storage. After 28 days, κ decreased slightly in all samples but remained higher in BC4 and BC5. Although the overall consistency declined with time, mainly associated to structural rearrangements within the gel and partial loss of firmness/stiffness of the protein matrix, the structural strength of BC4 and BC5 was better preserved than the control. A similar trend in the κ parameter during storage has also been reported in some yogurt matrices (Basak & Ramaswamy, 1994; Santillán-Urquiza et al., 2017). Overall, cold storage at 4°C caused moderate changes in flow behavior, while η values remained below 1, confirming the persistence of the pseudoplastic, shear-thinning nature of the samples. These findings show that BC4 and BC5 produced fermented milks with higher apparent consistency and more pronounced pseudoplastic behavior both immediately after fermentation and after 28 days of storage, whereas BC1 behaved more similarly to the control.

3.4. Free amino acids

Milk contains limited free amino acids and peptides, which are required by lactic acid bacteria to grow during fermentation. FAAs are

usually generated during milk fermentation as a result of proteolysis. In yogurt, *L. bulgaricus* shows higher proteolytic activity than *S. thermophilus*, hydrolyzing casein into peptides and amino acids that, in turn, stimulate the growth of *S. thermophilus*. The interaction between bacteria influences the levels of FAAs during fermentation (Akan, 2022; Ozcan et al., 2019). In our study, the addition of *L. crispatus* strains determined an increase of total FAAs leading to a clear differentiation between the control and probiotic samples (Fig. 4), in agreement with previous studies reporting species and strain-dependent variations in proteolytic activity and amino acid release during fermentation (Toe et al., 2019; Raveschot et al., 2018). In detail, while control samples showed ranges between 50.8 and 61.1 mg/Kg of FAAs, all the other samples presented a threefold increase, with the highest level observed in the BC4 sample at day 28 (214.5 mg/Kg) (Table 3). Over time, the FAA profile of fermented milks produced with BC4 and BC1 showed a continuous increase in concentration. In contrast, the BC5 sample exhibited an initial rise in FAA levels, followed by a more stable trend, suggesting partial utilization of amino acids during storage and their further transformation in aroma compounds. According to Germani et al. (2014) the proteolytic activity of bacteria persists in natural yogurt for the whole duration of the shelf-life even if it is slowed down by storage temperatures and it has been suggested as an index of the vitality of *Lactobacillus delbrueckii* subsp. *bulgaricus*. In our case, this may be associated to the vaginal strains applied. However, the higher increase in FAAs did not correspond to a higher number of viable cells of the probiotic strains or the starter culture. In fact, samples containing BC4 showed lower viability of both probiotics and starters over time, while displaying a higher accumulation of FAAs. This suggests that the proteolytic activity was not directly linked to microbial viability, but rather to proteolysis driven by released enzymes or by cells undergoing autolysis. In addition, it has to be considered that FAA are precursors of

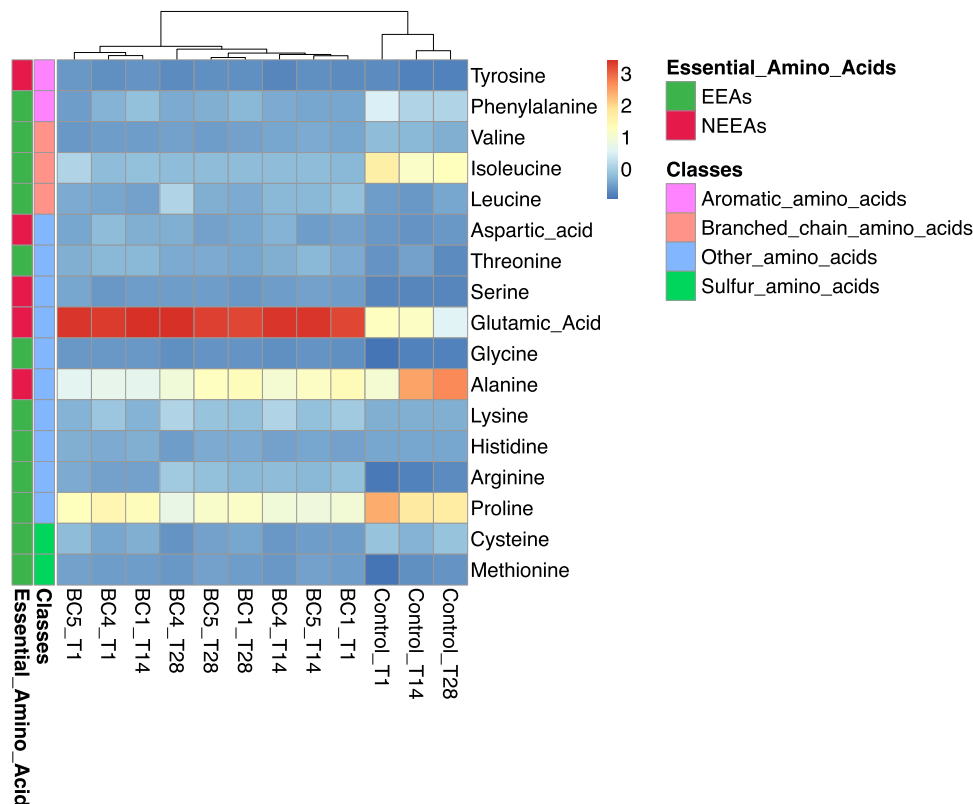


Fig. 4. Heatmap of free amino acids in fermented milks prepared with different *L. crispatus* strains (BC1, BC4, and BC5), analyzed after 1, 14 and 28 days (T1, T14, T28) of storage in refrigerated conditions. Control: fermented milk with only Lyofast ST440; BC1: fermented milk with Lyofast ST440 and *L. crispatus* BC1; BC4: fermented milk with Lyofast ST440 and *L. crispatus* BC4; BC5: fermented milk with Lyofast ST440 and *L. crispatus* BC5. Results represent the means of three independent replicates ($n = 3$).

Table 3

Free amino acids (FAAs) (mg/Kg) quantified in the different fermented milks after 1, 14 and 28 days (T1, T14, T28) of storage in refrigerated conditions. C: control, fermented milk with only Lyofast ST440; BC5: fermented milk with Lyofast ST440 and *L. crispatus* BC5; BC1: fermented milk with Lyofast ST440 and *L. crispatus* BC1; BC4: fermented milk with Lyofast ST440 and *L. crispatus* BC4. Values are expressed as mean \pm standard deviation (n = 3). Within the same time point, data with different lowercase letters were statistically different ($p < 0.05$) while the absence of letters indicates no significant differences among samples for a specific amino acid. Total free amino acids without ornithine (Total FAA), essential amino acids (Total EAA), and derived indices (bitter, sweet, and bitter-to-sweet ratio) were calculated as the sum of the mean values of the individual amino acids; therefore, no statistical analysis was applied to these derived parameters.

Free Amino Acids	T1				T14				T28			
	Control	BC5	BC4	BC1	Control	BC5	BC4	BC1	Control	BC5	BC4	BC1
Aspartic acid	1.19 $\pm 0.132^b$	0.99 $\pm 0.098^b$	5.03 $\pm 0.408^a$	3.36 $\pm 1.037^a$	0.96 $\pm 0.081^b$	2.84 $\pm 0.451^b$	5.93 $\pm 0.431^a$	2.49 $\pm 0.036^b$	0.96 $\pm 0.093^b$	2.61 $\pm 0.378^b$	7.28 $\pm 0.187^a$	2.69 $\pm 0.04^b$
Threonine	1.02 $\pm 0.087^b$	1.34 $\pm 0.098^b$	4.68 $\pm 0.967^a$	4.58 $\pm 1.46^a$	1.41 $\pm 0.184^b$	6.42 $\pm 1.252^a$	5.5 $\pm 1.069^a$	3.7 $\pm 0.754^{ab}$	0.35 $\pm 0.13^c$	3.93 $\pm 0.611^{ab}$	6.67 $\pm 0.848^a$	2.96 $\pm 0.343^b$
Serine	0.58 ± 0.212	0.96 ± 0.218	1.37 ± 0.242	2.99 ± 1.014	0.21 $\pm 0.21^b$	3.38 $\pm 1.157^a$	3.12 $\pm 0.3^{ab}$	1.05 $\pm 0.16^{ab}$	0.09 $\pm 0.148^b$	1.92 $\pm 0.043^b$	4.42 $\pm 0.187^a$	1.34 $\pm 0.194^{ab}$
Glutamic Acid	6.95 $\pm 2.322^d$	24.12 $\pm 1.595^c$	45.58 $\pm 1.31^b$	55.43 $\pm 5.979^a$	9.16 $\pm 1.282^d$	63.51 $\pm 7.764^a$	54.59 $\pm 3.115^b$	41.52 $\pm 2.115^c$	5.93 ± 1.034	53.67 ± 4.395	68.16 ± 2.402	45.5 ± 2.599
Glycine	0.04 ± 0.068	0.09 ± 0.076	0.97 ± 0.128	1.1 ± 0.258	0.03 ± 0.035	1.35 ± 0.498	1.64 ± 0.41	0.23 ± 0.005	0.03 ± 0.053	1.06 ± 0.535	2.01 ± 0.074	0.58 ± 0.063
Alanine	6.28 $\pm 0.408^d$	7.35 $\pm 0.561^c$	15.89 $\pm 0.574^b$	28.9 $\pm 0.371^a$	15.02 $\pm 0.438^c$	29 $\pm 4.074^a$	23.59 $\pm 0.987^b$	13.03 $\pm 0.353^c$	15.19 $\pm 0.519^b$	25.68 $\pm 0.996^a$	27.33 $\pm 0.782^a$	23 $\pm 0.976^{ab}$
Valine	2.37 ± 0.331	n.d.*	1.56 ± 0.215	4.23 ± 1.007	2.47 $\pm 0.071^{ab}$	4.67 $\pm 1.264^a$	4.28 $\pm 0.337^a$	1.06 $\pm 0.917^b$	1.81 $\pm 0.391^b$	1.99 $\pm 1.76^b$	5.25 $\pm 0.265^a$	2.11 $\pm 0.249^b$
Cysteine	2.64 ± 0.114	2.14 ± 0.642	2.77 ± 0.133	2.85 ± 0.207	2.31 ± 0.59	2.56 ± 0.021	2.75 ± 0.118	2.64 ± 0.037	2.79 ± 0.16	2.46 ± 0.218	2.83 ± 0.173	2.84 ± 0.091
Methionine	n.d.	0.52 ± 0.247	1.53 ± 0.52	2.94 ± 0.983	0.63 ± 0.062	3.04 ± 0.905	2.52 ± 0.292	0.87 ± 0.073	0.81 ± 0.159	2.62 ± 0.319	3.46 ± 0.23	1.9 ± 0.648
Isoleucine	8.33 $\pm 0.151^a$	4.09 $\pm 0.687^b$	5.1 $\pm 0.271^b$	6.87 $\pm 0.729^{ab}$	8.81 ± 0.209	7.68 ± 1.364	7.24 ± 0.249	4.49 ± 0.183	8.82 $\pm 0.438^a$	5.95 $\pm 0.217^{ab}$	9.1 $\pm 0.327^a$	5.37 $\pm 0.115^b$
Leucine	1.4 $\pm 0.289^b$	1.06 $\pm 0.526^b$	2.43 $\pm 0.474^b$	8.02 $\pm 2.733^a$	1.1 $\pm 0.093^b$	6.83 $\pm 1.2^a$	6.95 $\pm 0.464^a$	1.43 $\pm 0.107^b$	1.47 $\pm 0.292^c$	4.43 $\pm 0.171^b$	14.3 $\pm 2.009^a$	3.23 $\pm 0.164^b$
Tyrosine	0.74 ± 0.064	± 0	0.1 ± 0.167	0.31 ± 0.052	n.d.	0.57 ± 0.769	0.45 ± 0.081	0.09 ± 0.162	0.04 ± 0.065	n.d.	1.44 ± 0.496	n.d.
Phenylalanine	4.61 $\pm 0.157^a$	0.33 $\pm 0.566^b$	4.13 $\pm 0.272^a$	4.4 $\pm 1.404^a$	4.01 ± 0.117	3.87 ± 0.651	5.21 ± 0.286	4.44 ± 0.047	3.78 ± 0.183	4.51 ± 0.965	6.35 ± 0.171	4.78 ± 0.191
Lysine	1.95 $\pm 0.14^b$	1.57 $\pm 0.28^b$	6.69 $\pm 0.434^{ab}$	9.49 $\pm 1.872^a$	2.01 $\pm 0.098^c$	8.36 $\pm 1.519^b$	11.47 $\pm 0.815^a$	3.25 $\pm 0.028^c$	1.9 $\pm 0.195^c$	7.17 $\pm 0.114^b$	14.34 $\pm 0.459^a$	5.64 $\pm 0.437^b$
Histidine	1.66 ± 0.329	1.5 ± 0.117	3.21 ± 0.053	3.82 ± 0.231	1.6 ± 0.056	4.27 ± 0.65	3.69 ± 0.226	2.76 ± 0.101	1.47 ± 0.246	3.83 ± 0.294	4.33 ± 0.061	3.11 ± 0.044
Arginine	0.2 $\pm 0.339^b$	1.28 $\pm 0.261^b$	2.23 $\pm 0.126^b$	8.05 $\pm 0.874^a$	n.d.	6.61 $\pm 1.051^a$	7.3 $\pm 0.641^a$	1.42 $\pm 0.174^b$	0.32 $\pm 0.331^c$	6.53 $\pm 1.08^b$	12 $\pm 0.244^a$	4.95 $\pm 0.363^b$
Proline	10.88 $\pm 1.171^b$	11.37 $\pm 0.693^b$	23.99 $\pm 0.922^a$	23.35 $\pm 1.345^a$	11.5 $\pm 0.318^c$	24.67 $\pm 1.175^a$	22.51 $\pm 1.47^{ab}$	19.89 $\pm 0.28^b$	10.61 $\pm 1.099^c$	23.88 $\pm 1.37^{ab}$	25.19 $\pm 0.443^a$	20.96 $\pm 0.113^b$
Ornithine-Hcl	0.40 $\pm 0.142^b$	1.27 $\pm 0.120^a$	1.17 $\pm 0.214^a$	0.37 $\pm 0.192^b$	0.43 $\pm 0.098^b$	1.09 $\pm 0.192^a$	1.14 $\pm 0.212^a$	1.34 $\pm 0.202^a$	0.11 $\pm 0.121^b$	1.13 $\pm 0.292^a$	1.34 $\pm 0.234^a$	1.09 $\pm 0.282^a$
Total FAA	50.83	170.67	127.27	58.70	61.13	104.36	168.75	179.63	56.37	130.95	214.46	152.25
Total EAA	35.09	79.70	59.31	25.28	35.79	46.18	81.08	80.33	34.17	58.43	105.82	68.37
Bitter	19.30	38.64	20.30	8.77	18.53	16.56	37.64	37.53	18.52	25.45	56.22	29.87
Sweet	20.75	70.40	53.60	22.68	30.18	41.15	67.83	73.18	28.17	54.48	79.96	63.63
Bitter/sweet	0.93	0.55	0.38	0.39	0.61	0.40	0.55	0.51	0.66	0.47	0.70	0.47

* n.d., not detected.

many other compounds including several aroma compounds. Other than considering the behavior of total FAAs, it is important to look at their profile particularly for the product sensory and nutritional/functional properties. Considering the latter, essential amino acids (EAAs) support not only muscle metabolism but also bone health, glucose regulation, and gut function. Total EAAs (essential + conditionally essential) presented a twofold increase upon fermentation with the vaginal strains. All the single amino acids increased compared to control except for isoleucine, while tryptophan was not detected in all samples. Lysine and leucine were among the essential amino acids (EAAs) that showed the most pronounced increase during storage in samples containing vaginal strains. The enrichment in these amino acids is particularly relevant for women, who are more prone to age-related bone density loss and sarcopenia. Lysine plays a key role in calcium absorption and collagen synthesis, contributing to bone strength and potentially reducing the risk of osteoporosis. In addition, it is involved in energy metabolism, immune response, wound healing, and angiogenesis, highlighting its broad physiological relevance for women's health and metabolic balance (Aggarwal & Bains, 2022). At the same time, leucine is a key regulator of muscle protein metabolism and may help counteract

age-related declines in muscle mass, a condition that tends to affect women more markedly with aging (Smith-Ryan et al., 2022). Regarding the most abundant amino acids (above 10 mg/Kg), fermentation with vaginal strains determined an increase in glutamic acid, proline, and alanine (Table 3).

These amino acids were also dominant in the work presented by Bao et al. (2016), while Irigoyen et al. (2012) reported the dominance of other amino acids. According to the classical classification, these are non-essential amino acids (NEAAs), although this definition for animals and humans is increasingly considered physiologically outdated (Weiler et al., 2023). The non-proteinogenic amino acid ornithine also increased in all samples, from 0.1–0.4 mg/Kg in the control to 1.1–1.3 mg/Kg in those fermented with BC4 and BC5. L-ornithine has shown potential benefits such as stress relief, reduced HPA axis activity, and improved sleep quality related to fatigue (Armborst et al., 2018). In addition to their health benefits for the host, FAAs can also influence the final organoleptic profile of the product. Bitter-tasting amino acids (e.g., valine, leucine, isoleucine, methionine, arginine, histidine, phenylalanine, tryptophan and tyrosine) (Kato et al., 1989) can negatively affect the sensory quality of fermented products when present in excess, while

sweet amino acids (e.g., glycine, alanine, serine, threonine, proline and lysine) enhance palatability by contributing mild sweetness which can mask the bitterness. The ratio of bitter to sweet amino acids was higher in the control samples compared to those fermented with vaginal probiotic strains. Beyond their health and direct organoleptic roles, FAAs can be further metabolized into a range of flavor-active compounds, including aldehydes, amines, and alcohols (Chen et al., 2017).

3.5. Volatile molecule profiles

Volatile compounds produced during fermentation and storage may affect both the aroma and functionality of the final product. Therefore, the volatilome of the samples was analyzed. Approximately 19 compounds were identified mainly belonging to the class of alcohols, ketones, organic acids, and aldehydes. To better understand the effects of the different strains of *L. crispatus* on the volatile profile, a principal component analysis (PCA) was performed using volatilome data. The projection of the samples and the related molecules, analyzed after 1, 14, and 28 days of storage (Fig. 5), were able to explain 49.2 % of the total variance among the samples. A distinct cluster corresponding to control samples at all storage times was observed on the top of the figure. This cluster was characterized by a higher relative abundance of acetaldehyde (0.25-0.36 a.u. - area peak /area IS), 2,3-butanedione (diacetyl, 1.0-3.6 a.u.) and acetoin (1.61-2.12 a.u.). These compounds are key to yogurt's desirable flavors: acetaldehyde adds a fresh, green note and is crucial to its characteristic aroma; diacetyl imparts sweet, buttery, and creamy tones, enhancing buttery flavors; while acetoin softens the intense aroma of diacetyl, contributing a mild, creamy taste (Krastanov et al., 2023). The addition of the vaginal strains determined a modification of the volatilome. In fact, samples containing *L. crispatus* clustered together and in the lower quadrants. They were characterized by the higher presence of organic acids, mainly acetic acid (0.78-0.83 a.u. at T1), but also butyric, hexanoic and octanoic acid. During the storage period, acetic acid increased mainly in samples containing BC1 and BC5, reaching around 2.8-3.0 a.u. after 28 days of storage. However, during storage, the differences between the *L. crispatus*-containing fermented milks and the control samples progressively decreased. In fact, samples containing BC1 and BC5 stored for 14 days and those with BC4

and BC5 after 28 d of storage were separated from the control samples stored for 14 and 28 d only along the PC2 explaining 23.7% of the variance. According to Costantini et al. (2021) and Chang et al. (2001), *L. crispatus* possess a homolactic metabolism. However, the production of low amounts of acetic acid has been reported to depend on the specific strain and growth conditions. An increase of acetic acid in presence of *L. crispatus* was also described by Siroli et al. (2017) and D'Alessandro et al. (2023b) during its growth in milk and soy beverages. This production may occur through alternative metabolic routes from pyruvate, acetyl-phosphate or fatty acid biosynthetic pathways (Gänzle, 2015). The storage time determined a further increase in other organic acids but in a strain-dependent fashion. For instance, at T14, butanoic and hexanoic acids were higher in BC4 (0.33 and 0.48 a.u., respectively) and BC5 (0.20 and 0.29 a.u., respectively), compared to BC1 (0.14 and 0.23 a.u.), while lower values were observed in the control (0.09 and 0.15 a.u.). These organic acids, besides influencing the final aroma profile of the product in a concentration-dependent manner (Zhao et al., 2022), may also enhance its functional properties due to their antimicrobial activity (Coban, 2020). Moreover, SCFAs have been associated with several health-promoting effects (appetite regulation, anti-inflammatory properties, etc.) (Ragavan & Hemalatha, 2024).

3.6. Antimicrobial activity

Fermented milks were tested for their antagonistic activity towards the intestinal pathogens enterotoxigenic *E. coli* H10407, *S. enterica* and *Y. enterocolitica* by overlay assay (Table 4). All samples inhibited the growth of enteropathogens, especially against *E. coli* H10407 and *S. enterica*, as indicated by the formation of inhibition halos. The control displayed a moderate antagonistic activity towards the three enteropathogens, and such activity was kept stable over 21 days of refrigerated storage, except for *Y. enterocolitica* where the antimicrobial activity was reduced. Among the samples containing the vaginal strains, those with BC1 and BC5 were the best performing ones since they showed the widest inhibition zone which was maintained up to 28 days of storage. The improved performance of probiotic-containing samples may depend on metabolites produced during fermentation, such as lactic acid and SCFAs (Mani-López et al., 2022). It should be noted that part of the

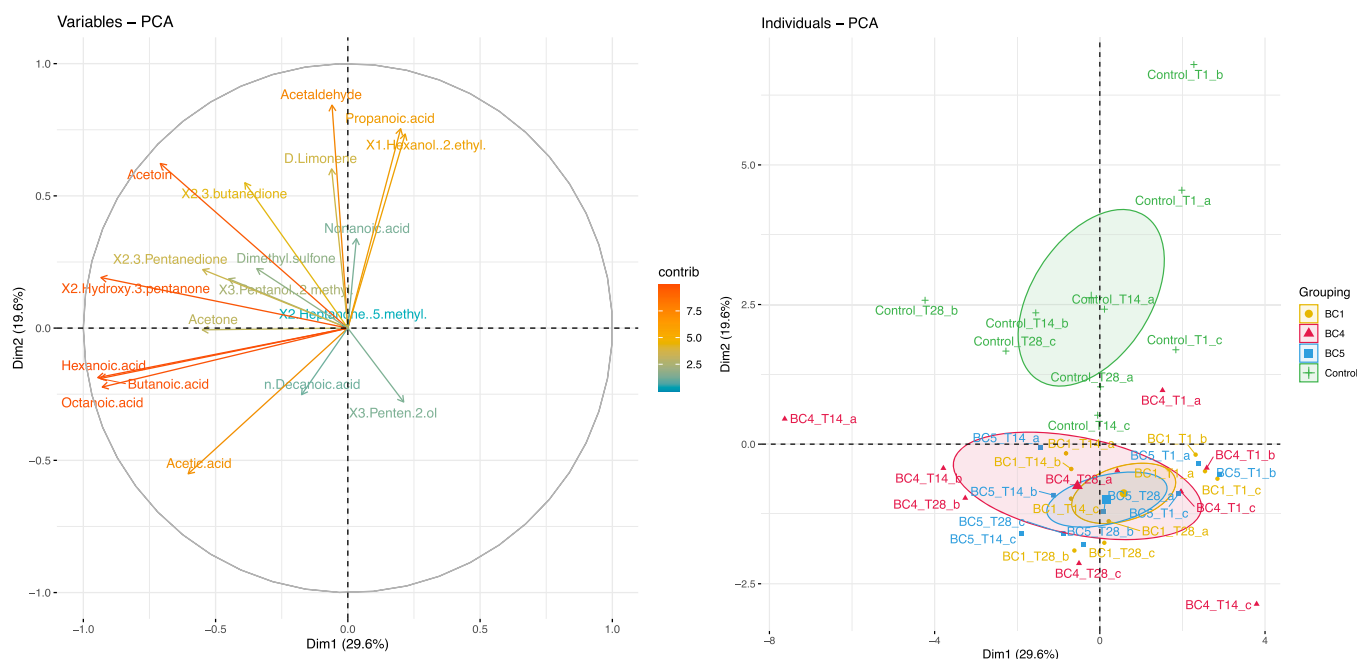


Fig. 5. Principal Component Analysis (PCA) of volatile compounds in fermented milks prepared with different *L. crispatus* strains (BC1, BC4, and BC5), analyzed after 1, 14 and 28 days (T1, T14, T28) of storage in refrigerated conditions. Control: fermented milk with only Lyofast ST440; BC1: fermented milk with Lyofast ST440 and *L. crispatus* BC1; BC4: fermented milk with Lyofast ST440 and *L. crispatus* BC4; BC5: fermented milk with Lyofast ST440 and *L. crispatus* BC5.

Table 4

Evaluation of the antagonistic activity of the fermented milks after 1 and 28 days (T1 and T28) of storage in refrigerated conditions against enterotoxigenic *Escherichia coli* H10407, *Salmonella enterica*, and *Yersinia enterocolitica*. Control: fermented milk with only Lyofast ST440; BC1: fermented milk with Lyofast ST440 and *L. crispatus* BC1; BC4: fermented milk with Lyofast ST440 and *L. crispatus* BC4; BC5: fermented milk with Lyofast ST440 and *L. crispatus* BC5. The antagonistic activity was expressed in relation to the observed diameter of inhibition: -, No inhibition; +, Diameter between 1 and 3 mm; ++, Diameter between 3 and 6 mm; +++, Diameter between 6 and 10 mm; +++++, Diameter > 10 mm. The diameter of inhibition, for each strain, was the average of three replicates.

	<i>E. coli</i> H10407 (ETEC)		<i>S. enterica</i>		<i>Y. enterocolitica</i>	
	T1	T28	T1	T28	T1	T28
Control	++	++	+++	+++	++	+
BC1	++++	++++	++++	+++	+++	+
BC4	+++	+++	+++	++	++	+
BC5	++++	++++	++++	+++	++	+

antimicrobial activity may be attributed to the acidic environment of the fermented matrix, which can inhibit the growth of pathogenic microorganisms. However, the enhanced inhibition observed in samples containing BC1 and BC5 compared to the control or BC4, despite similar pH values, suggests a relevant contribution of strain-specific antimicrobial compounds. In particular, samples prepared with BC1 and BC5 presented the highest relative abundance of acetic acid and, more generally SCFAs, which are known for their strong antimicrobial activity against both Gram-positive and Gram-negative microorganisms. This effect is primarily attributed to their ability to diffuse through microbial cell membranes, leading to intracellular acidification and disruption of key metabolic functions (Mani-López et al., 2022; Poppi et al., 2015). In addition, the possible production of other strain-dependent antimicrobial molecules, such as bacteriocin-like ones or other bioactive metabolites, cannot be excluded and may further contribute to the observed antagonistic effect (Santarelli et al., 2025). Notably, the observed antimicrobial effect against enterotoxigenic *E. coli* (ETEC) and *S. enterica* is relevant in the context of women's health, as the control of intestinal pathogens contributes to maintaining gut microbiota balance, which may in turn influence the urogenital ecosystem through the gut-vagina axis (Amabebe & Anumba, 2020).

4. Conclusions

This study demonstrated the potential of three *Lactobacillus crispatus* strains (BC1, BC4, and BC5) as adjunct cultures for the development of a fermented milk designed for women. The rationale for this formulation is supported by the strain-dependent functional features observed. The selected strains, originating from the vaginal niche, are known to contribute to vaginal microbial homeostasis and, when delivered through food or oral supplementation, may reach and influence the urogenital microbiota via the gut-vagina axis. Fermentation with these strains resulted in a product with improved technological and functional properties compared to the control, including modified rheological behavior and syneresis characteristics, increased levels of free amino acids, particularly lysine and leucine, and stronger antimicrobial activity, likely associated with the production of organic acids and short-chain fatty acids. In addition, the modification of the volatile profile suggests potential implications for both sensory quality and functionality. The increase in specific amino acids and the observed antimicrobial effects may have relevance for women's health, particularly in relation to metabolic regulation, bone health, and microbiota balance. Among the strains tested, BC1 showed the highest viability during storage, indicating its suitability for industrial applications, while BC5 exhibited the most pronounced technological and antimicrobial performance, although further optimization is required to improve its stability. Overall, these findings demonstrate the feasibility of developing a

fermented dairy product with promising technological and functional characteristics. Future research will focus on scaling up the production process and further validating the functional and sensory properties of the product through advanced approaches, including *in vitro* gut models and human intervention studies, to better elucidate the underlying mechanisms and substantiate potential health-related claims in the context of women's health.

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Ethical statement

No humans or animals were used in this work.

CRediT authorship contribution statement

Daide Gottardi: Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Margherita D'Alessandro:** Writing – review & editing, Investigation, Formal analysis. **Giuseppe Celano:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Leonardo Mancini:** Writing – review & editing, Investigation. **Maria Alessia Schouten:** Writing – original draft, Visualization, Investigation, Formal analysis. **Silvia Malavolta:** Writing – review & editing, Investigation. **Santina Romani:** Writing – review & editing, Supervision. **Carola Parolin:** Writing – review & editing, Investigation. **Beatrice Vitali:** Writing – review & editing, Resources. **Maria De Angelis:** Writing – review & editing, Supervision, Conceptualization. **Francesca Patrignani:** Writing – review & editing, Conceptualization. **Rosalba Lanciotti:** Writing – review & editing, Validation, Supervision, 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.

Data availability

<https://zenodo.org/records/17722497> (The datasets supporting the conclusions of this article are available in the Zenodo repository, in .)

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