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Whey as a substrate for biogenic production of exopolysaccharide

Lactosuero como sustrato para la producción biogénica de exopolisacárido

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Abstract

Whey, a byproduct of the dairy industry, is often discarded during cheese production despite its considerable protein and lactose content. One of the alternatives for revalorizing whey is obtaining compounds of industrial interest using fermentative microorganisms. Therefore, the objective of this project was to use sweet whey (SW) as a matrix for the biogenic production of an exopolysaccharide (EPS) using lactic acid bacteria. The strains used were *Lactobacillus delbrueckii* subsp *bulgaricus* NCFB 2772 and *Streptococcus thermophilus* SY-102 in monoculture and coculture (1:1 ratio). Microbial growth, changes in pH, EPS production, and the release of free amino groups were monitored to determine the degree of proteolysis over 48 hours of fermenting at 37 °C. The results showed that the coculture system had the highest microbial concentration, reaching 7 x 10^{11} CFU/mL. Likewise, it also had the lowest pH (3.77) with more outstanding production of EPS (1.49 mg/mL) and free amino groups (0.20 mg/mL) compared to the two monocultures. This difference can be attributed to the symbiosis between both strains, concluding that the protocooperation process could be involved in the enhanced production of exopolysaccharides.

Keywords: Whey, Lactic acid bacteria, Exopolysaccharides, Proteolysis, Symbiosis.

Resumen

El lactosuero es un subproducto de la industria láctea que se desecha a pesar de su buen contenido nutrimental. Algunas opciones para revalorizarlo incluyen la obtención de compuestos de interés industrial utilizando microorganismos fermentativos. Por ello, el objetivo de este proyecto fue utilizar el lactosuero dulce (SD) como matriz para la producción biogénica de un exopolisacárido (EPS) empleando bacterias ácido lácticas. Las cepas empleadas fueron *Lactobacillus delbrueckii* subsp *bulgaricus* NCFB 2772 *y Streptococcus thermophilus* SY-102*,* tanto en monocultivo como en cocultivo (proporción 1:1). Se monitoreó el crecimiento microbiano, cambios en el pH, producción de EPS y la liberación de grupos amino libres para determinar el grado de proteólisis a lo largo de 48 horas fermentando a 37 °C. Los resultados obtenidos determinaron que en el sistema de cocultivo se tuvo la mayor concentración microbiana alcanzando niveles de 7 x 10^{11} UFC/mL. Asimismo, también tuvo el menor pH (3.77) con una mayor producción de EPS (1.49 mg/mL) y de grupos amino libres (0.20 mg/mL) en comparación con los dos monocultivos. Esta diferencia puede atribuirse a la simbiosis entre ambas cepas, concluyendo que el proceso de protocooperación podría estar implicado en la mayor producción de exopolisacárido.

Palabras clave: Lactosuero, Bacterias ácido-lácticas, Exopolisacáridos, Proteólisis, Simbiosis.

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1 Introduction

A recurring issue in the dairy industry is the underutilization of whey, a liquid byproduct (approximately 93% water) from cheese making. Whey accounts for 85-90% of the industry's total waste. Despite its high content of proteins, lactose, and mineral salts, whey is often discarded as wastewater, contributing to environmental pollution (Zandona *et al.*, 2021). Whey can be classified into two types based on the cheese-making process: sweet whey (resulting from enzymatic coagulation) and acid whey (resulting from coagulation with organic acids). Acid whey poses significant usage challenges due to its acidity and associated technological difficulties (Wherry *et al.*, 2019).

Although various bioremediation strategies aim to dispose of whey with minimal environmental impact (Sebastián-Nicolás *et al.*, 2020), circular economy studies are particularly significant due to their potential economic benefits for the dairy industry (Smithers, 2015; Skryplonek *et al.*, 2019; Lavelli and Beccalli, 2022). Some approaches focus on separating specific components for industrial use through physicochemical processes such as drying, membrane separation, or ultrafiltration (Zhao *et al.*, 2023). Other methods leverage whey through biotechnological processes, such as microbial fermentations, to produce a wide range of products, including biofuels and new food products (Yildiz *et al.*, 2023). Functional foods are an example of products that can be developed from whey and right now have very high demand both from a commercial and a biotechnological perspective (Sebastián-Nicolas *et al*., 2024).

Examples of such products include EPS, which are polymeric carbohydrates some microorganisms produce during their growth cycle (Hernández-Rosas *et al*., 2021). In terms of cost and functionality, it has been reported that these compounds of biogenic origin present significant advantages and applications across multiple industries: in the food industry, they enhance the sensory characteristics of products (Brüls *et al.*, 2024); in pharmaceuticals and biomedicine, they offer therapeutic properties (Ahuja *et al.*, 2023); and in environmental applications, they assist in the detoxification of products (Kavitake *et al.*, 2020). Lactic acid bacteria, recognized as Generally Recognized as Safe (GRAS), have been used extensively by the food industry due to their various properties (Borrás-Enríquez *et al*., 2023) and have been shown to have great capacity for EPS production. These microorganisms can generate valuable compounds directly within a food matrix (Moradi *et al.*, 2021). Due to its composition and origin, whey is an excellent substrate for developing these microorganisms, thanks to its high lactose and protein content and the technological flexibility these components provide (Rama *et al.*, 2019). Given this context, this research aimed to utilize whey as a substrate for the biogenic production of EPS using lactic acid bacteria. The produced material could be used as an additive to enhance the rheological characteristics of food products, assist in toxin decontamination, serve as an encapsulation material, or function as a functional ingredient.

2 Materials and methods

2.1 Reagents

Picryl sulfonic acid, trichloroacetic acid, dextran from *Leuconostoc* spp. and phenol were purchased from Sigma Aldrich (St. Louis, MO, USA). Man, Rogosa, and Sharp (MRS) broth and agar, peptone water, and skim milk medium were purchased from the Difco Company (Detroit, Michigan, USA). Citric, sulfuric and hydrochloric acid were purchased from J.T. Baker (NJ, USA). Absolute ethanol and NaOH were purchased from Meyer (Mexico City). Bradford 1x dye reagent and glycine were purchased from BioRad, Hercules (CA, USA). Liquid rennet was purchased from CHR Hansen (Mexico City, Mexico).

2.2 Starter cultures

Starter cultures for both bacteria were prepared by adapting the methodology of Hernández-Riveros *et al.* (2024) with some modifications. The two working strains, *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 and *Streptococcus thermophilus* SY-102, were sourced from the strain collection of the Food Biotechnology laboratory at the Universidad Autónoma Metropolitana, Campus Iztapalapa. Initially, cryo beads of the microorganisms were conditioned in MRS broth at 37 °C for 48 hours. From this, 1 mL was used to inoculate 9 mL of skim milk medium, which was incubated at 37 °C for 24 hours. Following this, aliquots of pasteurized whey were inoculated with the culture at a 1:10 v/v ratio and incubated at 37 °C until reaching a microbial load of 1 x 10^7 CFU/mL, as determined by plate count. The starter was kept at 4-6 ºC until its use.

2.3 Sweet whey production

The sweet whey (SW) used as the basis of the project was produced by adapting the Panela cheese process described by Ochoa-Flores *et al.* (2021) as follows. Fresh pasteurized whole milk (Lala brand) was heated until it reached 30 ºC. Liquid rennet was added at a concentration of 0.2 mL/L of milk, and the mixture was incubated at 35 °C for one hour. The precipitated

casein was then separated using a sterile cloth for draining. Subsequently, SW was pasteurized at 65 °C for 30 minutes and rapidly cooled to 4 °C by placing it in an ice bath for 10 minutes. The resulting SW was stored at -18 °C until use. Lactose content was determined by the technique of Dubois *et al*. (1956) while the protein concentration was determined by the Bradford assay (1976).

2.4 Sweet whey fermentation

The conditions for the fermentation of the SW were adapted from the methodology used by Carrero-Puentes *et al.* (2021) with some modifications. Three different batches were designated for fermentation: one with *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 (LB), a second with *Streptococcus thermophilus* SY-102 (ST), and the third batch with the co-culture with both bacteria in a 1:1 ratio (LBST). Both the LB and ST batch were inoculated with their respective starter cultures at 5 % (v/v), while the LBST batch was inoculated with 2.5% (v/v) of each of the two starters. All fermentation systems were incubated at 37 °C for 48 hours. Cell growth, EPS production, pH decreases in the medium, and proteolysis (measured as the formation of free amino groups) were monitored during this period.

2.4.1 Microbial growth and pH

The growth of each bacterium was evaluated using the micro drop plate counting technique described by Morales-García *et al.* (2012). The samples' dilutions were prepared from 10^{-1} to 10^{-6} using peptone water, and 5 μL aliquots were plated on MRS agar medium. The plates were incubated at 37 °C for 24 hours, and the dilutions showing growth between 25 and 250 colonies were considered. The results for each sample were reported as log CFU/mL. Finally, the pH was monitored using 10 mL aliquots measured with a potentiometer (Conductronic pH120).

2.4.2 Determination of exopolysaccharides (EPS)

For this project, we aim to quantify the biogenic EPS production in each fermentation system during the incubation period, for a possible future application of the SW with EPS as a source for the development of a functional food product, as it has been previously reported (Carrero-Puentes *et al*., 2021). While the extraction or full characterization of the EPS produced was not of interest for this project, it can be explored in future studies.

A modified version of the method described by Domínguez-Soberanes (1998) was used to determine the EPS concentration by turbidity of the full polysaccharide. Six milliliters of f SW were mixed with 1 mL of 80% (w/v) TCA, vortexed for 30 seconds, and allowed to stand for 20 minutes at 4 °C. The mixture was then centrifuged at 15, 000 x g for 30 minutes at 4 °C (Beckman Coulter, J2-MI). Two milliliters of the supernatant were collected and mixed with 2 mL of absolute ethanol, vortexed for 30 seconds, and allowed to stand for 20 minutes at 4 °C. The absorbance of the sample was measured at 720 nm (Shimadzu UV-1800-120V) using a distilled water blank treated in the same manner as the sample. The exopolysaccharide concentration was determined using a dextran standard curve ranging from 0.2 to 1.0 mg/mL. Dextran was used because of its wide applications in various biotechnological industries (Castilla-Marroquín *et al*., 2020).

2.4.3 Proteolysis

Free amino groups were quantified using the picryl sulfonic acid (TNBS) method proposed by Adler-Nissen (1979). A 250 μL sample was mixed with 2 mL of phosphate buffer solution (0.2125 M, pH 8.2) in a test tube lined with aluminum foil. Then, 2 mL of TNBS solution (0.10% in 0.2125 M phosphate buffer, pH 8.2) was added. The mixture was homogenized and incubated for 1 hour at 50 °C in the dark. After incubation, the reaction was stopped by adding 4 mL of 0.1 N HCl. The mixture was homogenized again, and the absorbance was measured at 340 nm (Shimadzu UV-1800-120V). A blank was prepared by replacing the sample with distilled water. A glycine standard curve, ranging from 0.025 to 0.25 mg/mL, was used to calculate the final concentration of free amino groups.

2.5 Statistic analysis

The experiments were conducted in triplicate, and the data were analyzed using analysis of variance (ANOVA). Post hoc comparisons of means were performed using Tukey's rank test ($p < 0.05$). Furthermore, correlation analyses were conducted to examine the relationships between growth parameters, EPS production, and proteolysis within each fermentation system, with correlation coefficients compared accordingly. All error bars marked in the Figures are standard deviations from each experimental triplicate set.

3 Results and discussions

3.1 Cell growth and exopolysaccharide production

Some of the most important factors in EPS production by LAB are the carbon source, nitrogen source and the C/N ratio in the culture media for microbial

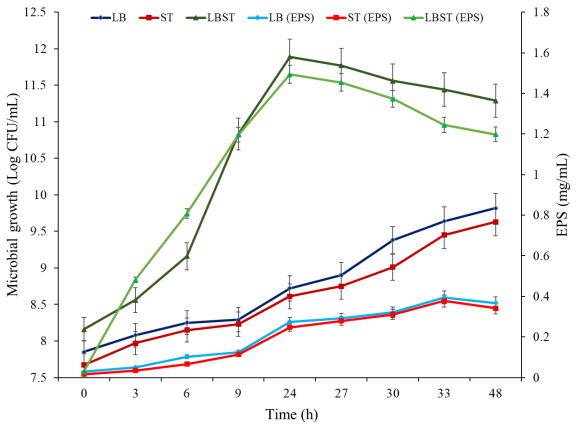

Figure 1. Cell growth and EPS production in the three fermentation systems for *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 (LB), *Streptococcus thermophilus* SY-102 (ST), and the co-culture of bacteria (LBST).

growth (Hernández-Rosas *et al*., 2021). Therefore, even though SW has been recognized as a suitable culture medium for microbial growth (Macwan *et al*., 2016) we evaluate the effect of its physicochemical composition in both the LAB growth and the EPS production in our three fermentation batches (Figure 1).

From the obtained lactose (44.56 ± 0.42 mg/mL) and protein (5.8 ± 0.14 mg/mL) concentrations, we calculated their C/N ratio to be 7.68. However, we didn't test different concentrations of lactose or protein since the main of this work is to evaluate the microbial development in an SW similar to that usually discarded from cheese factories in Mexico (Mazorra-Manzano *et al*., 2019).

The maximum EPS production for each system was 0.39 mg/mL for the LB system, 0.37 mg/mL for the ST system and 1.49 mg/mL for the LBST system. These findings show a different EPS production than those reported by other authors for the same LAB (Korcz *et al*., 2021; Srinivash *et a*l., 2023). This yield variation could be associated with the temperature (Oleksy-Sobczak *et al*., 2020), the carbon source (Srinivash et al.,2023) or the nitrogen source (Korcz et al., 2021) of SW. Another important factor to consider is the C/N ratio. Gayosso-Sánchez *et al* (2024) showed that a smaller (3 and 5) C/N ratio favored the production of EPS in *Enterobacter soli* and attributed that to the effect of this ratio on the enzymatic system responsible for the EPS production.

As can be seen in the microbial growth curves (Figure 1), both the LB and ST monocultures exhibited slow and limited development compared to the LBST system, which achieved its maximum cell concentration after 24 hours of incubation. This enhanced growth in the LBST system can be attributed to the symbiotic relationship between the two bacteria used, wherein *L. bulgaricus* cell wall proteases generate small peptides and free amino acids that support the growth of *S. thermophilus*. In return, *S. thermophilus* produces formic acid and CO_2, which promote the growth of *L. bulgaricus* (Smid and Lacroix, 2013). Calculations of the growth rate (μ) revealed values of 0.052 h^{-1} for the LB system, 0.049 h^{-1} for ST, and 0.123 h^{-1} for the LBST system.

These findings highlight the symbiotic interaction between both strains. In a medium where whey proteins are the primary nitrogen source, the LB monoculture develops faster than ST. These results align with those documented by Liu *et al.* (2016). Other studies, such as those by Sebastián-Nicolas *et al.* (2021) and Olvera-Rosales *et al.* (2023), underscore the protocooperation between lactic acid bacteria alongside *S. thermophilus*, emphasizing how this combination enhances growth rates over monoculture fermentation systems.

Similarly, the LBST fermentation system exhibited the highest exopolysaccharide (EPS) production throughout the fermentation period. The production of EPS correlates with the observed growth patterns, a phenomenon documented in various microorganisms, including LAB (Shukla *et al.*, 2019). However, in all studied fermentation systems, EPS production declines after reaching peak concentrations (0.39 mg/mL for LB, 0.37 mg/mL for ST, and 1.29 mg/mL for LBST). This trend is consistent with observations in other fermentation systems employing EPS-producing lactic acid bacteria, where microbial glycosyl hydrolases may contribute to polymer degradation (Tang *et al.*, 2017) and other authors have mentioned that EPS are considered a primary energy reserve and can be consumed as a carbon source for the microorganism (Gayosso-Sánchez *et al*., 2024).

The results obtained imply a link between growth and EPS production within each fermentation system; correlation analyses were conducted between these variables to verify this. The LB fermentation system showed a correlation coefficient of 0.92, ST had 0.91, and LBST exhibited 0.94. All systems demonstrated correlations exceeding 0.9, confirming a direct relationship between growth and EPS production. While influenced by factors like temperature, controlled acidity, and oxygen levels, this correlation has been consistently observed in various bacterial strains known for EPS production (Ruijgrok *et al.*, 2024).

3.2 pH changes

Lactic acid bacteria inherently prefer whey fermentation due to their high lactose content, a carbon source for their growth. They utilize various metabolic pathways, prominently generating lactic acid as an essential product (Bintsis, 2018). Therefore, the quantification of pH in SW directly correlates with microbial growth. The pH change over time is

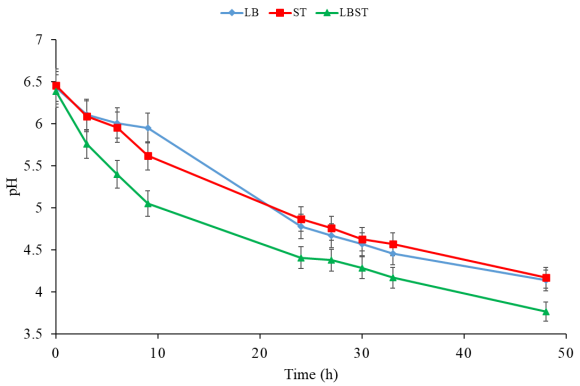

Figure 2. pH changes in the three fermentation systems for *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 (LB), *Streptococcus thermophilus* SY-102 (ST), and the co-culture of bacteria (LBST).

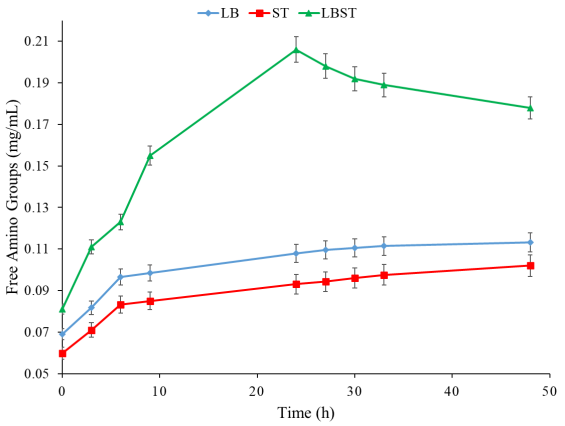

Figure 3. Free amino group concentration in the three fermentation systems for *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 (LB), *Streptococcus thermophilus* SY-102 (ST), and the co-culture of bacteria (LBST).

illustrated in Figure 2.

The initial pH of the three fermentation batches was maintained around 6.5, which was expected since SW has pH values close to those of milk (Shraddha and Nalawade, 2015). The pH decrease observed in each system approached 4, primarily attributed to lactic acid production by the respective cultures in the medium. This phenomenon is well-documented in various LAB cultivated in whey protein media (Solieri *et al*., 2022). However, the acidification observed in this study was more gradual than other researchers' findings (Soriano-Perez *et al.*, 2011). These results may be linked to each bacterial species-specific nutritional requirements and LAB efficiency in converting lactose to lactic acid (Østlie *et al.*, 2005).

Furthermore, whey proteins and the formation of peptides resulting from their hydrolysis influence pH changes. It has been documented that terminal amino and carboxyl groups of peptide chains exert a buffering effect against rapid pH fluctuations in the medium (Mennah-Govela *et al.*, 2019).

3.3 Proteolytic profile

A critical factor influencing microbial growth and development is their metabolic capacity to hydrolyze nitrogen sources within the medium for the acquisition of essential amino acids (Liu *et al*., 2020). To assess this proteolytic activity, the TNBS technique was employed to analyze the release of free amino groups. Established for its efficacy in serum protein analysis due to its interaction with cysteine residues (Spellman *et al*., 2003), this method was utilized for the present investigation. The results of this analysis are presented in Figure 3.

Both the LB and the ST system followed a similar trend in the release of free amino groups, with values remaining low but constant throughout the process. On the other hand, the LBST system increased rapidly from the onset of fermentation, peaking at 24 hours and with significantly higher concentrations observed at the end of the fermentation compared with the monoculture systems ($p < 0.05$). This pattern parallels the trends observed in cell growth, prompting a correlation analysis between these variables for each system. The results yielded correlation coefficients of 0.72 for LB fermentation, 0.82 for ST, and 0.97 for LBST. These findings confirm a stronger relationship, indicating greater nitrogen utilization in the medium when fermented in co-culture with both bacterial strains under study. Similar findings have been documented previously.

This process entails the release of peptides concurrently with lactic acid production, indicative of microbial metabolic activity in SW. Both *L. bulgaricus* and *S. thermophilus* have proteolytic systems involving a cell envelope protease (PrtB and PrtS respectively) that begin the hydrolysis process in the media. Both enzymes are pH-dependent (PrtB: 5.2 - 5.8, PrtS: 5.5 - 8.5), and thus, it is expected that their proteolytic activity changes as the medium becomes more acidic (Fernandez-Espla *et al*., 2000; Rodríguez-Serrano *et al*., 2018; Bendig *et al*., 2023).

Concerning monoculture growth, Pescuma *et al.* (2015) evaluated the proteolytic capabilities of various LAB in whey protein media, with *L. bulgaricus* strains showing greater proteolytic activity than *S. thermophilus*. Specifically, *L. bulgaricus* preferred to hydrolyze β-lactoglobulin, achieving a 12% hydrolysis rate after 24 hours of incubation. In contrast, *S. thermophilus* exhibited a stronger affinity for α-lactalbumin, hydrolyzing 23% within the same timeframe. These differences may be attributed to

distinct proteolytic systems, nutritional requirements, and adaptations of each microorganism to available nitrogen sources (Olvera-Rosales *et al.*, 2023).

In studies focusing on *L. bulgaricus* growth on whey proteins (Liu *et al.*, 2012), it has been noted that this bacterium exhibits a robust capability for hydrolyzing whey proteins, attributed to the expression of cell wall proteases that enhance its enzymatic activity. However, further research is needed to elucidate this phenomenon's precise mechanisms. Regarding *S. thermophilus*, its proteolytic behavior in the presence of whey proteins has been investigated in various fermentation systems (Sebastian-Nicolas *et al.*, 2021; Olvera-Rosales *et al.*, 2023). Studies have reported up to 0.69 mg/mL of free amino groups, notably achieved in media enriched with high concentrations of whey proteins (10% w/v). In contrast, this study focused on utilizing whole whey (aiming to enhance its utilization), achieving a protein concentration of 0.5%, thereby limiting the potential presence of free amino groups. Other studies have reported that the higher free amino group concentration observed in the coculture system not only is the sum of the amino acids released from each bacteria but also could be because the interaction between the two species triggers metabolic changes in the strains, resulting in the rearrangement of metabolic fluxes and stimulation of individual biosynthetic pathways in coculture that is not present in the monocultures (Ulmer *et al*., 2022).

Finally, as EPS is a significant product generated during fermentation, an exploration of its correlation with proteolysis was undertaken. This relationship has been previously documented for other compounds produced in analogous processes (Settachaimongkon *et al*., 2014). The analysis revealed correlation coefficients of 0.65 for LB fermentation, 0.75 for ST, and 0.89 for LBST. These findings suggest that in the coculture system, bacterial protocooperation enhanced nitrogen utilization within the medium, resulting in increased EPS production. This positive correlation observed in the LBST batch was also found by Amani *et al*. (2016) while measuring the characteristics of mixing different strains of *L. bulgaricus* and *S. thermophilus* growing in milk, although they mention that not all the possible combinations have this positive correlation and that this could impact the rheological characteristics of the EPS-medium system. Further studies on products made with the SW-EPS could prove or refute that.

Conclusion

Fermentation of whey by filamentous lactic acid bacteria, specifically *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772 and *Streptococcus thermophilus* SY-102 in coculture enhance protein hydrolysis, lower pH, and promote exopolysaccharide production. This process also proves conducive to maintaining and accumulating bacterial biomass, offering advantages in producing biogenically derived materials through synergistic utilization of these microorganisms' distinct carbohydrate metabolism and proteolytic systems. Such research represents a pivotal step towards obtaining valuable materials for applications in the food, pharmaceutical, and materials science industries, utilizing waste streams from the cheese industry and expanding their utilization. The outcomes highlight ways for further exploration, including the characterization of materials and peptides obtained through fermentation using cooperative interactions among lactic acid microorganisms.

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