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Characterization of technological properties of Enterococcus and Lactobacillus strains isolated from raw camel milk for potential use in dairy fermentations

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Abstract
This study evaluated the technological properties of 10 lactic acid bacteria strains belonging to the speices of Enterococcus faecalis, E. faecium, Lactobacillus brevis, and Lb. plantarum previsiouly isolated from raw camel milk and relevant for dairy fermentations. Enterococcal strains were found to be slow acid producers, although E. faecalis exhibited better acidifying activity after 24h of incubation. E. faecalis showed significantly higher proteolytic activity than E. faecium, increasing between 6 and 24h. Growth kinetics revealed an initial lag phase followed by robust growth for most strains, with potential limitation for E. faecium due to its low proteolysis. Autolytic activity demonstrated inter-strain variability, with some E. faecium strains being as autolytic as Lb. plantarum. Diacetyl production was confirmed in Enterococcus and Lb. plantarum, but absent in Lb. brevis. Finally, peptidase activities, essential for protein degradation and bitterness reduction, were highlighted, with lactobacilli being recognized for their diverse enzymatic repertoire. These findings contribute to a better understanding of the technological potential of these strains for specific applications in the dairy industry, by identifying their strengths and limitations.
Keywords: *Lactic acid bacteria, raw camel milk, growth, acidifying activity, proteolytic activity, aminopeptidase activity, autolytic activity, diacetyl production.*

INTRODUCTION

Camel milk is increasingly recognized for its economic value and unique nutritional properties, making it a staple food and a valuable resource in various regions worldwide (Zhao, 2020 ; Belkheir, 2025). Beyond its intrinsic qualities, camel milk is also considered an important natural reservoir for isolating new strains of lactic acid bacteria (LAB) with significant industrial and probiotic interest (Sharma, 2020 ; Mokhtari, 2025).

Fresh camel milk is characterized by a high bacterial diversity, including a wide range of lactic acid bacteria (Baubekova, 2015 ; Davati, 2015). Recent studies have identified numerous LAB species in camel milk, such as Lactobacillus paracasei, Enterococcus italicus, Enterococcus durans, Lactococcus lactis ssp. lactis, Weissella confusa, and Enterococcus faecium (Zhao, 2020). Other research has also highlighted the presence of genera like Lactobacillus, Lactococcus, Enterococcus, Leuconostoc, Pediococcus, and Streptococcus in camel milk from different geographical origins, including Algeria, Kazakhstan, Ethiopia, and Iran (Belkheir, 2025 ; Baubekova, 2015 ; Davati, 2015 ; Fguiri, 2016). This microbial richness underscores the potential of camel milk as a source of microorganisms with interesting technological and probiotic properties (Belkheir, 2025).

The genera Lactobacillus and Enterococcus are particularly important in the dairy fermentation industry due to their crucial roles in the development of flavors, textures, and the preservation of fermented products (Belkheir, 2025 ; Hawaz, 2016). The selection of these bacteria for use as starter cultures or probiotics relies on a thorough evaluation of their specific technological properties (Kishilova, 2025 ; Zeid, 2025).

Among these properties, acidifying activity is paramount. It reflects the ability of strains to produce lactic acid, which lowers the pH of the medium and contributes to milk coagulation, product preservation, and aroma development (Hawaz, 2016). The speed and intensity of acidification can vary considerably between

strains, directly influencing the duration of the fermentation process and the final characteristics of the product (Tlais, 2023 ; Atuna, 2025).
Proteolytic activity is another essential criterion. LAB degrade milk proteins into peptides and free amino acids, which influences the texture, rheology, and aromatic profile of fermented dairy products (Harper, 2022 ; Santiago-López, 2018). This activity is crucial for the release of aroma precursors and for cheese ripening. It has been observed that lactobacilli, in particular, often exhibit higher levels of proteolytic activity than other isolates (Du, 2024). Aminopeptidase activity, closely linked to proteolysis, plays a specific role in the release of individual amino acids from small peptides, thereby significantly contributing to flavor development and the reduction of bitterness in fermented products (Zhang, 2025).
Autolytic activity is also an important technological property. Autolysis of bacterial cells releases intracellular enzymes, including proteases and peptidases, into the medium, which can accelerate the ripening of fermented products and contribute to the formation of complex aromas (Wilkinson, 2020).
Finally, diacetyl production is an important selection criterion for starter cultures, as this compound is responsible for the characteristic buttery note of many fermented dairy products (Hernandez-Valdes, 2020). Although diacetyl production is not always explicitly detailed in all abstracts, the evaluation of acidifying and proteolytic activities is crucial for the development of aromas and texture in fermented products, and the ability to produce aromatic compounds like diacetyl is often sought after in selected strains (Hawaz, 2016). For instance, LAB strains isolated from Algerian dromedary milk have shown promising technological properties, making them good candidates for camel milk processing or other dairy fermentation processes (Latreche, 2025).
The purpose of the present work was to determine some technologically important traits in a series of enterococci and lactobacilli, such as biomass production, acidification ability, proteolytic and aminopeptidase activities, autolytic activity and production of diacetyl, which are relevant to their technological performance in fermented dairy foods. The evaluation of these properties in respect of origin and species would allow an initial selection of native strains from camel raw milk to be used as starters in food fermentations.

2. MATERIAL AND METHODS

2.1. Material and chemicals

The lactic acid bacteria strains used in this study were obtained from the Laboratory of Environment and Sustainable Development Research (University of Relizane - Algeria). These strains were originally isolated from raw camel milk collected in Algeria and identified by PCR amplification using E. faecalis, E.faecium, Lb. plantarum and Lb. brevis primers and 16S rDNA sequencing (Vincent, Roy, Mondou, & Dery, 1998). A search for homology of the DNA sequence was performed using the BLAST algorithm available at the National Center for Biotechnology Information (NCBI, USA). The cultures were maintained (kept frozen) at – 80 °C in MRS broth (Merck, Darmstadt, Germany) supplemented with 20 % (v/v) glycerol, and were revitalized in the same medium by overnight growth at 30°C. Phenolphthalein, ethylenediaminetetraacetic acid (EDTA), Folin & Ciocalteu's phenol regent, leucine-para-nitroanilide (leucine-pNa) and α-naphtol were from Sigma-Aldrich Co. (St. Louis, MO).

2.2. Technological characteristics of strains

2.2.1. Measurement of growth

To quantify bacterial growth, the optical density (OD) at 480 nm was measured at two time points (6h and 24h). Samples of incubated skim milk (Régilait UCA, St-Martin, Belle-Roche, France) were first diluted with an EDTA solution (0.2%, pH 12) to minimize matrix interference and ensure reliable optical measurements (Thomas et Turner, 1997).

2.2.2. Acidifying activity

The acid producing ability of each strain was tested by inoculating (1%, v/v) 18h culture into 10% skim milk and incubating at 30 °C. The pH was measured at 6h and 24h using the electrode of the pH-meter (inoLab, D-82362 Weilheim Germany). The acidification rate was calculated as ΔpH (ΔpH = pH at time — pH zero time) (Bradley et al., 1992).
Acidity produced by the strains during growth in reconstituted skim milk was also determined by the method of titration. Five (5) drops of 1% phenolphthalein in 95% ethanol were added to 10 mL of incubated milk (sampled at 6 and 24h), , and the mixture was titrated with NaOH (N/9) until a stable

pale pink color developed. The volume of NaOH required was then recorded (Accolas et al., 1971). The results are expressed in Dornic degrees (°D) according to the following formula : Acidity (°D) = n × 10, where n is the volume of sodium hydroxide solution used to titrate 10 mL of milk.

2.2.3. Proteolytic activity

The proteolytic activity of the strains grown in milk was measured by a method of Folin and Ciocalteu (1927) after 6h and 24h of incubation at 30°C. Proteolytic activity of the cultures was assessed by measuring the release of tyrosine and tryptophan from the milk substrate. These amino acids react with the Folin–Ciocalteu reagent, producing a blue color which was measured at 750 nm. The results were calculated from a calibration curve of tyrosine and were expressed as µg tyrosine mL-1.

The proteolytic activity of whole cells was also assayed by streaking the cultures on skim milk agar (4% skim milk, 1.5 % agar) plates according to the method of Fransen et al. (1997). The plates were incubated at 30°C for 48h and then at 4°C for three days prior the detection of proteolytic activity. A positive result was indicated by a clear halo around the bacterial colonie.

2.2.4. Aminopeptidasic activity of intact cells

Aminopeptidase (AP) activity was assayed using leucine-para-nitroanilide (leucine-pNa) as a substrate according to El Soda et Desmazeaud (1982) as modified by Thivierge (1999). The quantity of p-nitroanilide released was evaluated at 410nm during 6h and 24h using a Jasco V-530 UV/VIS Spectrophotometer.

The reaction was initiated by combining 400 µL of phosphate buffer potassium (0.1M, pH 7), 100 µL of cell suspension and 50 µL of Leu-pNa. Then, the reaction mixture was incubated at 30°C. The reaction is stopped by addition of an acetic acid solution prepared at 10%. After centrifugation at 16000xg for 10 min at 4 °C, the supernatants were recovered for readings by using a Jasco V-530 UV/VIS Spectrophotometer at 410 nm. One unit of aminopeptidase activity (U) was defined as the amount of enzyme required to release 1 µM/min p-nitroaniline under the assay conditions.

2.2.5. Autolytic activity

Autolytic activity was measured according to the method described by Lansgrud et al (1987). Cells from exponentially growing cultures (Optical Density, OD640nm = 1) in MRS broth were harvested by centrifugation at 16000xg (Sigma GmbH, Model 6K15, Gottingen, Germany) for 10 min at 4°C. The biomass pellets were washed twice and resuspended in 0.1M potassium phosphate buffer (pH 5.8) containing 4% NaCl. Lysis was monitored during 24h at 30°C by recording the decrease in OD640nm. Percentage of lysis was determined as (A0 – At) x 100/A0 where A0 = initial absorbance, and At = absorbance measured after t days of incubation as described by Boutrou et al (1998).

2.2.6. Diacetyl production

Diacetyl production was determined by mixing 1 mL of the strain culture (1% inoculated in skim milk and incubated for 24h at 30°C) with 0.5 mL of α-naphtol (1%) and KOH (16%). After incubation at 30°C for 10 min, diacetyl producer strains showed a red ring at the top of the tubes (King, 1948).

2.3. Statistical analysis

Mean separation and significance were analysed using the IBM SPSS® software (SPSS Statistical Software, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and the Tukey post-test were carried out to determine statistical differences ($P < 0.05$ was considered significant).

3. RESULTS AND DISCUSSION

Lactic acid production

Acidification is a fundamental parameter in dairy fermentation processes. In cheese manufacturing, it plays a dual essential role; it contributes to the development of organoleptic properties (taste, texture, aroma), and it ensures microbiological control by inhibiting the growth of pathogenic or spoilage flora. A good mesophilic fast acid producing starter culture will reduce the pH of the milk from its initial value of 6.6 to 5.3 in 6 h at 30 °C (Cogan et al., 1997). The acidifying activity of the strains was determined by measuring pH changes (ΔpH) after 6h and 24h of incubation in reconstituted skim milk. All strains are slow acid producers since their growth in reconstituted skim milk in 6h of incubation at 30°C (Figure 2). After 24h of growth, E. faecalis CHM16, E. faecalis 18, E. faecalis 19 and E. faecalis 20 strains had shown the higher acidifying activity (1.99, 1.98, 1.96, 1.81) followed by Latobacillus brevis CHTD27 and Lactobacillus plantarum BH14 (1.74, 1.33) which it can be concluded that these strains would be of little benefit as starter cultures. Similar observations were made by Aspri et al. (2017) and Elzeini et al. (2021)

who also reported that enterococcal strains examined in their study were slow and weak acid producers. Morever, in a study which assessed the technological properties of enterococcal strains isolated from artisanal Jben cheese, the most acidifying strains belonged to the E. faecalis strains which is in agreement with our study (Azzou et al., 2025). However, Tuncer (2009) reported that E. faecium strains had higher acidifying ability than E. faecalis when he examined enterococci from Turkish Tulum cheese for their technological properties, whereas authors in other studies did not find differences in acidifying activity between E. faecalis and E. faecium strains after 24 hours (Jaouani et al. 2015) and Aspri et al. 2017). Therefore, while authors of the aforementioned studies agree that enterococcal strains are poor acidifiers, it is not completely clear whether acid production by enterococci is species specific, which requires other studies to establish to which species the most acidifying strains belong (Graham et al. 2020).

Proteolytic activity

The proteolytic activity of dairy lactic acid bacteria is essential for bacterial growth in milk and it is involved in the development of organoleptic properties of fermented milk products (Ayad et al., 2004 ; Flambard et al., 1998). Our tested strains were characterized by different caseinolytic breakdown ability (Figure 3). This figure lets see a very weak proteolytic activity for strains after 6h of growth at 30°C. After 24h of growth, this activity increases for CHM16 (169 µg tyrosine/mL) CHM18 (235 µg tyrosine/mL) CHM19 (275 µg tyrosine/mL) and CHM20 (212 µg tyrosine/mL). These same strains exhibited high proteolytic activity in MRS agar plates (Figure 4 and 5).

Our results showed that E. faecalis exhibits higher proteolytic activity than E. faecium which confirm a general trend observed in numerous studies. For example, Tuncer (2009) reported in his study that E. faecalis strains were generally more active than E. faecium and E. durans strains, with the highest proteolytic activity value (100.5 µg tyrosine/mL) coming from E. faecalis. Macovei (2009) reported that genes such as gelE (gelatinase) and sprE (serine protease), regulated by the fsrABDC operon, are associated with strong protease phenotypes in E. faecalis. Another study even indicated that, among the tested strains, 53% of Enterococcus faecalis exhibited proteolytic activity, whereas no Enterococcus faecium strains demonstrated such activity (Gagnon, 2020). Although some strains of E. faecium may show proteolytic activity, it is often less pronounced or absent compared to E. faecalis (Leuschner, 1999 ; Rampanti, 2024). This difference is significant because proteolysis by enterococci plays an important role in the maturation of fermented products, contributing to the development of flavours and texture (Suzzi, 2000).

Furthermeore, the increase in proteolytic activity observed between 6 and 24 hours of growth for our E. faecalis strains is a frequently documented phenomenon. This kinetics suggests regulation of the expression or activity of proteolytic enzymes depending on the phase of bacterial growth as was reported by Worsztynowicz (2019) and Baran (2022). The growth-proteolysis relationship observed in this study could be attributed to the initial utilization of naturally occurring amino acids and peptides in milk to initiate bacterial metabolism. Subsequently, the degradation of milk proteins, specifically caseins and soluble proteins becomes crucial for sustained bacterial growth.

Growth kinetic

The results showed initial growth for all strains of Enterococcus faecium, Enterococcus faecalis, Lactobacillus brevis and Lactobacillus plantarum after 6 hours of incubation, with optical densities (OD) ranging from 0.13 to 0.61. However, after 24 hours of incubation, cultures of Enterococcus faecalis, Lactobacillus brevis and Lactobacillus plantarum reached high optical densities, ranging from 2.11 to 4.5. The low bacterial growth observed after 6 hours of incubation (OD from 0.13 to 0.61) for all strains suggests that these bacteria may be in a latent phase or at the beginning of an exponential phase under the experimental conditions used. The lag phase is a period of adaptation during which microorganisms adjust to their new environment before beginning rapid cell division (Zuleta, 2023). The duration of this phase can vary considerably depending on the bacterial strain, the composition of the culture medium, the temperature and other environmental factors (Pereira, 2023).

The significant increase in optical densities after 24 hours (OD from 2.11 to 4.5) for Enterococcus faecalis, Lactobacillus brevis and Lactobacillus plantarum indicates that these strains have reached a robust exponential growth phase, or even the stationary phase, where the bacterial population is at its maximum. This observation is consistent with the behaviour of many lactic acid bacteria and enterococci, which are known for their ability to proliferate rapidly under favourable culture conditions (Bonger, 2023; Zuleta, 2023).

It is interesting to note that the results for E. faecium are not explicitly mentioned for the 24-hour period with high DO levels. The low capacity of E. faecium to break down milk proteins means that it has limited access to the peptides and amino acids necessary for its proliferation. In an environment rich in proteins such as milk, but poor in free amino acids, insufficient proteolytic activity can become a factor limiting growth compared to Enterococcus faecalis which possess strong proteolytic activity. Although some strains of E. faecium may be associated with technological and probiotic properties, their growth performance in milk may be affected by this enzymatic limitation (Sakoui, 2024; Darwish, 2022).

Autolytic activity

Our data revealed significant variability in autolytic activity among the strains tested, even within the same species. For example, the autolytic activity of Enterococcus faecium varies from 26% (CHM11) to 40% (CHM12), and that of Enterococcus faecalis from 17% (CHM18) to 30% (CHM20). For lactobacilli, Lactobacillus brevis shows 27% and Lactobacillus plantarum 39%. This variability is a well-documented feature of autolysis in LAB, which is often strain-specific (Cruciata, 2014; Dalca, 2018).

In comparision with to more recent studies, Dalca (2018) identified LAB isolates with high autolytic activity, ranging from 34% to 54% (Dalca, 2018). Our values, in particular 40% for E. faecium CHM12 and 39% for L. plantarum, fall within this range of reported high activity. Other strains, such as E. faecalis CHM18 with 17%, show more moderate activity, which is also expected given the diversity of autolytic profiles (Cruciata, 2014).

It has generally been observed that lactobacilli can exhibit higher proteolytic and autolytic activity than other isolates, including enterococci (Hassaine, 2007; Hassaïne, 2008). Although Lactobacillus plantarum (39%) is among the most autolytic strains, Enterococcus faecium CHM12 (40%) exhibits activity comparable to, or even slightly higher than, that of L. plantarum. This highlights the importance of evaluating strains individually, as some Enterococcus strains can be as autolytic as lactobacilli.

Incubation conditions (0.1M phosphate buffer, pH 5.8 and 4% NaCl) are environmental factors known to influence autolytic activity. The literature indicates that autolysis may increase with temperature and pH, but decrease at high NaCl concentrations (Dalca, 2018). However, some strains, such as E. faecium and L. plantarum, have shown high autolysis even when exposed to low pH and high NaCl concentrations (Dalca, 2018). For example, optimal conditions for autolysis of Lactobacillus casei have been reported at pH 5.2 and with 2-4% NaCl (Dako, 1995). Your conditions (pH 5.8 and 4% NaCl) are relatively close to these conditions, which could explain the levels of autolysis observed for some of our strains. The presence of 4% NaCl is considered a high concentration, and the fact that some of your strains maintain significant autolytic activity under these conditions is relevant.

Diacetyl production

Diacetyl is an important flavour compound, particularly in dairy products, where it contributes to buttery notes (Azari, 2025). Its production by lactic acid bacteria is a desirable technological trait in many fermentations.

The results obtained for Enterococcus faecalis and Enterococcus faecium strains are consistent with the scientific literature. Recent studies have identified Enterococcus species as primary producers of diacetyl among lactic acid bacteria (Azzouz, 2025). For example, isolates of Enterococcus faecalis and Enterococcus faecium from animal rennet have been reported to produce diacetyl (Cruciata, 2014). Another study also confirmed the production of diacetyl by Enterococcus strains isolated from cheese (Azzouz, 2025).

With regard to Lactobacillus plantarum BH14, the results are also consistent with recent studies. The genus Lactiplantibacillus, which includes L. plantarum, has been identified as a major producer of diacetyl (Azzouz, 2025). Although, Azari (2025) and Azzouz (2025) noted that L. plantarum produced acetaldehyde and that another Lactobacillus strain (L. delbrueckii subsp. lactis) showed the highest diacetyl production, the general identification of Lactiplantibacillus as the main producer of diacetyl supports your observations (Azari, 2025; Azzouz, 2025).

However, the non-production of diacetyl by Lactobacillus brevis CHTD27 is a specific observation. Although Lactobacillus brevis is a lactic acid bacterium commonly isolated from various fermented products, including dairy products (Yu, 2011 ; Hoorde, 2008 ; Pulido, 2005 ; Castro, 2016 ; Tzora, 2021), the sources consulted do not provide direct information on whether or not it is capable of producing diacetyl. Variability in diacetyl production may exist at the strain level, meaning that some strains of the same species may produce diacetyl while others may not.

Peptidase activity

The peptidase activities of lactic acid bacteria play a crucial role in the breakdown of proteins into peptides and amino acids, which are essential for bacterial growth and contribute to the development of flavours in fermented foods (Liu, 2010 ; Khalid, 1990). Aminopeptidase activity also plays a crucial role in the hydrolysis of bitter peptides, thereby reducing bitterness in cheeses. Studies have shown that lactobacilli possess a more diverse and comprehensive set of peptidases than other lactic acid bacteria, including lactococci, streptococci and leuconostocs (Liu, 2006). The increase in peptidase activity in Canestrato Pugliese cheese has been correlated with the highest levels of lactobacilli during maturation (Cagno, 2004). Although enterococci also exhibit proteolytic activity, as observed in Enterococcus faecalis (Dağdemir, 2008), lactobacilli are often highlighted for their significant contribution to peptidase activities, particularly in contexts such as cheese maturation (Khalid, 1990; Cagno, 2004).

4. CONCLUSION

This study characterized the technological properties of various bacterial strains, including Enterococcus faecalis, Enterococcus faecium, Lactobacillus brevis, and Lactobacillus plantarum, relevant to dairy fermentation processes. Regarding lactic acid production, all strains were found to be slow producers, with E. faecalis showing superior acidifying activity after 24 hours of incubation, although enterococci are generally considered weak acidifiers. The inter-species and inter-strain variability in acidification highlights the complexity of this trait.

Proteolytic activity revealed a clear distinction, with E. faecalis exhibiting significantly higher activity than E. faecium, a phenomenon that intensified with incubation time. This protein degradation capacity is crucial for bacterial growth and flavor development. Growth kinetics showed an initial lag phase followed by robust exponential growth for E. faecalis, L. brevis, and L. plantarum after 24 hours, while the growth of E. faecium might be limited by its lower proteolytic activity in milk medium.

Autolysis demonstrated considerable variability among strains, even within the same species, with some E. faecium strains displaying activity comparable to, or even higher than, that of L. plantarum. Incubation conditions, particularly pH and NaCl concentration, also influence this activity. Concerning diacetyl production, a key aroma compound, Enterococcus species and L. plantarum were identified as primary producers, although L. brevis CHTD27 did not show this capability, illustrating strain specificity. Finally, peptidase activity, essential for bacterial growth and flavor formation, is generally more diverse in lactobacilli, although E. faecalis also contributes to this function.

These results confirm the diversity of technological properties within the studied strains and underscore the importance of in-depth characterization for the selection of starter cultures adapted to the specific requirements of fermented dairy products.

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Results

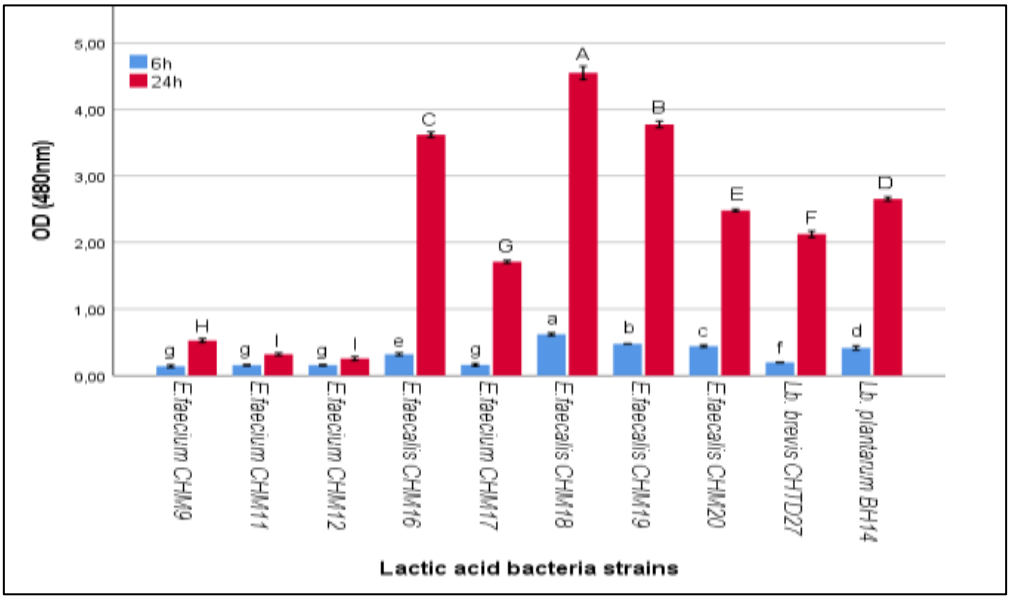

Figure 1. Growth of lactic acid bacteria strains in skimmed milk at 30°C after 6h and 24h

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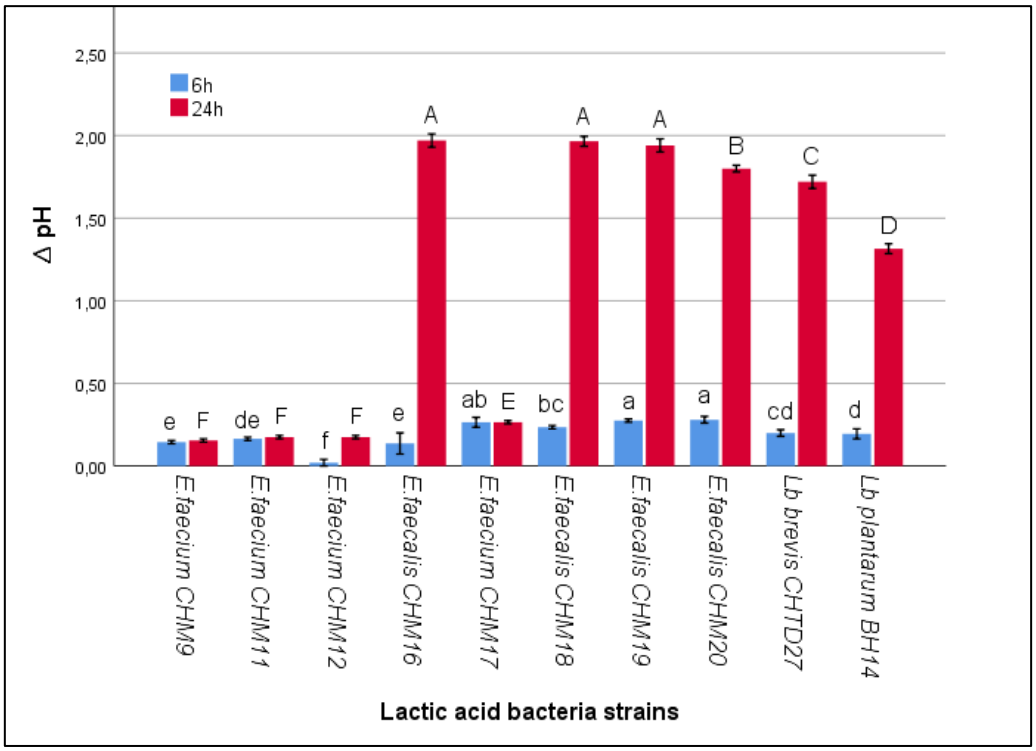

Figure 2. pH variation of skimmed milk inoculated with lactic acid bacteria strains at 30°C

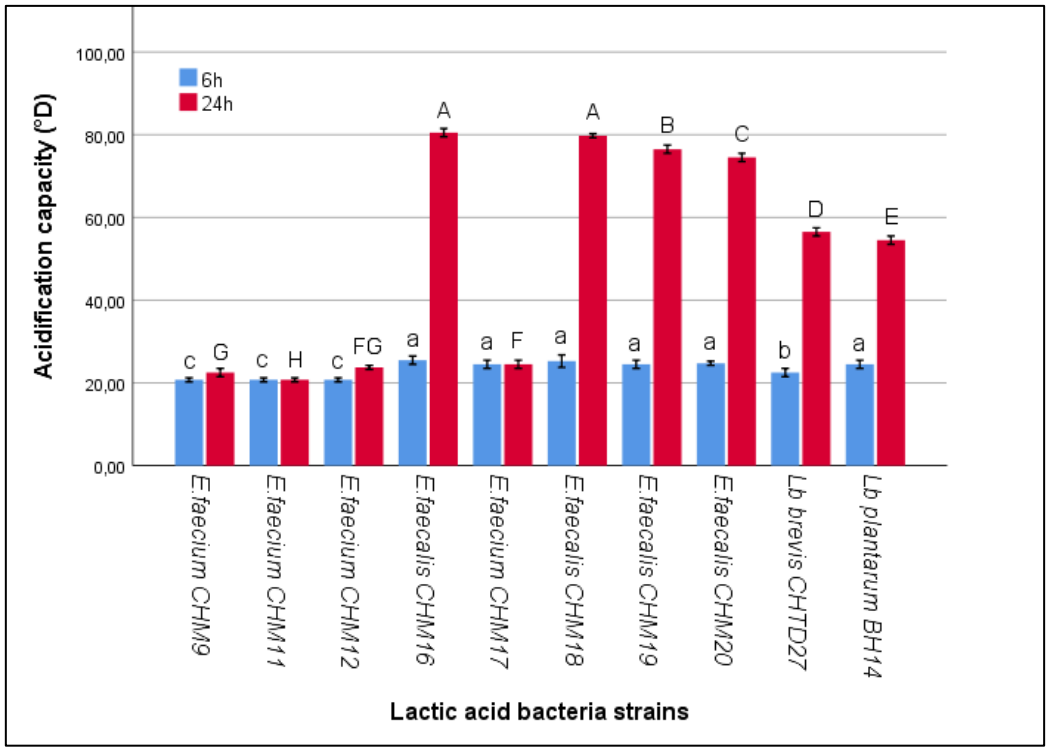

Figure 3. Acidification capacity of lactic acid bacteria strains in skimmed milk at 30°C

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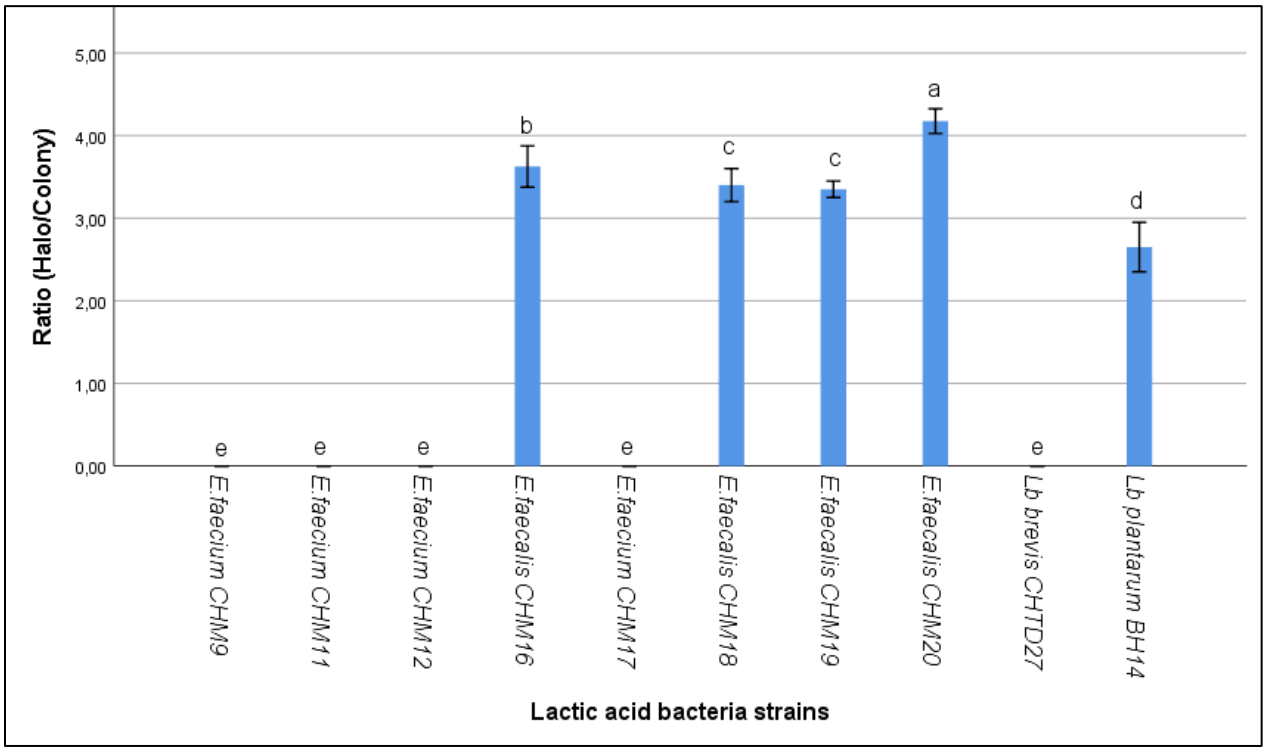

Figure 4. Proteolytic activity of lactic acid bacteria strains in milk-agar (4%) at 30°C

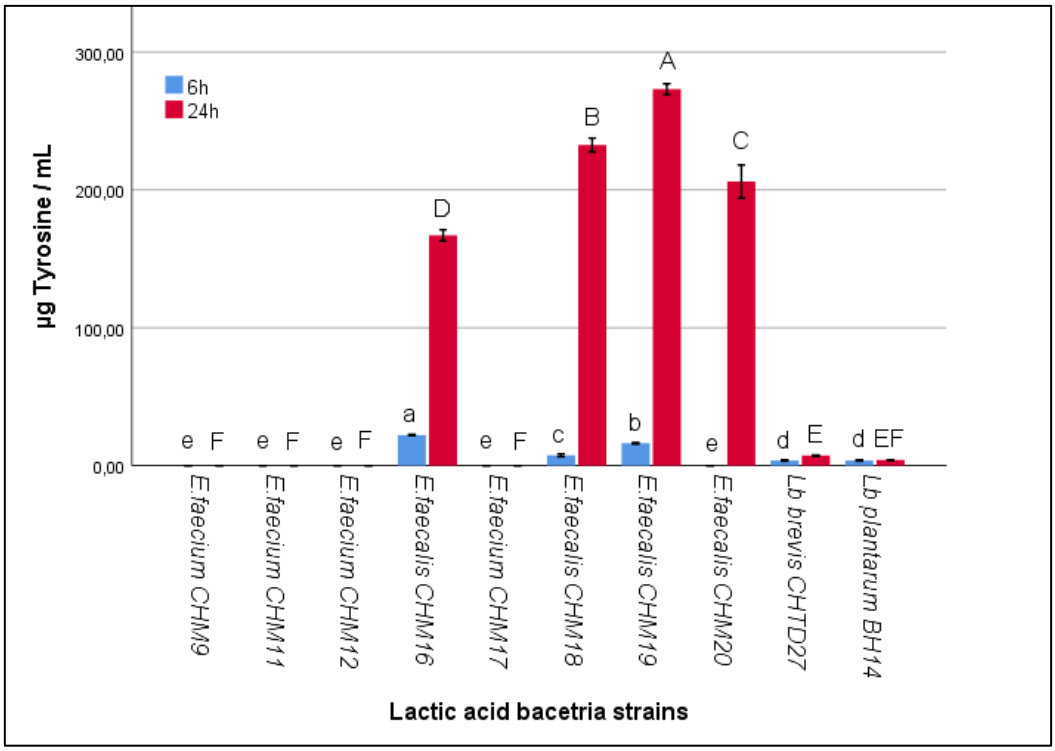

Figure 5. Evaluation by Folin-Ciocalteu titration of the milk proteins degradation by lactic acid bacteria strains at 30°C

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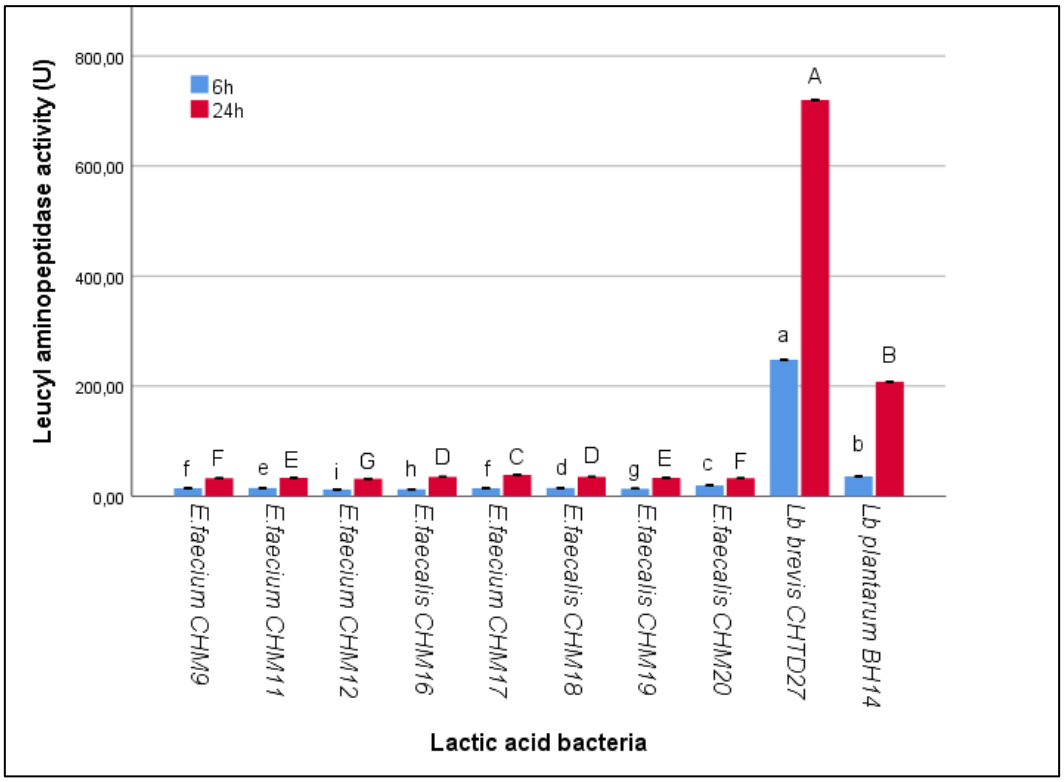

Figure 6. Leucyl-aminopeptidase activity of lactic acid bacteria strains at 30°C

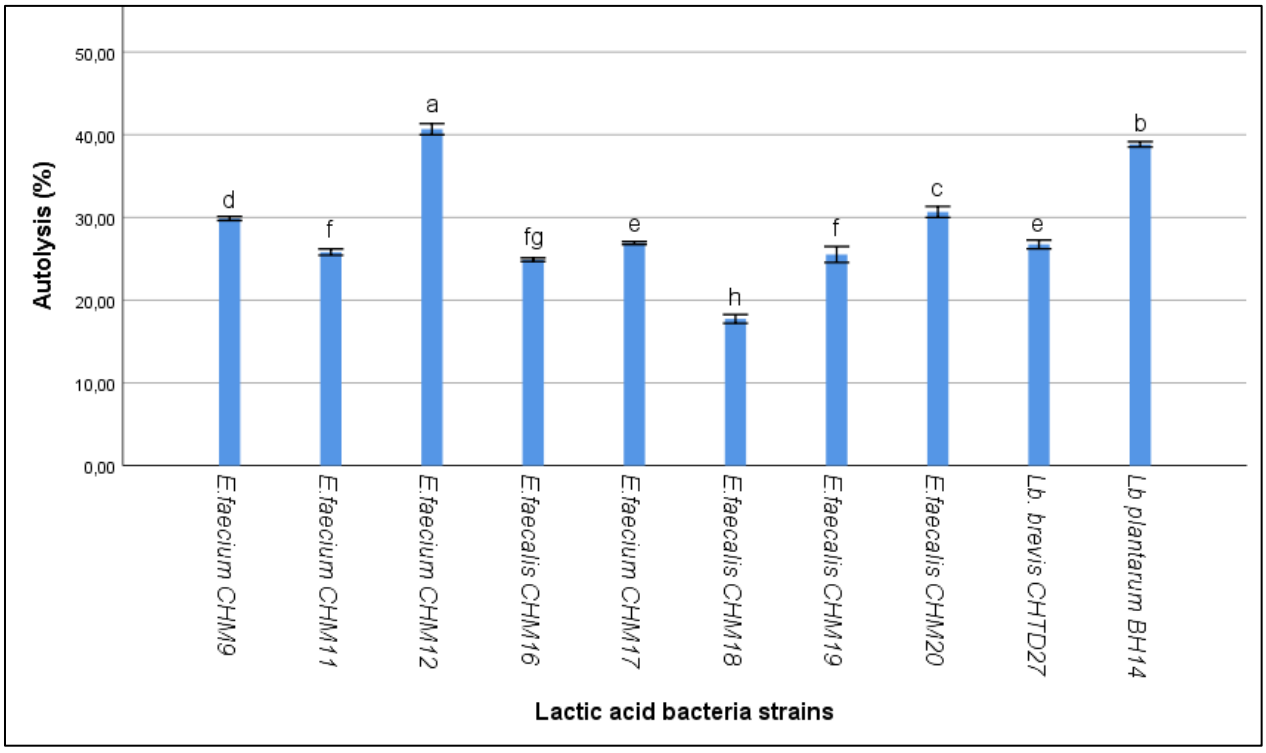

Figure 7. Autolytic activity of lactic acid bacteria strains measured at 30°C after 24h