Enhancement of waste from the dairy industry: production of a fermented product based on Buffalo cheese whey with nutraceutical properties.

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1. Summary

Buffalo cheese whey, a byproduct of mozzarella and ricotta production, has emerged as a valuable substrate in biotechnological research due to its remarkable nutritional and functional properties. Composed primarily of water (93–94%), it contains lactose, proteins, lipids, vitamins, and bioactive peptides, making it a valuable resource for transformation into high-value products. Once considered waste, cheese whey is now recognized as a secondary raw material with applications in food science, biotechnology, and sustainable waste management. The recovery and valorization of this by-product using sustainable waste management processes has become a challenge of increasing importance for the promotion of a circular economy. The use of biotechnological processes such as fermentation, which exploits the metabolism of selected strains, is still one of the most studied methods to be applied for this purpose. The microbial community inhabiting cheese whey, influenced by the origins of the milk, the working environment and the addition of starter cultures during production, is important because it makes the product unique and plays a pivotal role in the transformations that occur during cheese making process. Bacteria, yeasts and fungi are key microbial agents capable of synthesizing or increasing molecules that characterize the flavor, aroma, texture and taste of the product, but they have also an impact on the production of bioactive peptides, which have a wide range of beneficial effects on the human body. High-throughput techniques such as metabarcoding analysis have facilitated the identification and characterization of microbial diversity in Cheese Exhausted Whey (CEW) and Ricotta Cheese Exhausted Whey (RCEW), revealing species with probiotic and prebiotic potential. Complementary culturomics approaches have led to the isolation of bacterial and yeast strains that have been studied for their phenotypic characteristics and effects on the human organism as probiotics. Some of these strains were employed in fermentation processes, demonstrating the capacity to enhance bioactive peptide concentrations while maintaining the substrate's natural origin. The resulting bioactive peptides exhibit diverse health benefits, including antioxidant properties, modulation of immune responses, oxidative stress mitigation, and enzymatic activity regulation. Their therapeutic potential extends to managing chronic conditions such as hypertension, diabetes, and cardiovascular diseases. By leveraging the metabolic activity of microorganisms, cheese whey can be transformed into innovative, high-value nutraceutical products, contributing to sustainable solutions for the disposal of dairy industry byproducts while addressing global health and environmental challenges.

2. Synopsis

The progressive increase in the global population has led to a rising demand for food, which in turn results in an increased production of waste from the food industry. Proper management and disposal of such waste are crucial to prevent environmental pollution and its potentially severe consequences on human health (FAO, 2019). Beyond disposal, the recovery and valorization of food industry by-products have become essential strategies for promoting sustainable food production and promoting an efficient circular economy. This study focuses on the dairy industry, which generates significant quantities of by-products every year. Due to their high content of organic molecules, these by-products are considered highly polluting. However, they also represent a valuable source of bioactive compounds with potential applications not only in human health but also in other industries (Pires et al., 2021). Specifically, this research aims to explore an alternative and sustainable biotechnological method for recovering Campania buffalo milk whey, a by-product of Mozzarella di Bufala Campana DOP production. This PDO (Protected Designation of Origin) product is a hallmark of Southern Italy, strictly regulated to enhance its quality and preserve the unique characteristics of buffalo milk. Buffalo cheese whey is primarily composed of water but also contains bioactive molecules such as proteins, peptides, vitamins, and fatty acids. Currently, it is predominantly utilized in protein supplements for athletes, often in powdered form, while its application in cosmetic formulations is steadily increasing (Smithers, 2008). Among the various biotechnological recovery methods, fermentation presents a promising approach, although further studies are needed to validate its effectiveness and scalability at an industrial level. Fermentation, mediated by bacteria and yeasts, facilitates the bioconversion of substrates into secondary metabolites with enhanced functional and biological properties.

The objective of this study was to develop a nutraceutical product—a term derived from "nutrition" and "pharmaceutical"—indicating natural bioactive molecules capable of exerting beneficial effects on human health. Such products have been investigated for their potential role in managing chronic diseases and other health conditions (Pandey et al., 2010). Valorizing buffalo milk whey not only contributes to reducing waste but also supports economic and scientific advancements, as demonstrated by prior research that has led to publications and the filing of a European patent on its bioactive potential (Patent No. EP3988169A1).

In this study, buffalo whey from Campania was characterized for its microbiological composition, revealing a unique dairy-associated microflora shaped by environmental and nutritional factors. Using metabarcoding analysis and direct microbial isolation, several bacterial and fungal species with known and potential probiotic properties were identified. Among the isolated bacterial strains, *Leuconostoc mesenteroides* RCEW1, *Enterococcus faecalis* CEW1, and *Enterococcus durans* RCEW2 were tested for their probiotic properties. Each strain exhibited distinct beneficial traits, with potential applications in encapsulated probiotic formulations to enhance gastrointestinal passage and stability (Papadimitriou et al., 2015). However, further studies are required to assess the pathogenic potential of *Enterococcus* species due to the possible presence of virulence factors (Franz et al., 2011).

The yeast strains, *Saccharomyces cerevisiae* strain 25, *Saccharomyces cerevisiae* strain 26 and *Kluyveromyces marxianus*, in addition to their probiotic characteristics, were studied for their phenotypic and robustness characteristics and for the potential prebiotic effect on the viability of known probiotic strains. Yeasts were selected to be used in single culture for the fermentation of buffalo cheese whey under controlled conditions. This is due to the good results regarding their probiotic, prebiotic characteristics and their greater suitability to ferment a substrate of this type. The bioactive potential of buffalo whey was assessed before and after fermentation, focusing on nutraceutical properties such as antioxidant activity, antihypertensive effects (ACE inhibitory activity), antidiabetic properties (DPP-IV inhibitory activity), and B-vitamin content. The results indicated that fermented whey exhibited superior functional properties compared to unfermented whey, with specific strains enhancing distinct bioactive attributes. These findings reinforce the potential of fermentation as a viable strategy for transforming dairy by-products into high-value functional foods.

3. Aims of the work

The primary aim of this research is to develop innovative strategies for the valorization of Buffalo Campania cheese whey, with applications in biotechnology, food science, and environmental sustainability, focusing on its transformation into high-value-added products. Cheese whey, a byproduct of the dairy industry, is abundant and rich in organic components such as proteins, peptides, lactose, lipids, vitamins, and minerals, many of which exhibit bioactive properties. While its nutritional composition makes it a valuable resource, its high organic content also poses environmental challenges, emphasizing the need for effective recovery and valorization strategies (Smithers, 2008; Prazeres et al., 2012). This study aims to fully exploit the potential of cheese whey, contributing to waste reduction and the creation of sustainable bioproducts. With its diverse biochemical composition, cheese whey is increasingly recognized for its potential applications, particularly as a substrate for microbial fermentation and as a functional food ingredient.

This study was conducted using advanced genomic techniques to analyze Buffalo Campania cheese whey, such as metabarcoding—a high-throughput sequencing method that allows for the identification and characterization of microbial communities by targeting specific genetic markers (Porter & Hajibabaei, 2018) and providing a detailed snapshot of microbial diversity (Caporaso et al., 2010). This approach involves amplifying the specific region of 16S rRNA for bacteria and the internal transcribed spacer (ITS) region for fungi and yeasts, followed by sequencing and bioinformatics analysis (Tedersoo et al., 2015). This approach was complemented by a culturomic strategy, which involved plate isolation techniques to validate and expand the database of microbial species inhabiting Buffalo Campania cheese whey. Among the isolated species, those belonging to the genera *Enterococcus*, *Leuconostoc*, *Saccharomyces*, and *Kluyveromyces* provided valuable insights into the microbial diversity present in cheese whey. The scientific community has highlighted the significant technological and functional potential of different strains belonging to these genera. Tests on probiotic and prebiotic activity, phenotypic characteristics, and robustness enabled the classification of their suitability and potential applications in fermentation processes. However, certain strains, particularly those from the *Enterococcus* genus, require further investigation due to their potential pathogenic traits (Franz et al., 2011). Despite this, their properties make them promising candidates for use as dietary supplements (Sánchez et al., 2017).

The isolated yeast strains underwent phenotypic characterization and robustness studies, after which they were employed as starter cultures for the fermentation of Buffalo Campania cheese whey. This process aimed to enrich the product with bioactive compounds and molecules. The fermented Buffalo Campania cheese whey

was subsequently analyzed for its nutraceutical properties, including its potential antioxidant, antihypertensive, antidiabetic, and immunomodulatory activities, as well as its content of B-group vitamins (Nagpal et al., 2012).

The originality of this work lies in the unique microbial and molecular composition of Buffalo Campania cheese whey, which makes it an attractive product in the markets for dietary supplements, health-promoting products, and specialized nutrition (Smithers, 2008; Macori et al., 2018). This study highlights the unexplored potential of Buffalo Campania cheese whey as a valuable source for the isolation of probiotics and as substrate for the yeast fermentation, to bridge the gap between sustainable waste management and the development of innovative bioproducts.

4. Buffalo cheese whey: microbial characterization, isolation and identification of potential probiotic strains and phenotypic characterization

Introduction

The growing global emphasis on sustainability and waste management has underscored the necessity of innovative approaches for utilizing by-products in various industries, including dairy. Cheese whey, a by-product of cheese production, represents a valuable resource with significant potential for biotechnological applications. This project investigates the recovery and valorization of two types of buffalo cheese whey named respectively Cheese Exhausted Whey (CEW), the by-product of Buffalo mozzarella cheese production process and Ricotta Cheese Exhausted Whey (RCEW) obtained from ricotta cheese production process, through biotechnology processes to develop nutraceutical products with enhanced functional properties.

Historically, Cheese Whey, has been considered as waste due to its high Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD), necessitating regulated disposal to avoid environmental damage (Maragkoudakis et al., 2016). However, its rich biochemical composition—including lactose, proteins, minerals, and bioactive compounds—has shifted this perception, highlighting its potential as a "secondary raw material" (Brandelli et al., 2015). Buffalo milk whey from Campania has been studied and characterized for its properties and nutraceutical composition and a European patent has been filed on it with the name “Nutraceutical composition comprising buffalo milk whey and use thereof in the reduction of the intestinal permeability” (EP 3988169 A1). **Table 1** reports the data present in the Tenore and Novellino patent (2022) which shows the composition of Campana buffalo cheese whey which is approximately 93-94% water and retaining about 55% of milk nutrients, buffalo cheese whey is particularly notable for its high lactose content (4g/100g) which makes it a promising candidate for fermentation processes, for the presence of protein fractions (1,1g/100g), minerals (0,5g/100g includes calcium and phosphorus), lipids (1,2 g/100g including phospholipids), vitamins (A, B, D) and bioactive peptides, which confer multiple health benefits such as antioxidant, immunomodulatory, and antihypertensive effects (Hernández-Ledesma et al., 2011; Ashaolu, 2020).

Chemical-physical composition	Cow's milk	Campana Buffalo milk	Campana Buffalo cheese whey
Water	87-89 %	81,5 %	93-94 %
Proteins	3,2-3,5 %	4-5 %	1,1 g/100g
Lactose	4,7-5 %	5 %	4g/100g
Fats	3-4 %	7,2-9,6 %	1,2g/100g
Calcium (mg/100g)	120-130	180-240	180-240
Mineral salts	0,7-0,8 %	0,9-1,0 %	0,5g/100g

Table 1 Average chemical-physical composition of cow's milk, Campana buffalo milk and Campana Buffalo cheese whey. Consorzio Per La Tutela Del Formaggio Mozzarella Di Bufala Campana, Tenore, G. C., & Novellino, E. (2022). *Nutraceutical composition comprising buffalo milk whey, and use thereof in the reduction of the intestinal permeability* (EP 3988169 A1). European Patent Office.

Due to the high quantities of cheese whey produced by the dairy industry, and to overcome the problem of disposal, different techniques are used to convert whey into value-added products. For this aim, cheese whey can be processed directly to produce whey powder, whey protein, whey permeates etc., or it can be fermented to obtain new functional products with functional/nutritional proteins and bioactive peptides (Yadav et al., 2015), These peptides, composed of 3-20 amino acid residues, remain inactive within parent proteins but can be released through enzymatic hydrolysis, gastrointestinal digestion, fermentation by Generally Recognized as Safe (GRAS) bacteria and yeasts, or during food processing (Mora & Hayes, 2015). The activity and functionality of bioactive peptides can be affected by various factors such as process conditions, structure, sequence, amino acid composition, hydrophobicity, molecular weight and pH (Chen et al., 1998, de Castro and Sato, 2015). To exert a positive health effect, peptides must cross the intestinal barrier and survive enzyme degradation in the GI tract following consumption. They are considered therapeutic agents for treating and preventing certain diseases; they can have a wide range of beneficial effects on immune system (immunomodulatory), nervous system (opioid effect), cardiovascular system (anti-hypertensive, anti-thrombotic, anti-inflammatory), gastrointestinal track system (antioxidant peptides, anti-ulcerative, satiety effect), metabolic health (Hypocholesterolemia e Hypotriglyceridemic, Glucose control, Insulinotropic effect) (Mehra et al., 2021). Furthermore, the lactic acid and bioactive compounds present in cheese whey are of particular interest for their potential to support gut health by promoting the growth of beneficial microbiota. The potential of cheese whey as a functional food ingredient is particularly significant given the growing interest in sustainable food production and circular economy practices.

This work explores microbial diversity of Cheese exhausted whey (CEW) and Ricotta cheese exhausted whey (RCEW), with a focus on identifying and characterizing

bacterial and yeast strains through the metabarcoding analysis and culturomics. This is particularly important because the microbial community present in cheese whey can significantly influence its potential applications, such as in probiotic or nutraceutical formulations. The composition of the microflora is not only influenced by the environmental conditions of the dairy or from the origin of the milk but also by the addition of selected starter cultures which play an essential role in the ripening process. Natural whey starter, added following the production specifications (G.U. No. 258, 6-11-2003), consists of a natural starter resulting from the previous day's preparation contributes to the development of the product's characteristic aromas and flavors through their metabolic activities. High-throughput DNA sequencing enabled the identification and quantification of microorganisms present in a sample without the need for cultivation, providing a detailed snapshot of microbial diversity (Caporaso et al., 2010). This approach involves amplifying a specific region of the DNA barcode, often the 16S rRNA gene for bacteria or the internal transcribed spacer (ITS) region for yeast and fungi, followed by sequencing and bioinformatics analysis (Tedersoo et al., 2015). The resulting data allows for a comprehensive taxonomic classification, revealing both dominant and rare species within the sample. This approach is particularly valuable in microbial community analysis, as it enables the detection of species that are difficult to culture, thus providing a more accurate representation of the microbiome. However, it is important to note that while metabarcoding offers high sensitivity, the results can be influenced by microbial community diversity, sequence read length, intrapopulation sequence and gene diversity, as well as the algorithms and population abundance used for analysis (Ramos-Barbero et al., 2019). Despite these potential limitations, metabarcoding remains a powerful tool for the in-depth characterization of microbial communities in complex samples such as Buffalo Campana cheese whey.

In addition to metabarcoding analysis, a culturomics approach was important for the isolation of bacterial and yeast species; this technique combines traditional culture-based microbiology with high-throughput molecular methods, such as DNA sequencing, to identify and characterize the microorganism's diversity in a sample. By using selective media and optimal growth conditions culturomics enables the isolation of a wide range of microbial species. Once isolated, species are identified through sequencing techniques which provide a more detailed picture of the microbial community (Lagier et al., 2012). Both bacterial and fungal community were studied in this work to this aim. These strains were evaluated for their potential probiotic activity, robustness and their phenotypical characteristics; some of them were selected as starter inoculum for fermentation tests on RCEW with the aim of obtaining a

nutraceutical value-added product, which was subsequently tested to verify its nutraceuticals properties.

Results

Taxonomic profiling of microbial community in the Cheese Exhausted Whey and Ricotta Cheese Exhausted Whey

In CEW the bacterial community is represented by 96% of bacteria belonging to the order Lactobacillales. The bacterial composition of CEW was predominantly characterized by the *Streptococcaceae* family, accounting for 86%, where *Streptococcus* genus was the dominant (72% of bacteria), followed by *Lactococcus* (17 %). *Streptococcus salivarius* (54.8%); *Streptococcus thermophilus* (15.6%), and *Lactococcus lactis* (12.9%) were identified as the primary species. Yeasts and fungi dominated the microbial community where *Saccharomyces cerevisiae* was the predominant species, accounting for 98.14%. The remaining 1.86% consisted of a heterogeneous group of strains, each present in minimal quantities, among which also appeared a strain of *Kluyveromyces marxianus*.

The bacterial profile of RCEW exhibited a different distribution, with 68% of bacteria belonging to the *Lactobacillus* genus and 30% to the *Streptococcaceae* family. Within the *Lactobacillaceae* family, *Lactobacillus delbrueckii* was predominant species, with *Lactobacillus delbrueckii subsp. indicus* accounting for 36.6% of the population. This species is a thermophilic lactic acid bacterium typically isolated from dairy products. It plays a role in milk fermentation, contributing to acidification and exerting many potential probiotic benefits such as surviving gastric conditions, adhere to intestinal cells, and produce antimicrobial compounds (Chaudhari et al., 2024). Notably, *Streptococcus thermophilus* was the sole representative of the *Streptococcaceae* family, constituting 31% of the bacterial community in RCEW. In RCEW fungi and yeast collectively constituted approximately 91% of the microbiota (**Figure 1 panel B**).

Comparative analysis of the sequencing results from the two samples revealed that the number of species decreases in the RCEW than CW giving a lower microbial variety, likely attributable to the heat treatment applied to CW during the Ricotta manufacturing process. The microbial composition analysis revealed significant changes between CW and RCEW samples. A marked reduction in the *Streptococcus* genus was observed, decreasing from 72% in CW to 30% in RCEW. Conversely, *Lactobacillus* demonstrated a considerable increase, rising from 10% in CEW to 68% in RCEW. Among the species identified, *Streptococcus thermophilus* was uniquely present in both sample types. Notably, the *Lactobacillus* species detected in CW were not found in RCEW (**Figure 1 panel A**).

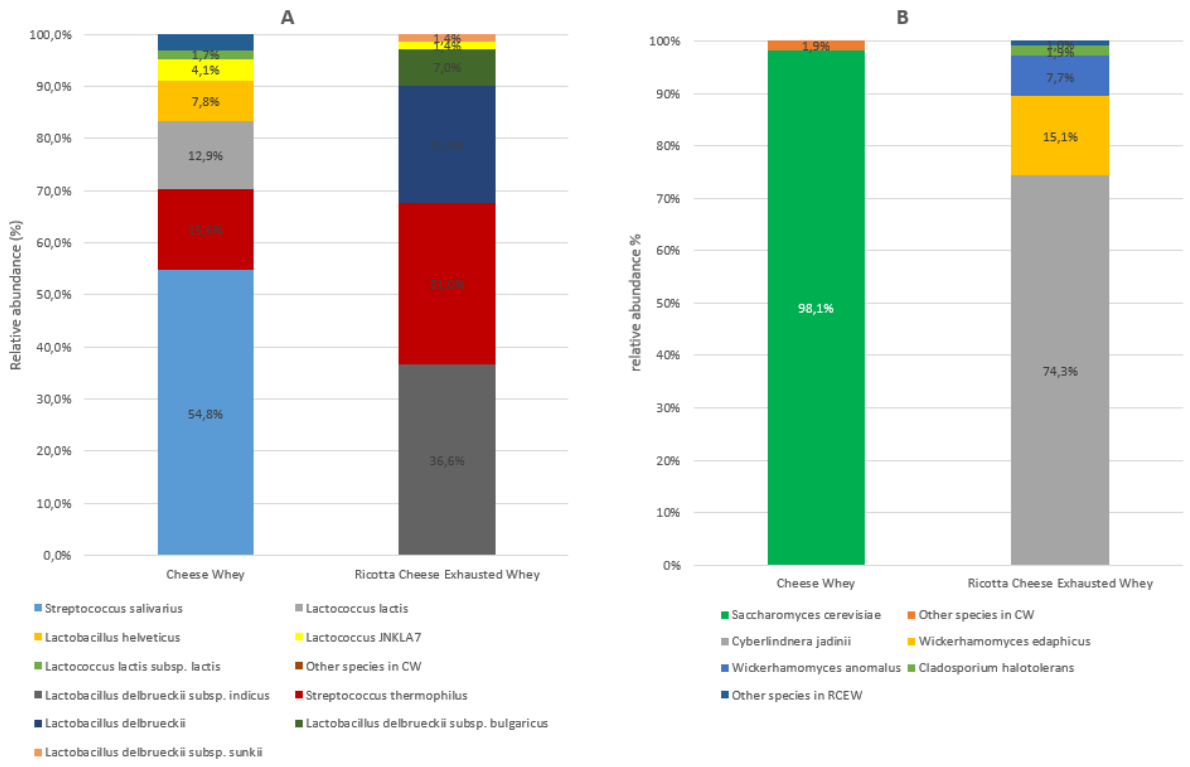


Figure 1. Microorganism diversity of Cheese Whey and Ricotta Cheese Exhausted Whey. Bacteria species-level (**A**) and Yeast and fungi species-level (**B**) expressed as Relative abundance (%); species with Relative abundance < 1% are collected in "Other species".

Culturomics approach: Isolation and identification of bacterial strains

CW and RCEW showed a rich and diverse microbial population distinguished and identified through the application of different growth temperature (28 and 37 °C) conditions on MRS, rich medium which promote the growth of lactic acid bacteria and YPD which promote the growth of fungi and yeasts. **Table 2** showed a different microbial load between the two samples with RCEW having a significantly higher load than CW. CEW shown the carachteristics reported in table 1 with a pH=4.7, while pH of RCEW was 4.3.

	CW		**RCEW**	
	28 °C	37 °C	28 °C	37 °C
MRS	5,37 ± 0,12	5,11 ± 0,1	8,15 ± 0,03	8,05 ± 0,15
YPD	6,14 ± 0,06	6,36 ± 0,02	8,05 ± 0,19	8,04 ± 0,01

Table 2 Microbial load in Cheese exhausted whey (CEW) and Ricotta cheese exhausted whey (RCEW). Results are reported as mean ± sd expressed in Log(CFU/mL).

Among the 26 bacterial colonies isolated by MRS cultivation, 1 from CW (CW1) and two from RCEW (RCEW1 and RCEW2) were analyzed at the molecular level by the amplification of 16S rDNA. The sequences obtained from sequencing were compared with those in the BLASTN database, so that *Leuconostoc mesenteroides* (RCEW1), *Enterococcus faecalis* (CW1) and *Enterococcus durans* (RCEW2) were identified (**Table 3**). In figure 2 is reported the phylogenetic analysis based on the 16S rRNA genes of the above bacteria, to determine relationships among the whey isolates and the other bacterial strains. Among the 31 colonies isolated by YPD, 3 from CW were analyzed at the molecular level by the amplification of internal transcribed spacer (ITS). The sequences obtained from sequencing were compared with those in the BLASTN database, so that *Saccharomyces cerevisiae* CEW25, *Saccharomyces cerevisiae* CEW26 and *Kluyveromyces marxianus* CEW1 were identified (**Table 3**).

Microbial strain	Isolation source	Growth condition
Leuconostoc mesenteroides RCEW1	RCEW	MRS, 28 °C
Enterococcus durans RCEW2	RCEW	MRS, 28 °C
Enterococcus faecalis CW1	CW	MRS, 28 °C
Saccharomyces cerevisiae CW25	CW	YPD, 28 °C
Saccharomyces cerevisiae CW26	CW	YPD, 28 °C
Kluyveromyces marxianus CW1	CW	YPD, 28 °C

Table 3 Strains isolated from Cheese exhausted whey (CEW) or Ricotta cheese exhausted whey (RCEW), cultivated in De Man-Rogosa-Sharpe agar and YPD (2% Glucose) agar and growth condition.

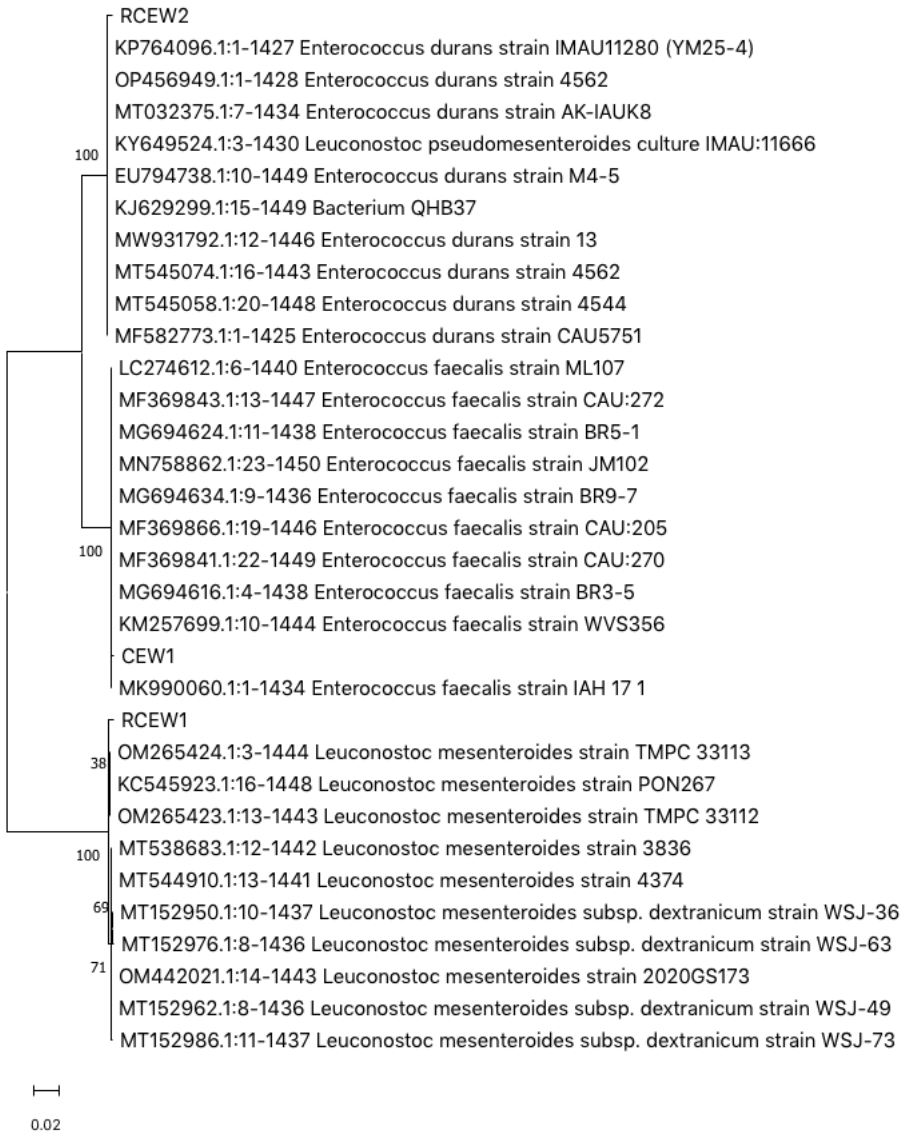


Figure 2. Phylogenetic tree of the bacteria identified from Buffalo cheese whey (CW1, RCEW1 and RCEW2) and the first ten most similar sequence obtained from a Blast analysis for each isolate. The 16S rRNA sequences were aligned, and the phylogenetic relationships among them were constructed using the neighbor-joining analysis through MEGA version 11.0. Numbers at nodes represent bootstrap values obtained with 1000 replications. The GenBank accession numbers for 16S rRNA gene sequences are shown. Bar 0.02 indicates 2 % nucleotide substitution.

Probiotic activity assay on isolated bacteria

Resistance to lysozyme

The high concentration of lysozyme in the oral cavity represents the initial barrier that probiotic strains must survive. Lysozyme works by breaking down the peptidoglycan in bacterial cell walls, weakening their structure. This leads to osmotic imbalance and eventual cell lysis. **Figure 3A** presents the bacterial survival data after 30 and 180 min of treatment with 50 mg/L lysozyme. All strains exhibited substantial resistance to lysozyme after 30 min of incubation (79%, 74%, and 76%, respectively), with survival percentages decreasing after 180 min. Notably, *L. mesenteroides* RCEW1 demonstrated the least resistance (49%) compared to *S. faecalis* CW1 (60%) and *E. durans* RCEW2 (66%) after 3h of exposure. Conversely, **Figure 3B** (100 mg/L of lysozyme) indicates that *Ln. mesenteroides* RCEW1 had significantly reduced viability (35%) after 30 min, that decreas around 6 % after 180 min; whereas *S. faecalis* CW1 maintained a viability of 61% after 30 min and 23% after 180 min. *E. durans* RCEW2 exhibited viability of 58% after 30 min and 32% after 180 min of exposure.

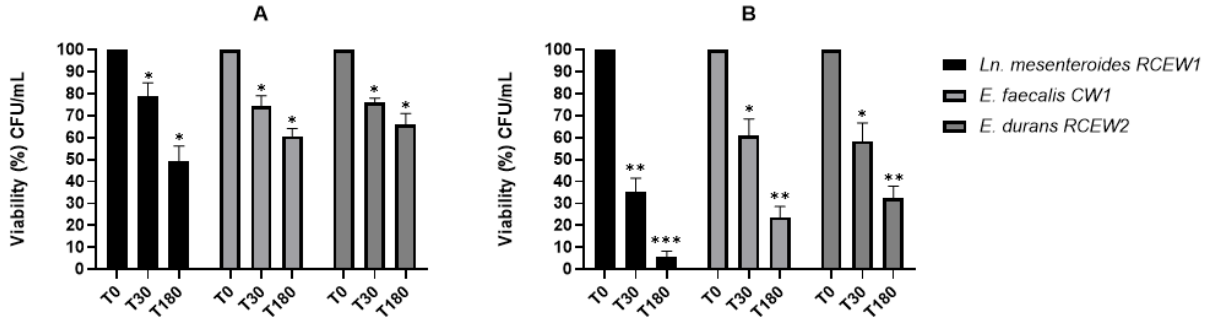


Figure 3. In vitro tolerance to lysozyme. (A) % viability CFU/mL of bacteria after 0, 30 and 180 min of exposure in 50 mg/L of lysozyme SES buffer. (B) % viability CFU/mL of bacteria after 0, 30 and 180 min of exposure in 100 mg/L of lysozyme SES buffer. % viability was calculated by comparing the CFU/mL after 30 and 180 minutes with the CFU/mL at T_0 considering it as 100%. Statistical analysis was performed using Tukey's multiple comparisons test. Significant differences are indicated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

Tolerance to acid pH and bile salts in bacterial strains

Evaluation of resistance to low pH and bile salts is essential for assessing the potential survival of these strains in the gastrointestinal environment. As the passage through the stomach typically lasts approximately 3 hours, the strains were evaluated for their resistance to pH 2 and 3 and for resistance to bile salt concentrations of 0.3 and 0.5%. At pH 3, no significant reduction in bacterial viability was observed (**Figure 4A**). In contrast, at pH 2, the viability of *Ln. mesenteroides* RCEW1 decreased to 55% after 1

hour, reaching approximately 45% after 3 hours (**Figure 4B**) similar to what was stated by Diana et al., 2015. *E. faecalis* CW1 showed 59% viability after 1 hour and 44% after 3 hours. *E. durans* RCEW2 demonstrated a similar trend, with viability decreasing from 62% after 1 hour to 42% after 3 hours in accordance with studies conducted by Hussein et al., 2020. The effect of bile salts was evaluated at two different concentrations, using the same methodology. At a concentration of 0.3%, bacterial cell viability remained satisfactory after 3 hours (**Figure 5A**). However, at 0.5% bile salts (**Figure 5B**), bacterial viability declined substantially, with *Ln. mesenteroides* RCEW1 and *E. faecalis* CW1 showing reductions to 4.34 ± 0.06 and 3.99 ± 0.17 log(cfu/ml), respectively, after 3 hours. *E. durans* RCEW2 demonstrated superior resistance under these conditions compared to what is shown by Comerlato et al., 2022. (**Figure 5B**).

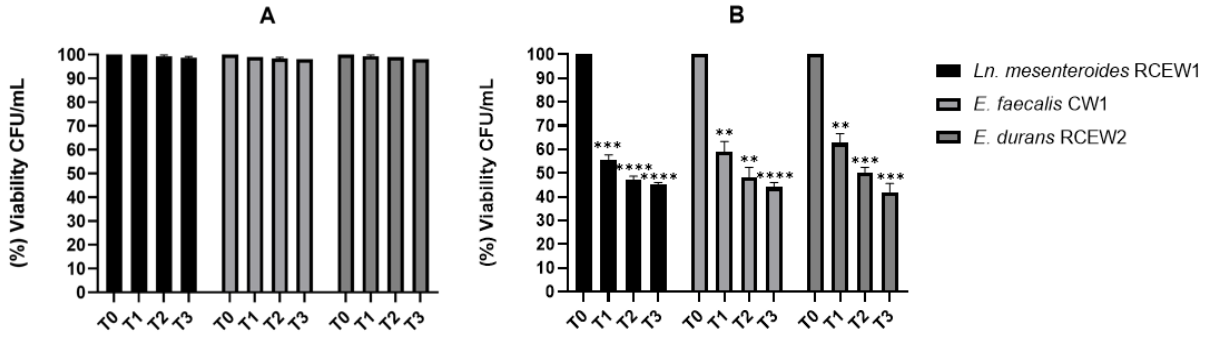


Figure 4 In vitro tolerance to gastric pH. Viable bacteria after 3 h of incubation in PBS adjusted to pH 3 (A) and pH 2 (B). % viability was calculated by comparing the CFU/mL after 30 and 180 minutes with the CFU/mL at T_0 considering it as 100%. Statistical analysis was performed using Tukey's multiple comparisons test. Significant differences are indicated by asterisks (*p < 0.05, **p < 0.01, ****p < 0.001, *****p < 0.0001).*

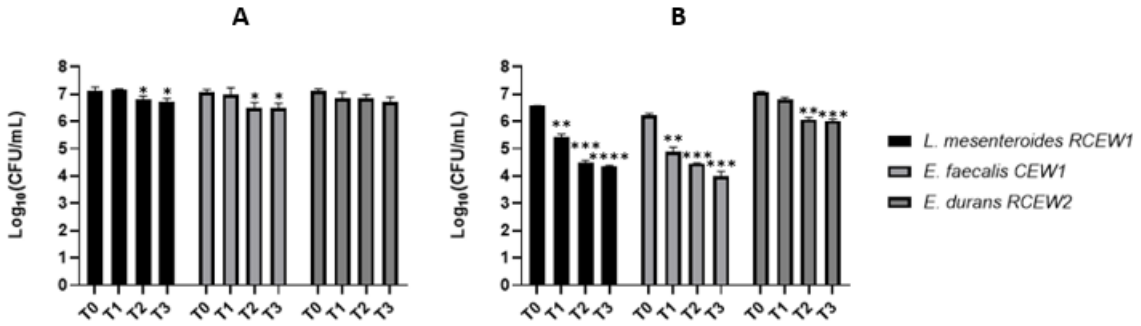


Figure 5 Number of cells (log CFU/mL) following incubation in 0.3% (A) and 0.5% (B) bile salts in PBS (mean ± SD) Statistical analysis was performed using Tukey's multiple comparisons test. Significant differences are indicated by asterisks (*p < 0.05, **p < 0.01, ****p < 0.001, *****p < 0.0001).*

Tolerance test to antibiotics

Antibiotic resistance in potentially probiotic bacteria is a crucial trait, as it ensures their survival in the presence of antimicrobial compounds. Probiotic supplements are often administered alongside antibiotic treatments to support gut microbiota balance. However, the safety of these strains must be rigorously assessed, as they could act as reservoirs of resistance genes, potentially transferring them to pathogens and contributing to the spread of antibiotic resistance (Montassier et al., 2021). Thus, antibiotic resistance is not inherently problematic but becomes a concern when there is a risk of horizontal gene transfer. *Leuconostoc mesenteroides RCEW1*, exhibits resistance to cell wall synthesis inhibitors, including Cefuroxime, Cephalothin, Ampicillin, Cefotaxime, Aztreonam, Vancomycin, and Penicillin G (MS) as shown in Table 3. *Leuconostoc mesenteroides RCEW1* was susceptible to Inhibitors of protein synthesis erythromycin, chloramphenicol, clindamycin and tetracycline, as confirmed by Salvetti et al., 2021. In this category, resistance was shown only for Amikacin while the strain was Moderately Susceptible (MS) to Gentamicin and Streptomycin. Inhibitors of cell wall synthesis were not effective against *Enterococcus faecalis CW1* as shown by resistance to Cefuroxime, Cephalothin, Cefotaxime and Aztreonam (Beta-lactam antibiotics), while was susceptible to Vancomycin and Moderately susceptible to Ampicillin and penicillin G. Resistance for Inhibitors of protein synthesis occurred for Gentamicin, Amikacin and Streptomycin while shown susceptibility to Chloramphenicol, Tetracycline, Clindamycin and Erythromycin. *Enterococcus durans* RCEW2 exhibited resistance to cefuroxime, cephalothin, cefotaxime, and vancomycin, while remaining susceptible to ampicillin and penicillin G. A moderate susceptibility was observed for aztreonam. Among protein synthesis inhibitors, most antibiotics were highly effective, with resistance detected only for amikacin. All tested strains shown susceptibility to Rifampicin (Inhibitors of nucleic acid synthesis) (**Table 4**).

Antibiotic	Interpretative zone diameters		
	Ln. mesenteroides RCEW1	*E. faecalis CW1*	*E. durans RCEW2*
Inhibitors of cell wall synthesis			
Cefuroxime	R	R	R
Cephalothin	R	R	R
Ampicillin	R	MS	S
Cefotaxime	R	R	R
Aztreonam	R	R	MS
Vancomycin	R	S	R
Penicillin G	MS	MS	S
Inhibitors of protein synthesis			
Gentamicin	MS	R	S
Chloramphenicol	S	S	S
Amikacin	R	R	R
Streptomycin	MS	R	S
Tetracycline	S	S	S
Clindamycin	S	S	S
Erythromycin	S	S	S
Inhibitors of nucleic acid synthesis			
Rifampicin	S	S	S

Table 4. Results are expressed as R (resistant), MS (moderately susceptible) or S (susceptible)

Antimicrobial activity in bacterial isolated strains

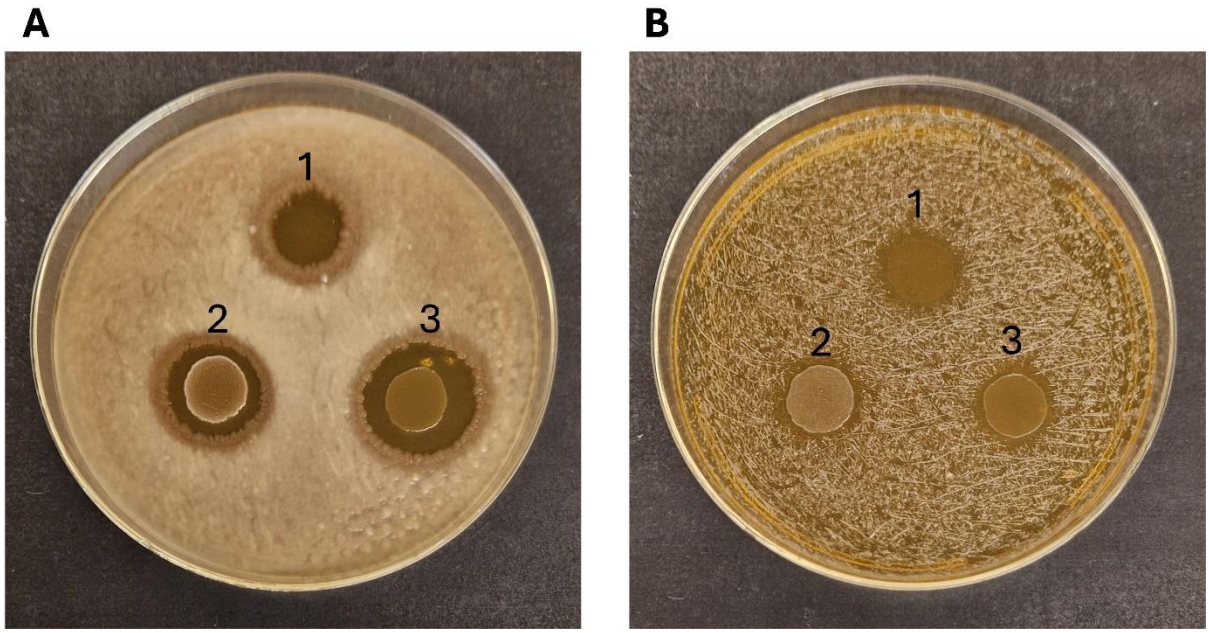


Figure 6. Antimicrobial activity of *Ln. mesenteroides RCEW1* (1), *E. faecalis CW1* (2) and *E. durans RCEW2* (3) towards *B. cereus NCIMB9373* (A) and *S. aureus NCIMB949* (B)

Probiotic strains can exhibit antibacterial activity primarily through the production of bacteriocins that inhibit the growth of pathogenic bacteria. This trait enhances their competitiveness within the gastrointestinal tract and contributes to maintaining microbial balance. The ability to produce bacteriocins is considered a significant probiotic characteristic, offering potential therapeutic applications in preventing and treating infections (Dobson et al., 2012). The inhibition halo is measured and indicates how efficient these strains are in producing molecules that inhibit the growth of Gram+ and Gram-. All the LAB strains tested showed relevant antimicrobial activity towards the Gram-positive *B. cereus* NCIMB9373 (**Figure 6A**) and, to a lesser extent, against *S. aureus* NCIMB949 (**Figure 6B**). No effect was observed on *P. aeruginosa* PA01 and *E. coli* NCIMB11943. The supernatants of the bacterial cultures collected after 24 and 48 h did not show any antibacterial effect. In **table 5**, the halo inhibition expressed in mm.

Microorganisms	*L. mesenteroides RCEW1*	*E. faecalis CEW1*	*E. durans RCEW2*
B. cereus NCIMB9373	11,00 ± 1,02	18,00 ± 1,05	20,00 ± 1,52
S. aureus NCIMB949	17,00 ± 1,15	15,00 ± 1,08	15,00 ± 1,00

Table 5 Inhibition zone diameter of tested strains expressed in mm

Proteolytic activity

The proteolytic activity test was evaluated at 28, 37 and 42 °C for 3 days to test the activity of protease system of the strains. *Streptococcus thermophilus* was used as control.

As shown in **Figure 7**, proteolytic activity in *E. faecalis* CEW1 was best at 28 and 37 °C, showing growth even at 42 °C like *S. thermophilus*. *L. mesenteroides RCEW1* showed lower proteolytic activity values than the previous strain. *E. durans RCEW2* showed a clear proteolytic halo both at 28 and 37 °C. A smaller halo also formed at 42 °C. **Table 6** reported the diameters (mm) of protein zone degradation of milk proteins that indicated the proteolytic activity of tested strains. Under these conditions, although the media did not support the growth of *L. mesenteroides* RCEW1 and *E. durans* RCEW2, these strains still showed modest proteolytic activity.

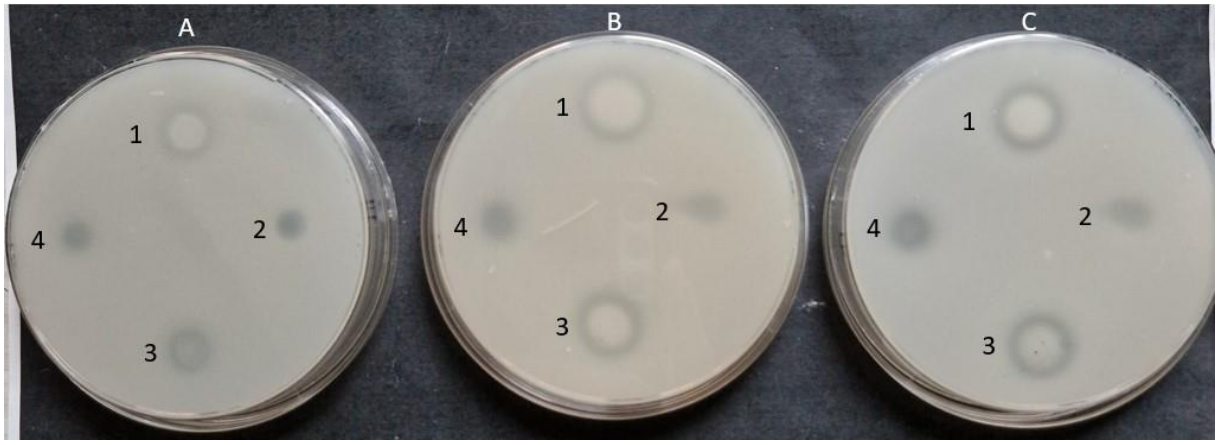


Figure 7 Protease activity test evaluated the diameters of protein zone degradation after 72 h at 42 °C (A), 37 °C (B) and 28 °C (C). (1) *Enterococcus faecalis CEW1,* (2) *Leuconostoc mesenteroides RCEW1,* (3) *Streptococcus thermophilus,* (4) *Enterococcus durans RCEW2*

72 h incubation	*E. faecalis CEW1*	*L. mesenteroides RCEW1*	*S. thermophilus*	*E. durans RCEW2*
28 °C	15	6	13	9
37 °C	16	7	14	8
42 °C	11	7	7	6

Table 6. Diameters of clear zone expressed in mm at different temperatures

Early adhesion and biofilm formation in bacterial isolated strains

Adhesion capacity is a crucial determinant of biofilm formation, influencing bacterial colonization and persistence on surfaces. The evaluation of biofilm formation was carried out following the protocol suggested by Di Domenico et al., 2016 which is based on the measurement of the ability of bacteria to trap magnetic microparticles within the biofilm matrix. The biofilm-forming capabilities of the tested strains were evaluated at 5 and 24 hours. At 5 hours, *Ln. mesenteroides* RCEW1 showed no detectable biofilm formation. At the same time, *E. durans* RCEW2 exhibited limited biofilm production compared to *L. paracasei* I 1688, a strain with minimal biofilm formation capacity at 5 hours, that was employed as a negative control (Truglio et al., 2024).

(**Figure 8**). In contrast, *E. faecalis* CW1 demonstrated significant biofilm formation, comparable to *S. aureus* ATCC 6538, which was used as a positive control for early biofilm production. After 24 hours, most of the tested strains displayed robust biofilm formation, except for *Ln. mesenteroides* RCEW1, which maintained consistently low biofilm formation throughout the assay.

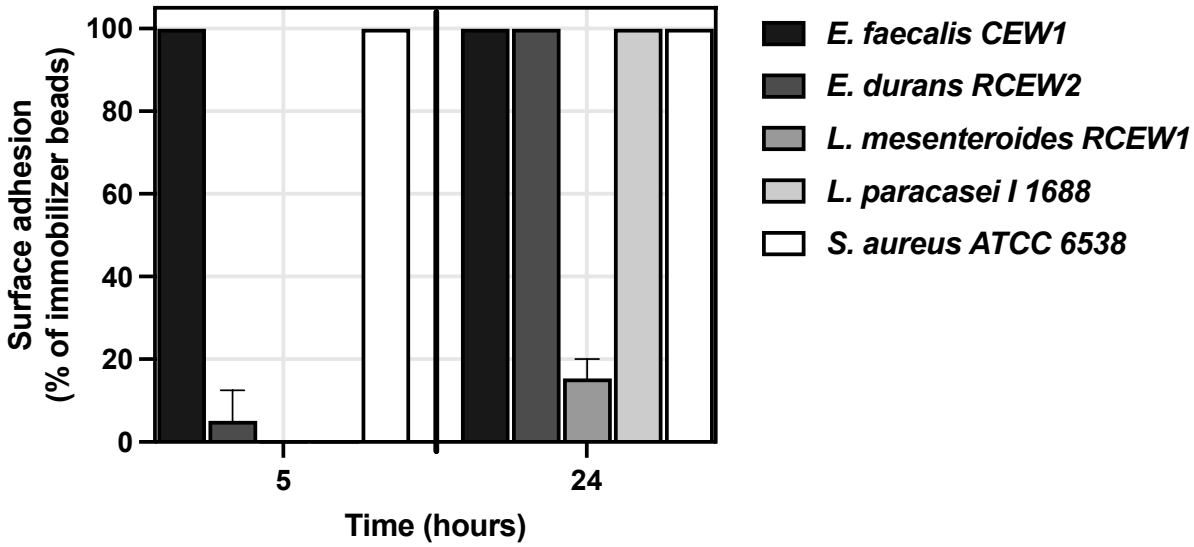


Figure 8 Biofilm formation was assessed at 5 hours (early adhesion) and 24 hours (mature biofilm) using the Clinical Biofilm Ring Test (cBRT) for *Enterococcus faecalis* CEW1, *Enterococcus durans* RCEW2, *Leuconostoc mesenteroides* RCEW1, *Lactobacillus paracasei* I 1688, and *Staphylococcus aureus* ATCC 6538. Error bars indicate the standard error of the mean from three independent experiments, with each experiment conducted duplicate.

Study of the characteristics of yeast strains isolated for use in the fermentation process

Physiological characterization of yeast strains: Influence of temperature on the use of different sugars on yeast growth and lactic acid as stressor

Tested strains showed consistent growth on glucose at both 30 °C and 37 °C (**Figure 9**). The results obtained from galactose were similar, although slightly lower. Notably, *S. cerevisiae* CEW25 and *S. cerevisiae* CEW26 exhibited unexpected growth on 2% S-lactose. The presence of LAC4 genes, encoding for b-galactosidase and the presence of 4 isoforms of LAC12 encoding for permease were assessed on tested strains for the presence of genes that encoded for the enzyme involved in lactose consumption; *K. marxianus* CEW1, shown the presence of 3 isoform of LAC12 (respectively LAC12-1, LAC12-2 and LAC12-3 and LAC4 as shown in **Figure 10**; presence of these 3 isoform for the lactose permease could be changeable from one strain to another belonging to the same species depending on different factors such as gene duplication in sub

telomeric regions, sequence variations affecting lactose transport efficiency or and environmental adaptation (Varela et al., 2017). The absence of these genes was confirmed in both *S. cerevisiae* strains even if some faint bands are present at LAC12-1 and LAC12-4 but at an unexpected position caused by the possible aspecific amplification. The consumption of lactose is probably due to the splitting of a small amount of lactose during the preparation stages of the medium.

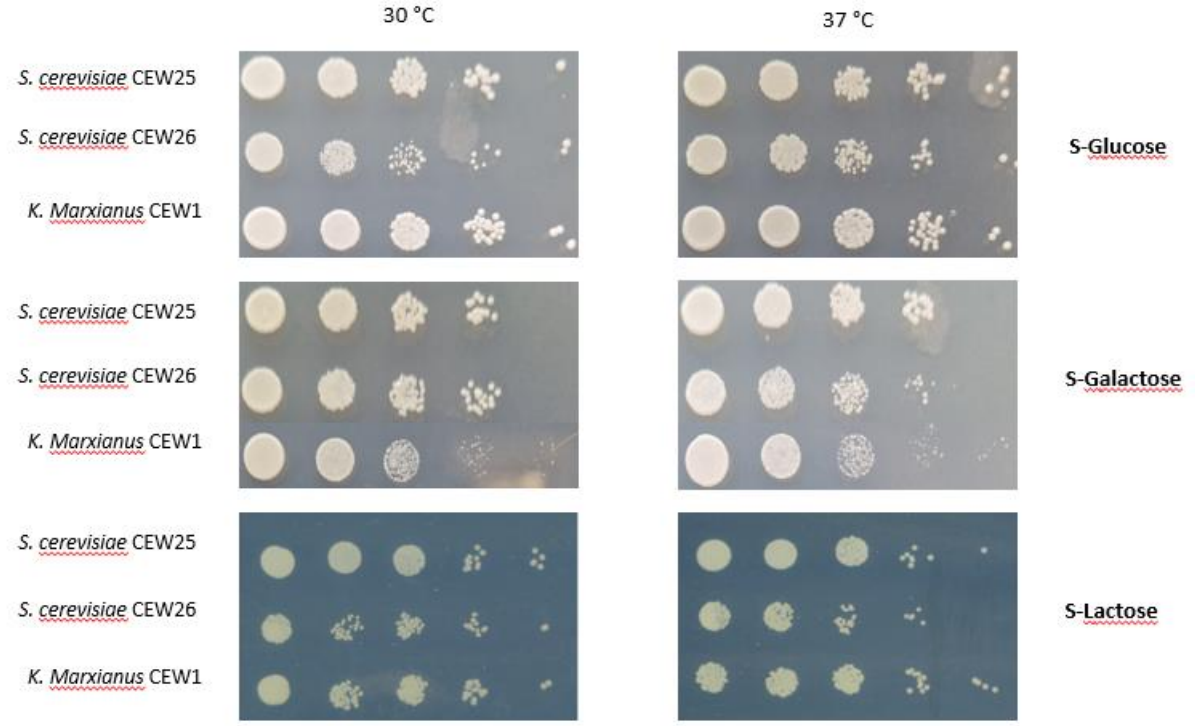


Figure 9 Growth of isolated strain on minimal media with different carbon source; GLU–glucose, GAL-galactose and LAC-lactose at 30 and 37 °C.

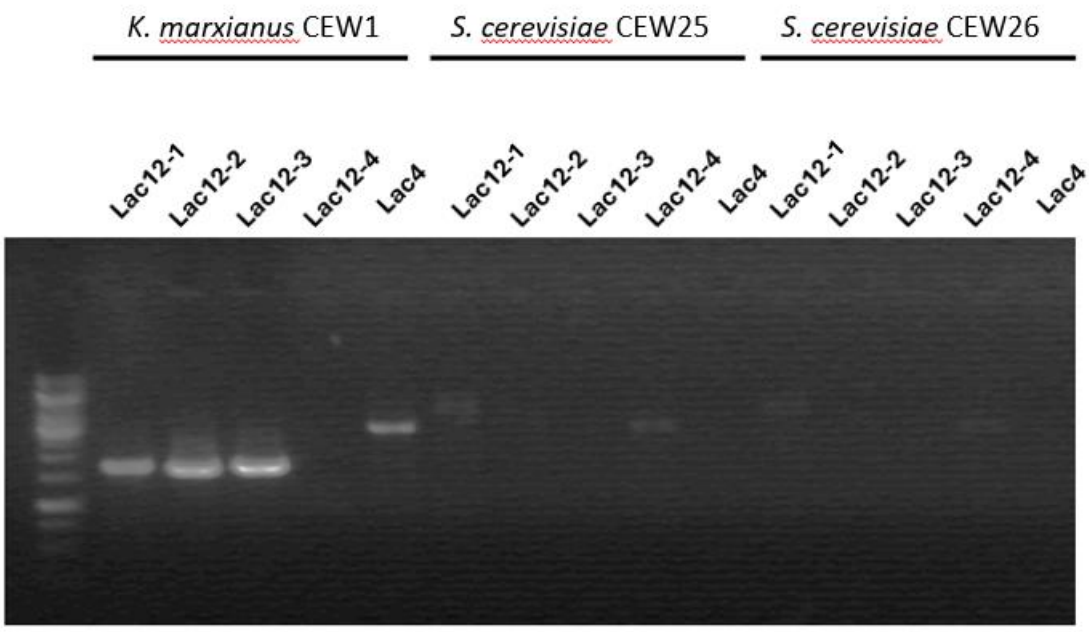


Figure 10 Electrophoresis analysis on agarose gel for screening for the presence of genes responsible for the lactose consumption

Increasing concentrations of lactic acid at pH 3 were added to S-glucose, S-lactose and S-galactose up to a final concentration of 50, 75, 100 and 200 mM to test the resistance of the strains to the presence of this acid which can be a stressor, present in cheese whey due to the metabolism of LAB which could either inhibit or promote the growth of certain species, or influence the flavor and texture of the dairy product (Viljoen, 2001). The acidity of the media containing lactic acid was approximately pH=4. *K. marxianus* CEW1 demonstrated robust growth up to a concentration of 200 mM, regardless of the carbon source tested. In contrast, *S. cerevisiae* CEW25 exhibited growth in the presence of lactic acid up to 100 mM in both S-glucose and S-galalactose media; however, no growth was observed in S-lactose media at any lactic acid concentration. *S. cerevisiae* CEW26 showed growth in S-glucose and S-galactose up to 75 mM lactic acid, with a decline in growth at 100 mM in S-glucose and complete inhibition in S-galactose. Moreover, growth in S-lactose was fully inhibited at all tested concentrations of lactic acid (**Figure 11**).

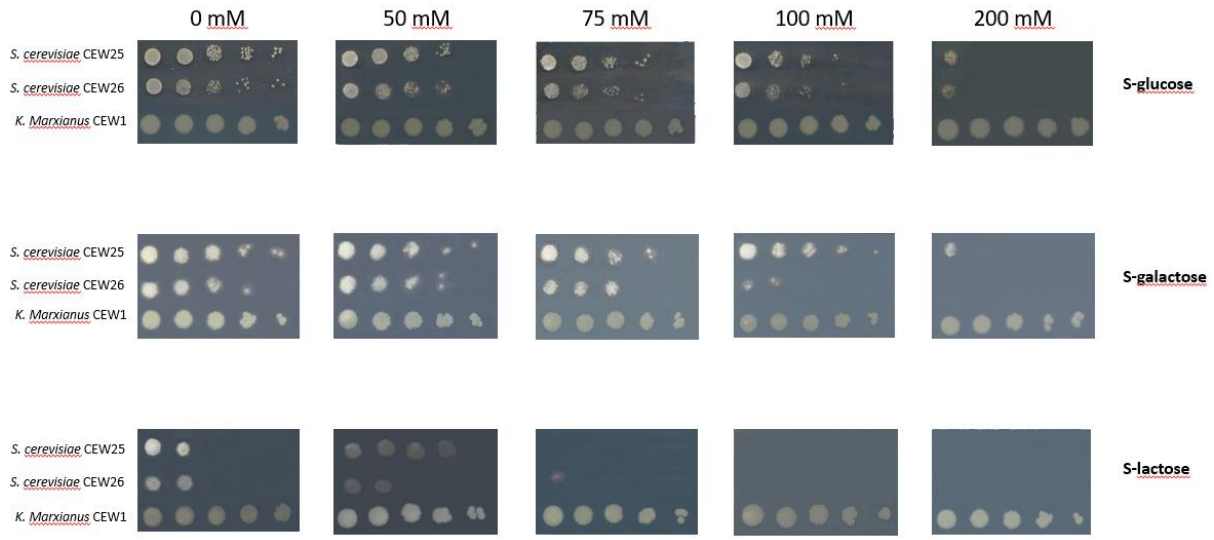


Figure 11 Growth in minimal media with GLU–glucose, GAL-galactose and LAC-lactose with lactic acid.

Kinetics growth and lactose consumption of yeast strains on minimal media and spray-dried cheese whey

Knowing and studying the growth kinetics and lactose consumption of the isolated strains is important to evaluate their suitability for use as starter cultures for the fermentation of substrates containing lactose as a carbon source. *Kluyveromyces marxianus* CEW1 exhibited robust growth in 2% S-lactose medium, reaching an optical density (OD_{600}) of 27.8 after 36 h of growth. Complete lactose consumption was observed after 36 hours of incubation, but already after 18 hours lactose concentration was 0,5 % demonstrating its high efficiency in utilizing this carbon source. The results were compared with those obtained from *K. marxianus* CBS 397 used as a control that consumes all lactose after 26 hours but its growth was slightly lower (OD_{600}=24,8) (**Figure 12A and 12B**). **Figure 12C** shows the production of ethanol by *K. marxianus* CEW1 and *K. marxianus* CBS 397; it is evident that the production begins after 12 hours for both strains and continues to grow steadily up to 20 hours where *K. marxianus* CEW1 reaches a concentration of about 18 g/L while *K. marxianus* CBS 397 reaches a concentration of 25.8 g/L. After 24 hours a rapid consumption of ethanol produced occurs which for *K. marxianus* CEW1 stops after 30 hours up to a concentration of 3 g/L, while for *K. marxianus* CBS 397 the consumption of ethanol stops at about 8 g/L. *Saccharomyces cerevisiae* CEW25 and CEW26 displayed significantly lower growth in 2% S-lac medium, reaching maximum ODs of 0.907 and 0.50, respectively, furthermore, their growth stopped after approximately 12-14 hours; lactose consumption, as expected, slowly decreased from 20 g/L to 15 g/L and stabilized after about 10 hours and remained constant with no further decreased over time suggesting

and confirming a limited ability to utilize lactose as carbon source. *S. cerevisiae* CEN.PK was used as a control since it is known that it does not consume this carbon source. (**Figure 13**). This experiment was repeated under the same conditions using RCEW to evaluate the growth ability of the strains in this complex and rich matrix. Similarly, *K. marxianus* CEW1 showed massive growth in RCEW reaching OD_{600} values above 50 after 24 h and 60 after 32 h of growth with a total lactose consumption after 20 h, confirming its metabolic versatility and ability to utilize a wide range of substrates (**Figure 14A and 14C).** However, *S. cerevisiae* CEW25 and CEW26 showed improved growth in RCEW (**Figure 14B**), reaching OD_{600} of 17 and 16 respectively after 24 h; the lack of lactose consumption over time is confirmed and indicates the presence of additional nutrients in this substrate that supports the growth of these yeasts, even in the absence of efficient lactose fermentation (**Figure 14C**). However, it is important to note that the initial lactose concentration in S-lac and RCEW is lower due to the cleavage that occurred during the preparation phases of the growth medium and heat treatment.

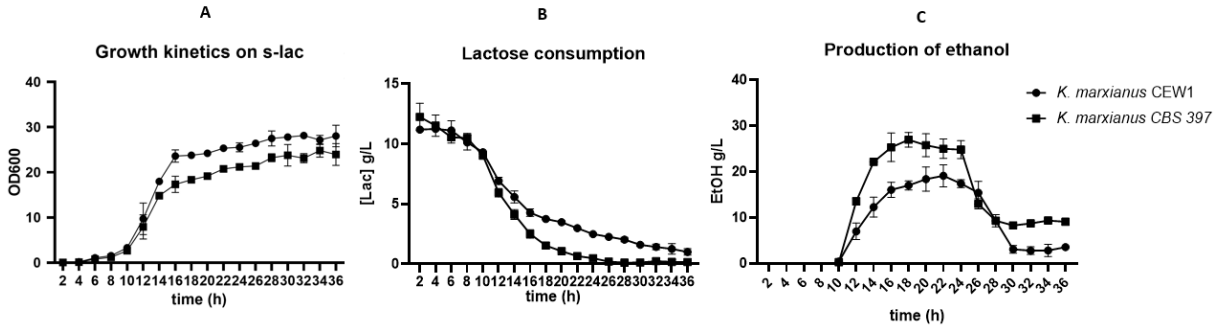


Figure 12 Kinetic growth expressed as OD_{600} (A) and lactose consumption expressed as [Lac] g/L (B) on time (h) of *K. marxianus* CEW1 strain in S-lac 2% medium. Ethanol production (C) expressed as g/L of *K. marxianus* CEW1 in RCEW as a substrate. Results were expressed as mean ± SD. *K. marxianus* CBS 397 was used as control.

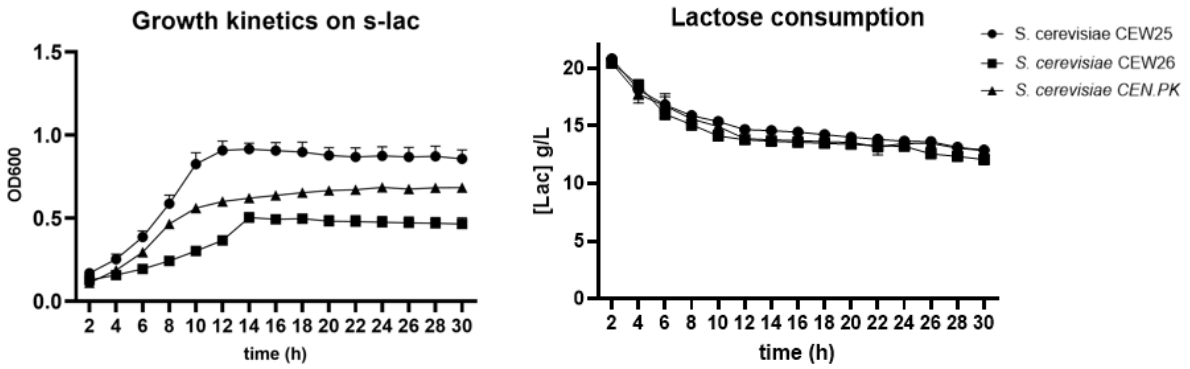


Figure 13 Kinetic growth is expressed as OD_{600} and lactose consumption expressed as [Lac] g/L on time (h) of *S. cerevisiae* CEW25 and CEW26 in S-lac 2% medium. *S. cerevisiae* CEN.PK was used as control.

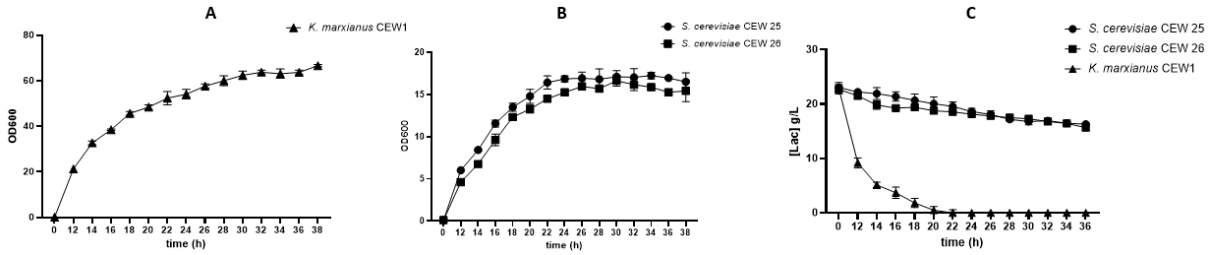


Figure 14 Kinetics growth expressed as OD_{600} of *K. marxianus* CEW1 (**A**) and *S. cerevisiae* strains (**B**) and lactose consumption [Lac] g/L (**C**) of the strains in RCEW as a substrate.

Probiotic and prebiotic assessment on isolated yeast strains

Assessing resistance to acidity and bile salts is important because these traits are required in the classification of a strain as a probiotic to ensure survival during transit through the gastrointestinal system and remain alive as long as possible to exert its beneficial effects on the human organism. Among the yeast strains, *K. marxianus* CEW1 displayed a survival rate around 91% after 3 hours at both pH 3 and pH 2, indicating low sensitivity to low pH. Also *S. cerevisiae* CEW25 exhibited acid tolerance, with a reduction in survival to approximately 95% after 3 hours at pH 3, and to around 94% at pH 2. Similarly, *S. cerevisiae* CEW26 showed the strongest acid resistance, with survival rates remaining above 98% at pH 3, although a slight decrease to approximately 92.5% was noted after 3 hours at pH 2 (**Figure 15A, 15B**). These results highlight the robust acid tolerance of the yeast strains under gastric pH conditions. Yeast strains, in contrast, exhibited good resistance to bile salts at both concentrations (**Figure 16A, 16B**).

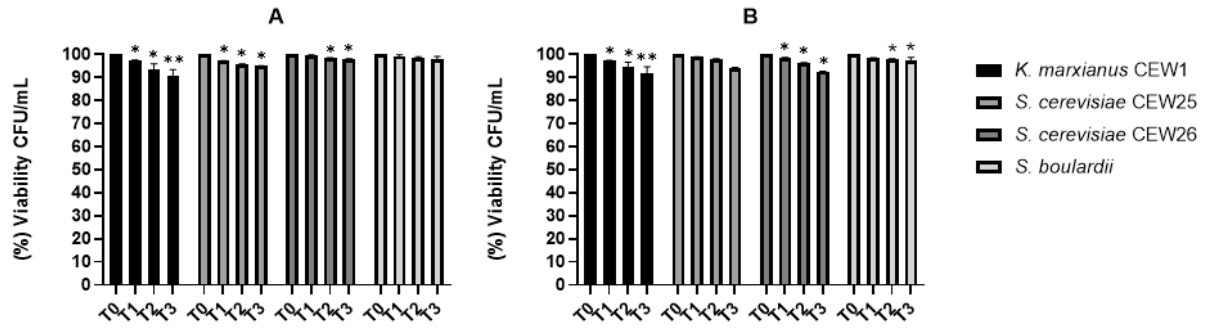


Figure 15 In vitro tolerance to gastric pH. Viable yeasts after 3 h of incubation in PBS adjusted to pH 3 (A) and pH 2 (B). Statistical analysis was performed using Tukey's multiple comparisons test. Significant differences are indicated by asterisks (*p < 0.05, **p < 0.01, ****p < 0.001, ******p < 0.0001).*

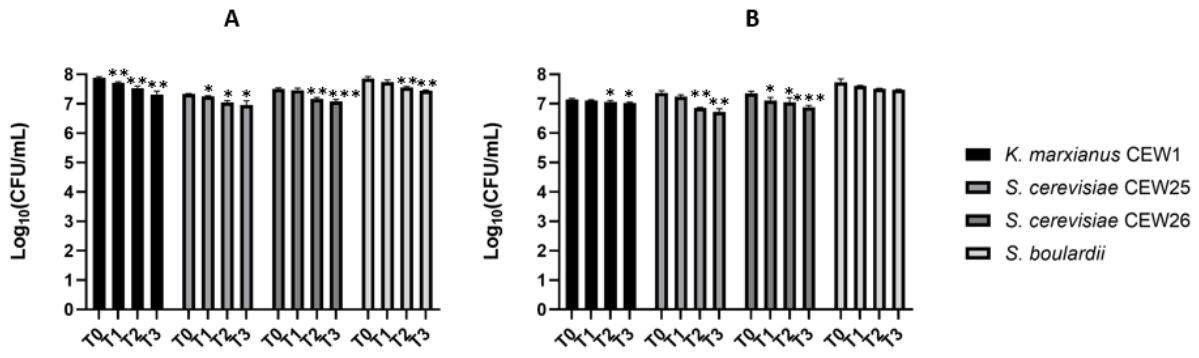


Figure 16. Number of cells (log CFU/mL) following incubation in 0.3% (A) and 0.5% (B) bile salts in PBS (mean ± SD). *S. boulardii* was used as control. Statistical analysis was performed using Tukey's multiple comparisons test. Significant differences are indicated by asterisks (*p < 0.05, **p < 0.01, ****p < 0.001, ******p < 0.0001).*

This study about prebiotic activity aimed to evaluate the beneficial potential of isolated yeast strains on the recognized probiotic strain *Lactobacillus rhamnosus* ICIM03 by assessing changes in its viability over time when co-cultured with yeasts. The prebiotic activity of a strain is related to its ability to positively influence the growth and activity of beneficial microorganisms in the human gut, such as probiotics, through the release of specific nutrients and/or metabolites. Co-cultures were established with yeast:bacterium ratios of 1:1 and 1:10. Single bacterial culture of *Lactobacillus rhamnosus* ICIM03 were assessed to a initial concentration of 10^6 cell/mL and 10^7 cell/mL to monitoring the growth in 24h and compared the growth with the co-culture. Among the tested strains, *S. cerevisiae* CEW25 exhibited the most significant effect, enhancing the viability of *L. rhamnosus* ICIM03. Notably, the cell numbers of *L. rhamnosus* ICIM03 increased already after 6 hours (**Figure 17A**) in co-culture ($1{,}02*10^9$ cell/mL) compared to ones in single culture ($4{,}05*10^8$ cell/mL) and continued to improve after 24 hours (co-cultivation $1{,}22*10^{10}$ cell/mL; single culture $1{,}47*10^9$ cell/mL) (**Figure 17B**). Similarly, *S. cerevisiae* CEW26 also promoted the cell number and growth of *L. rhamnosus* ICIM03, with measurable improvements observed after 6 hours in co-culture ($7{,}53*10^8$ cell/mL) compared to single culture ($4{,}05*10^8$ cell/mL) (**Figure 18A**), which were sustained and confirmed after 24 hours (co-culture: $1{,}99*10^9$ cell/mL; single culture: $1{,}47*10^9$ cell/mL) (**Figure 18B**). The most substantial improvements in *L. rhamnosus* ICIM03 cell numbers in co-culture were observed when the yeast:bacteria ratio was 1:10. *Kluyveromyces marxianus* CEW1 strain did not enhance the growth of *L. rhamnosus* ICIM03 (data not shown).

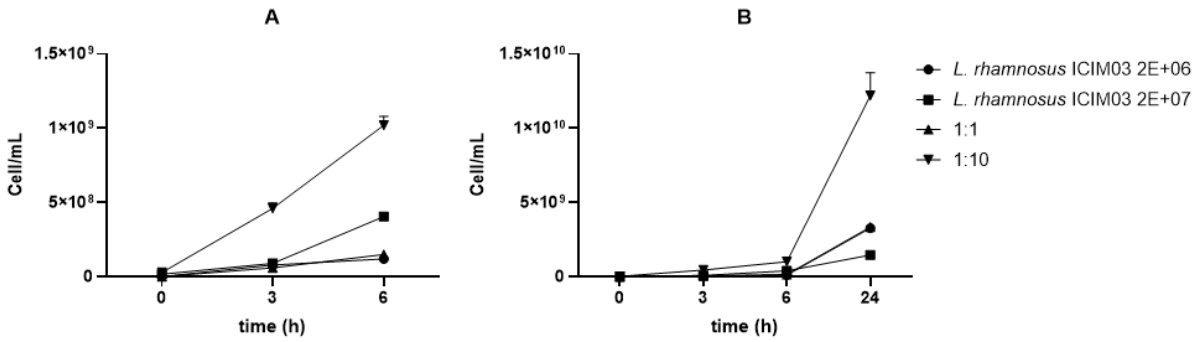


Figure 17 Bacterial growth kinetics in co-culture with *S. cerevisiae* CEW25 after 6 h (A) and 24 h (B) of incubation. *L. rhamnosus* ICIM03 was inoculated with a cell count of 10^6 (●) and 10^7 (■) cell/mL in single culture and at the same concentration in co-culture with a yeast:bacteria ratio 1:1 (▲) and 1:10 (▼).

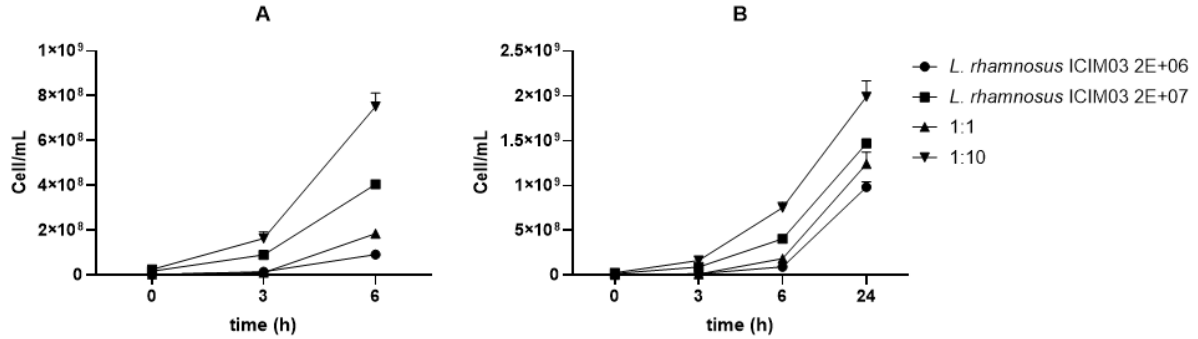


Figure 18 Bacterial growth kinetics in co-culture with *S. cerevisiae* CEW26 after 6 h (A) and 24 h (B) of incubation. *L. rhamnosus* ICIM03 was inoculated with a cell count of 10^6 (●) and 10^7 (■) cell/mL in single culture and at the same concentration in co-culture with a yeast:bacteria ratio 1:1 (▲) and 1:10 (▼).

Discussion

The microbiota of Cheese Exhausted Whey (CEW) and Ricotta Cheese Exhausted Whey (RCEW) were characterized in this work to understand the composition and the abundance of microbial population.

The 16S metabarcoding analysis of CEW shows a specialized bacterial community dominated by lactic acid bacteria (LAB). The Streptococcaceae family's dominance indicates an environment favoring these microorganisms. *Streptococcus salivarius*, the most abundant species, is known for its probiotic properties and bacteriocin production enhancing food safety and shelf life in dairy products (Wescombe et al., 2009). This makes it a promising natural preservative, reducing the need for synthetic additives and contributing to microbial balance in CEW (Damoczi et al., 2024). The presence of *Streptococcus thermophilus*, could be associated with fermented dairy products, highlights its technological significance. The bacterial composition of RCEW differently from that of CEW, was dominated by Lactobacillaceae, *Streptococcus salivarius* was absent due to its known sensitivity to heat treatments while *Streptococcus thermophilus* was the sole Streptococcaceae representative in common with CEW due to its known termotolerance.

Also the fungal composition, was notably different between CEW and RCEW. In CEW, *Saccharomyces cerevisiae* was the dominant species. The presence of *Kluyveromyces marxianus*, although minimal, is notable due to its ability to metabolize lactose, a primary sugar in CEW. The extreme dominance of *S. cerevisiae* prompts investigation into factors like substrate composition, pH, temperature, or inhibitory compounds, such as killer factor, that might suppress other microorganisms. The data from Ricotta Cheese Exhausted Whey (RCEW) reveals a diverse and complex microbial community composition, providing valuable insights into the microbiological profile of this dairy by-product. *Cyberlindnera jadinii*, formerly known as *Candida utilis* or *Torula*, is the most abundant species. This yeast is widely used in biotechnology and food production due to its beneficial properties (Sousa-Silva et al., 2021). The second most aboundant, was the *Wickerhamomyces* genus of the family Wickerhamomycetaceae in the class Ascomycetes. These fungi can survive in a variety of substrates and environments and perform many valuable roles in both industrial processes and the natural ecosystems (Chai et al., 2024).

The observed difference in the composition and relative abundance of microbial species within the CEW and RCEW samples is probably due to the alterations that occurred during the samples' "lifecycle" and their subsequent impact on the microbial

community. Notably, the diminished biodiversity in RCEW may be attributed to the heat treatment (80-90 °C) during the ricotta cheese production process, resulting in the elimination of numerous microbial species.

The results obtained from the metabarcoding analysis highlight the presence of many species that have probiotic characteristics present in cheese whey and this suggests that it can be a source for the isolation of these microorganisms; for this reason, a culturomics approach led to the isolation of bacterial and fungal species, which allowed to partially confirm the data obtained from metabarcoding analysis. Some studies suggest that metabarcoding may not always capture the full metagenome of a sample, potentially omitting specific species due to various factors (Meziti et al., 2021). These limitations are addressed through a more direct and reliable approach, which offers a clearer understanding of the species present in cheese whey. Furthermore, a review of the literature and previous studies supports the identification of the isolated species as characteristics of the sample's origin environment. The species isolated through the amplification of the 16S rRNA region for bacteria, *L. mesenteroides* RCEW1, *E. durans* RCEW2, and *E. faecalis* CEW1, were extensively studied for their potential probiotic characteristics. The species isolated through the amplification of the ITS region for yeast and fungi, *S. cerevisiae* CEW 25, *S. cerevisiae* CEW 26 and *K. marxianus* CEW1 were characterized phenotypically and for their robustness and tested for their probiotic and prebiotic activity to determine their suitability for use in fermentation or as supplements and nutraceuticals.

These microorganisms are responsible for the transformations that occur during cheese production and contribute to its sensory characteristics. *Leuconostoc mesenteroides*, one of the most extensively studied strains in the food industry, imparts distinctive aroma and flavor to fermented products through lactic acid production and the formation of flavor compounds (Gänzle, 2015). Additionally, it plays a preservative role in packaged foods and is widely utilized as a probiotic strain in dietary supplements, with its safety well established in the food sector. The role of lactic acid bacteria, including *Leuconostoc mesenteroides*, in food preservation further highlights its importance in the dairy industry. *Enterococcus* spp. are emerging as promising probiotic candidates, although their application has not been sufficiently investigated due to safety concerns (Franz et al., 2011). The genus *Enterococcus* has acquired a negative reputation primarily because of its association with serious healthcare-associated infections, raising concerns about the potential risks of utilizing enterococcal strains in food supplements, particularly regarding the dissemination of antimicrobial resistance and virulence genes (Jahan et al., 2015). The safety of

Enterococcus spp. for human consumption remains under investigation. Recent studies have indicated that some *Enterococcus* strains, following comprehensive characterization, may be suitable as safe probiotics in supplements (Comerlato et al., 2022; Han et al., 2024; Haibo et al., 2024).

		***L. mesenteroides* RCEW1**	***E. faecalis* CEW1**	***E. durans* RCEW2**	***S. cerevisiae* CEW25**	***S. cerevisiae* CEW26**	***K. marxianus* CEW1**
Lysozyme	50	P	P	P	ND	ND	ND
	100	-	P	P	ND	ND	ND
pH	3	P	P	P	P	P	P
	2	-	-	-	P	P	P
Bile salts	0,3 %	P	P	P	P	P	P
	0,5 %	-	-	P	P	P	P
Antimicrobial activity	*B. cereus* NCIMB9373	P	P	P	ND	ND	ND
	S. aureus NCIMB949	P	P	P	ND	ND	ND
	P. aeruginosa PA01	-	-	-	ND	ND	ND
	E. coli NCIMB11943	-	-	-	ND	ND	ND
Antibiotic susceptibility	*In. cell wall synth.*	-	-	P	ND	ND	ND
	In protein synth.	P	P	P	ND	ND	ND
	In. nucleic acid synth.	P	P	P	ND	ND	ND
Adhesion ability	5 h	-	P	-	ND	ND	ND
	24 h	-	P	P	ND	ND	ND

Table 7 Summary of the probiotic characteristics of the isolated microbial strains indicating the presence (P) or missing (-) of the tested probiotic characteristics; ND means "not detected".

Table 7 summarizes the probiotic characteristics tested for the isolated bacterial strains, indicating with (P) when the strain shows probiotic activity and with (-) when the strain does not show probiotic activity for that tested parameter.

The present study evaluates the survival and functional characteristics of selected microbial strains under simulated gastrointestinal conditions and their potential probiotic properties. *Enterococcus* species shown better lysozyme resistance compared to *Leuconostoc mesenteroides* RCEW1 while all tested strains shown good resistance to moderate acid pH, and moderate bile salts concentration. At lower pH all strains are sensitive while only *E. durans* RCEW2 showed good resistance to the higher bile salts %.

The isolated bacterial strains exhibited some antibiotic resistance, which, in the context of probiotics, can be considered a potential asset when carefully managed. Probiotic strains with intrinsic or non-transferable resistance can survive antibiotic treatments, thereby helping maintain gut microbiota balance during and after antibiotic use. This property may enhance therapeutic effectiveness, particularly in supporting recovery from infections or disturbances caused by antibiotic treatments. However, it is critical to ensure that resistance traits are not transferable to pathogenic bacteria, as this could contribute to the spread of antibiotic resistance (Li et al., 2020).

Key characteristics of probiotic microorganisms include adhesion capacity, which is crucial for host colonization and the production of bacteriocin against human pathogens (Tegegne et al., 2022). Bacterial strains showed different behavior where *E. faecalis* CEW1 exhibited early high biofilm production, while both *E. faecalis* CEW1 and *E. durans* RCEW2 demonstrated higher surface adhesion after 24 hours. *L. mesenteroides* RCEW1 was classified as a weak biofilm producer even after 24 hours. While adhesion and biofilm formation are beneficial traits for many probiotic strains, they are not universally required for all therapeutic applications. Some probiotics exert their effects through transient interactions within the gastrointestinal tract, producing antimicrobial substances or modulating the host's immune response without permanent colonization. For instance, certain strains can inhibit pathogen adhesion through competitive exclusion and the production of antimicrobial compounds, effectively reducing pathogen load without the necessity for biofilm formation or prolonged adherence (Tuomola et al., 1999).

The tested strains were effective in producing bacteriocin against gram-positive bacteria, such as *B. cereus* NCIMB9373 and *S. aureus* NCIMB949. The yeast strains, on the other hand, did not show any production of bacteriocins against pathogenic bacteria.

Furthermore, proteolytic activity has been evaluated, which is an important attribute for a dairy strain, by assessing the ability to break protein bonds to contribute to curd formation and the release of significant flavor molecules for the product's taste and aroma during the ripening process. Bacterial proteolytic activity influences also the release of bioactive peptides by enzymatically degrading proteins into smaller fragments with different beneficial effects. This process usually occurs in food fermentation or in gastrointestinal digestion and depending on the specificity of proteases involved (Korhonen & Pihlanto, 2006). *E. faecalis* CEW1 demonstrated the highest proteolytic activity compared to the other species.

Saccharomyces cerevisiae is the most common yeast species which is of great importance for various biotechnological applications due to its unique biological characteristics, i.e., its fermentation capacity with the production of alcohol and CO_2 and its resilience to adverse conditions of osmolarity and low pH (Parapouli et al., 2020). It is used mainly in baking processes but also in the beverage industries, particularly wine, and biofuel production. Interesting insights are still underway in this work regarding behaviours found in these strains isolated from cheese whey.

Kluyveromyces marxianus is a non-conventional food-grade yeast often isolated from dairy product with various beneficial traits, such as thermotolerance, the ability to utilize a broad range of sugars including lactose, secretion of lytic enzymes, the highest growth rate than other eukaryotes, and the production of fuel ethanol by fermentation (Lane and Morrissey, 2010) that make it useful for different industrial food and biotechnological applications. In addition, it has also achieved "Qualified Presumption of Safely" (QPS) and GRAS status in European Union and United States respectively, due to its safety use with dairy products, which makes it particularly suitable for commercial applications in the pharmaceutical, food, and feed industries (Löser et al., 2013; Morrissey et al., 2015).

The isolated yeast strains were studied in depth for their phenotypic characteristics and robustness in order to know their growth characteristics in media with different carbon sources and in the presence of stress factors such as lactic acid, with the aim of determining their suitability for use as inoculum for cheese whey fermentation. The strains exhibited good growth at both 30°C and 37°C, with an unusual growth pattern observed in *Saccharomyces cerevisiae* on lactose. This is surprising, as *S. cerevisiae* typically cannot utilize lactose due to the absence of the required enzymes, specifically the LAC4 and LAC12 genes as has also been confirmed for these strains. Possible explanations for this unexpected behavior include partial hydrolysis of lactose during medium preparation, which releases glucose and galactose monomers readily consumed by the strain, while an adaptation of these strains to the dairy environment from which they were isolated, which could facilitate the acquisition of these genotypic traits over time, allowing them to utilize lactose, has been excluded.

Furthermore, resistance to the presence of lactic acid as a stress factor combined with different carbon source was tested because it is produced by the metabolism of LAB that dominates within cheese whey; lactic acid could inhibit or promote the growth of some microorganisms, so resistance to it is important to understand the impact it has on the inhabiting microflora. Tests confirmed that *S. cerevisiae* strains are sensitive to lactic acid, which, even at a modest concentration, inhibited their growth on S-glu and S-gal. In contrast, when lactose was provided as a carbon source, lactic acid totally

inhibited the growth. *S. cerevisiae* CEW26 consistently exhibited weaker growth compared to *S. cerevisiae* CEW25 under all conditions tested. *Kluyveromyces marxianus* CEW1 demonstrated good growth on all carbon sources and robust growth in presence of lactic acid, even at the highest concentrations, confirming its potential as an exceptionally versatile strain for biotechnological applications, with a broad functional range.

Since lactose is the primary carbon source in cheese whey, growth kinetics in S-lac medium were assessed to evaluate strain growth and lactose consumption. *Kluyveromyces marxianus* CEW1 demonstrated remarkable growth, consuming lactose up to 0.5% when it slows down and consumes it completely in a longer time than *K. marxianus* CBS397 which is faster in consumption. This indicates that the lactose consumption system in *K. marxianus* CEW1 becomes less efficient as the concentration decreases. Regarding ethanol production, both strains start producing it at the same time but *K. marxianus* CBS397 was found to be more efficient in production; ethanol production stopped at a certain point and began to decrease in concentration because it is consumed by the yeast itself; this ability to consume the ethanol produced was found to be better in the *K. marxianus* CEW1 strain leading to an almost total consumption. In contrast, *S. cerevisiae* strains showed limited lactose consumption and a minimal increase in OD_{600}, suggesting the utilization of other nutrients present in the medium at lower concentrations. This was further confirmed by the reduced lactose consumption, which is likely due to partial hydrolysis of lactose over time during the incubation. RCEW was used as a growth medium to test the growth kinetics, as it will serve as the substrate for the fermentation of the isolated strains. *Kluyveromyces marxianus* confirmed the previous results, exhibiting significant growth and complete lactose consumption. The *S. cerevisiae* strains showed good growth kinetics, confirming that lactose consumption was absent, as it remained unchanged. However, RCEW proved to be a nutrient-rich medium, containing various compounds that supported the growth of the strains, making it suitable for fermentation applications.

Considering the potential of these strains as a novel probiotic, in this work are reported the results of some in-vitro tests useful to understand their potential probiotic activity. To be classified as probiotics, the tested strains must not be pathogenic, must be generally recognized as safe (GRAS), and must remain viable within the human organism after ingestion (Binda et al., 2020), thereby enabling colonization and the exertion of beneficial effects. Some important advantages of yeast probiotics, compared to bacteria, are that they are highly resistant against gastrointestinal enzymes, pH, bile salts, organic acids, and variations in temperature. Moreover, yeasts,

as eukaryotic cells, are resistant to bacteriophages and most antibiotics, and cannot transfer antibiotic-resistance genes to bacteria. Finally, yeasts have shown to have immunostimulatory effects in different animal taxa (Ortuño et al., 2002) (Wu et al., 2021). Furthermore, yeasts could have prebiotic activity; with the term of prebiotic yeast refers to non-pathogenic yeast strains that promote gut health by stimulating the growth and activity of beneficial microbiota. Unlike probiotics, prebiotic yeasts function primarily through the production of substrates, such as β-glucans and mannooligosaccharides, which enhance the proliferation of commensal bacteria. These compounds modulate immune responses, improve gut barrier integrity, and contribute to overall host well-being (Tanihiro et al., 2024).

The probiotic and prebiotic characteristics of the yeasts were evaluated by testing their resistance to acid pH and the presence of bile salts, which had no significant effect on the growth of the strains, and by evaluating the impact on the growth of the *Lactobacillus rhamnosus* ICIM03 a well-known probiotic bacteria. Prebiotics are essential for maintaining digestive health, enhancing immune function, and potentially contributing to metabolic well-being (Gibson & Roberfroid, 1995). Given their therapeutic potential, prebiotics are increasingly explored in both clinical and biotechnological research. In this study, the prebiotic effects of the isolated yeast strains showed an enhancement of the growth of probiotic by both *S. cerevisiae* CEW25 and CEW26. These results allowed to choose the yeast strains for the fermentation of RCEW with the aim of obtaining a product with nutraceutical characteristics. The results are presented in the next section.

5. Food waste recovery by fermenting cheese whey: development of a new product with nutraceutical properties

Introduction

The aim of this study is to explore sustainable alternatives for cheese whey disposal through biotechnological processes, such as fermentation, to enhance the value of this by-product and to obtain a product with nutraceutical properties. Currently an officially shared and accepted definition of nutraceuticals is missing but a nutraceutical can be defined as food-derived item or supplement that provides health benefits beyond basic nutritional value, often contributing to the prevention or management of chronic diseases or supporting overall well-being. These products typically contain bioactive compounds, such as vitamins, minerals, amino acids, fatty acids, probiotics, prebiotics, or plant-derived substances. Nutraceuticals can be classified into categories like dietary supplements, functional foods, or medicinal foods, depending on their form and intended use. They represent the intersection of nutrition and pharmaceuticals, aiming to enhance health outcomes while maintaining a natural origin (Kalra E. K. 2003) (Pandey et al., 2010). Cheese whey is categorized as a "secondary raw material," referring to material resulting from a recovery process that becomes an input in a new production. As widely discussed, cheese whey is a product with high Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) levels of approximately 50 and 80 g/L, respectively (Maragkoudakis et al. 2016), that requires alternative recovery methods to mitigate environmental pollution and health risks. Direct discharge of cheese whey into the environment or surface waters is prohibited, and transportation must adhere to waste or organic material transport regulations. Given the substantial costs associated with current waste treatment methods, the disposal of cheese whey presents a significant challenge for dairy producers. With increasing consumer awareness of the importance of maintaining a healthy and sustainable diet, both the scientific community and the agri-food industry have intensified efforts to develop innovative, high-quality food products. Particular attention has been directed on incorporating functional components, such as prebiotics, probiotics, microbial metabolites, and bioactive compounds, into these products (Terpou et al., 2019; Cunningham et al., 2021), while ensuring the sustainability of production processes.

In this context, the potential of cheese whey as a possible ingredient to produce food and beverages has been explored by several authors (**Table 9**). Main compounds of cheese whey are lactose (approximately 4-5%) and proteins (around 1%, w/v), such as

β-lactoglobulin (β-LG), α-lactalbumin (α-LA), bovine serum albumin (BSA), and immunoglobulins (IGs). Minor proteins, including lactoperoxidase (LP), lactoferrin (LF), and proteose-peptone (PP), are also present (Brandelli et al., 2015). From the peptide bond breaking activity of these proteins, bioactive peptides are derived, which are initially present in their parent proteins, to then be activated by enzymatic, fermentative or gastrointestinal digestive processes (Ashaolu, T.J., 2020). Bioactive peptides have between 2 to 20 amino acids in their primary sequence, which are always implicated in their activities. These peptides have significant health benefits listed in Table 8. Figure 19 shows an overview of the dietary route of the bioactive peptide to exert their functions.

Peptides derived from whey proteins, specifically from α-lactalbumin (α-LA) and β-lactoglobulin (β-LG), have demonstrated significant bactericidal activity (Pellegrini et al., 1999, 2001). These peptides are produced through proteolytic digestion using enzymes and the hydrolysates exhibit antimicrobial effects primarily against Gram-positive bacteria like *Bacillus subtilis*. However, when specific amino acids in peptides were modified (e.g., replacing D98 with R and adding K at the C-terminus), their efficacy against Gram-negative bacteria, such as *Escherichia coli* and *Bordetella bronchiseptica*, improved. Some peptides also show homology to naturally occurring antimicrobial peptides like human opsin, enhancing their bactericidal capabilities. These activities often involve disrupting microbial membranes or altering cellular integrity (Madureira et al., 2010).

The immunomodulatory activity of bioactive peptides derived from whey proteins involves the modulation of both adaptive and innate immune responses. For example, synthetic peptides YG and YGG, which correspond to sequences within α-lactalbumin (α-LA), have been shown to enhance lymphocyte proliferation and protein synthesis in vitro. Similarly, cheese whey protein isolates and purified β-lactoglobulin (β-LG) have been demonstrated to increase the production of immunoglobulins such as IgG and IgA, which are essential components of the immune response (Saint-Sauveur et al., 2009). Furthermore, these peptides may induce oral tolerance, thereby reducing allergic responses to dietary antigens. Hydrolysis of cheese whey proteins by probiotics, such as *Lactobacillus paracasei*, releases peptides that promote regulatory T-cell activity, increasing IL-10 secretion while suppressing pro-inflammatory cytokines such as IFN-γ and IL-4 (Prioult et al., 2004). Additionally, peptides such as GLF have been shown to enhance macrophage phagocytosis, providing protection against infections (Berthou et al., 1987).

The antioxidant activity of bioactive peptides derived from cheese whey proteins is closely linked to their ability to neutralize reactive oxygen species (ROS) and reactive nitrogen species (RNS), thus preventing oxidative stress and associated cellular damage (Ashaolu, 2020a). These peptides, typically generated through enzymatic hydrolysis of α-lactalbumin (α-LA) and β-lactoglobulin (β-LG), demonstrate radical-scavenging properties due to their amino acid composition, particularly the presence of hydrophobic and aromatic residues (Hernandez-Ledesma, Ramos, Recio, & Amigo, 2006). The antioxidative mechanisms are influenced by factors such as peptide size, sequence, and the method of hydrolysis. Studies have shown that these peptides can inhibit lipid peroxidation and protect cellular components from oxidative degradation. This activity makes whey-derived peptides valuable for health applications, particularly in reducing the risks associated with chronic diseases linked to oxidative stress, including cardiovascular diseases and aging-related disorders.

Furthermore, cheese whey peptides also play a role in reducing high blood pressure by inhibiting the angiotensin-converting enzyme (ACE) which prevents the conversion of angiotensin I to the vasoconstrictor angiotensin II. Specific peptides, including α-lactorphin (YGLF) from α-LA and β-lactorphin (YLLF) and β-lactotensin (HIRL) from β-LG, were effective in reducing blood pressure in hypertensive rat models, with YLLF demonstrating the highest ACE inhibition. The activity of these peptides is influenced by their amino acid sequences and stability during gastrointestinal digestion (Hernandez-Ledesma, Ramos, and Gomez-Ruiz, 2011).

Cheese whey peptides have demonstrated significant hypocholesterolemic, anti-diabetic, and anti-obesity effects. A key mechanism underlying these benefits is their ability to inhibit dipeptidyl peptidase IV (DPP-IV), an enzyme involved in various physiological processes, including glucose metabolism. Inhibition of DPP-IV prevents the degradation of incretins, such as GIP and GLP-1, thereby extending their half-life (Nongonierma & FitzGerald, 2013). This prolongation stimulates glucose-dependent insulin secretion postprandially, reduces food intake, and enhances satiety. Additionally, cheese whey hydrophilic peptides and amino acids have demonstrated insulinotropic properties, supporting glucose regulation (Madadlou, A., & Abbaspourrad, A. 2018). Moreover, DPP-IV inhibitory peptides derived from β-LA were reported to decrease blood glucose level in in-vitro and in vivo studies (Uchida et al., 2011, Silveira et al., 2013). Whey peptides also exhibit cholesterol-lowering effects, as demonstrated in rat models where β-LA peptides increased fecal steroid excretion, suggesting improved bile salt chelation and micellar cholesterol solubility (Hartmann, R., and Meisel, H. 2007).

Source of protein	Enzyme used	Amino acid sequence	Bioactivity
β-lactoglobulin	Corolase PP	W_{19}YSLAMAASDI$_{29}$	Antioxidant
β-lactoglobulin	Trypsin	V_{15}AGTWY$_{20}$ A_{25}ASDISLLDAQSAPLR$_{40}$ I_{78}PAVFK$_{83}$ V_{92}LVLDTDYK$_{100}$ V_{92}LVLDTDYK$_{100}$	Antimicrobial
β-lactoglobulin	Chymo-Trypsin	H_{146}IRL$_{149}$	Opioid activity
Lacto-ferrin	Pepsin	Lactoferricin: F_{17}KCRRWQWRMKKLGAPSITCVR RAF$_{41}$	Antimicrobial, Antitumoral
α-lactalbumin β-lactoglobulin	Protease from flower of Cynara cardunculus	K_{16}GYGGVSLPEW$_{26}$ D_{97}KVGINYW$_{104}$ D_{33}AQSAPLRVY$_{42}$	ACE inhibition
Bovine serum albulmin	Proteinase K	F_{221}P$_{222}$	ACE inhibition
Bovine lactoferrin	Pepsin and trypsin	GILRPY REPYFGY	ACE inhibition
β-lactoglobulin	Alcalasa	IDALNEK HIRLS KTKIPAVF	Antioxidant
β-lactoglobulin	Corollase	MAASDISLL VEELKPT	Antioxidant
β-lactoglobulin	Pepsin	KVAGT IRL VRT LMPH EKF	Antimicrobial
α-lactalbumin	Pepsin	KVGIN	Antimicrobial
α-lactalbumin	Flavorzyme	VGINYWLAHK	Antioxidant

Table 8 Cheese whey bioactive peptides with their sequences and biological activities (Zhao, C., & Ashaolu, T.J. 2020)

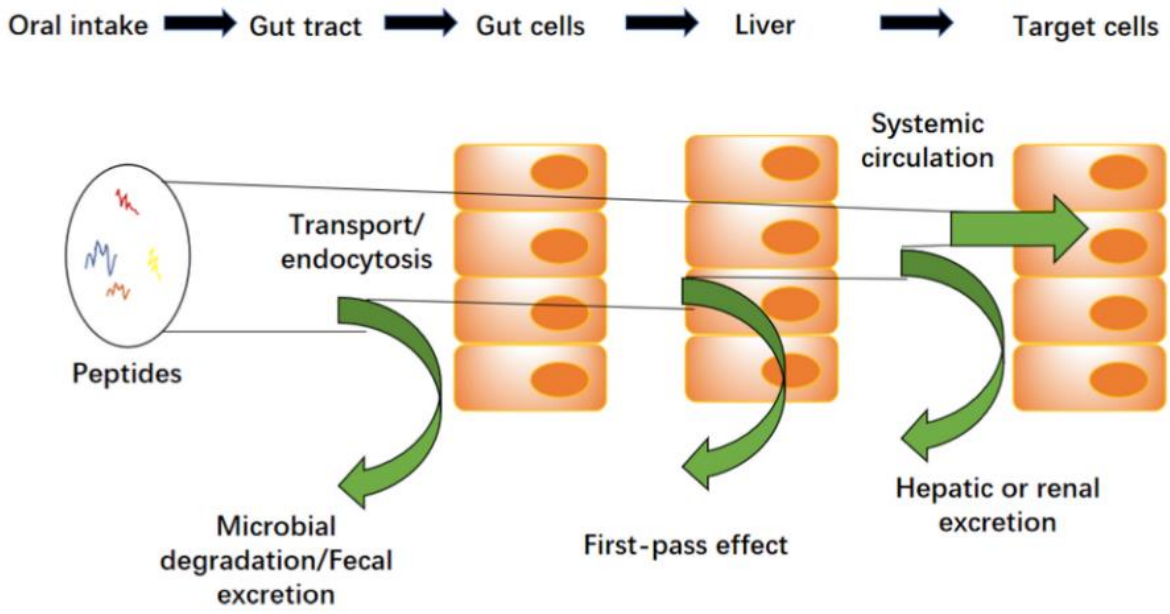


Figure 19 An overview of cheese whey bioactive peptide mechanisms. Peptides are digested to amino acids by gastrointestinal enzymes before absorption, of which di-peptides and tri-peptides can be directly absorbed by peptide transporters. A small number of oligopeptides are transported mainly by endocytosis. The peptides are then metabolized in the liver (first-pass effect) with some moving into the blood stream for circulation. Some bioactive peptides with penetrating ability can further enter certain tissues to exert functions with the others excreted (Zhao, C., & Ashaolu, T.J. 2020).

Results

Isolated yeast strains were used in single culture for the fermentation of Ricotta cheese exhausted Whey RCEW. Fermentation, which lasted 24 hours, was carried out at 28 °C under continuous aeration conditions; the pH and the O_2 concentration were constantly monitored.

Antioxidant activity test: ABTS Radical Scavenging Assay

The ABTS assay is an antioxidant protocol based on the ability of the molecules to react with $ABTS^{\bullet+}$ radicals. Fermented RCEW were tested to evaluate the increase in antioxidant activity following fermentation expressed as % inhibition. The fermented RCEW was freeze-dried for stabilization and preservation, but this process can modify the product, which, due to the stresses suffered, can lose or reduce some molecules with important bioactive effects. Freeze-drying did not cause any significant reduction in antioxidant effect (Freeze-dried RCEW) (23 %) when compared to RCEW (20 %). Fermentation, on the other hand, improved the antioxidant activity of RCEW as shown in **Figure 20A**, where the results showed improvement in this effect with a significant increase in RCEW fermented with *S. cerevisiae* CEW25 (72,2 %), *S. cerevisiae* CEW26 (34,1 %) and *K. marxianus* CEW1 (62,8 %). **Figure 20B** shown results expressed as µmol Trolox/mL, a strong antioxidant molecule used as control. The increase in antioxidant activity after fermentation is confirmed by the EC_{50} value, which indicates the concentration of a molecule that produces 50% of the maximal effect. The RCEW value is 32.14 ± 1.047 mg/mL, while the fermented RCEW with *S. cerevisiae* CEW25, *S. cerevisiae* CEW26, and *K. marxianus* CEW1 showed values of 6.17 ± 1.0095, 27.28 ± 1.047, and 10.38 ± 1.0228, respectively (**Figure 21**).

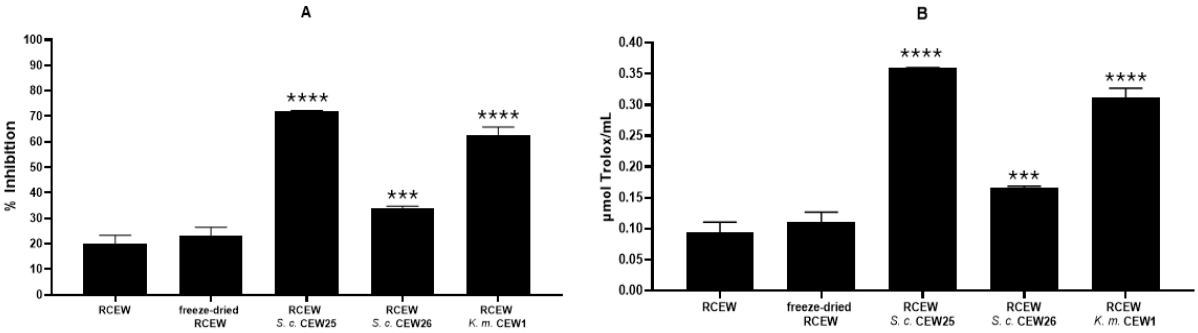


Figure 20 Antioxidant activity of Fermented RCEW with ABTS method. Results were expressed as % inhibition (A) and µmol Trolox/mL (B). Values are presented as the mean ± SD of 3 replicates; Statistical analysis was performed using Tukey's multiple comparisons test. Significant differences are indicated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs RCEW group).

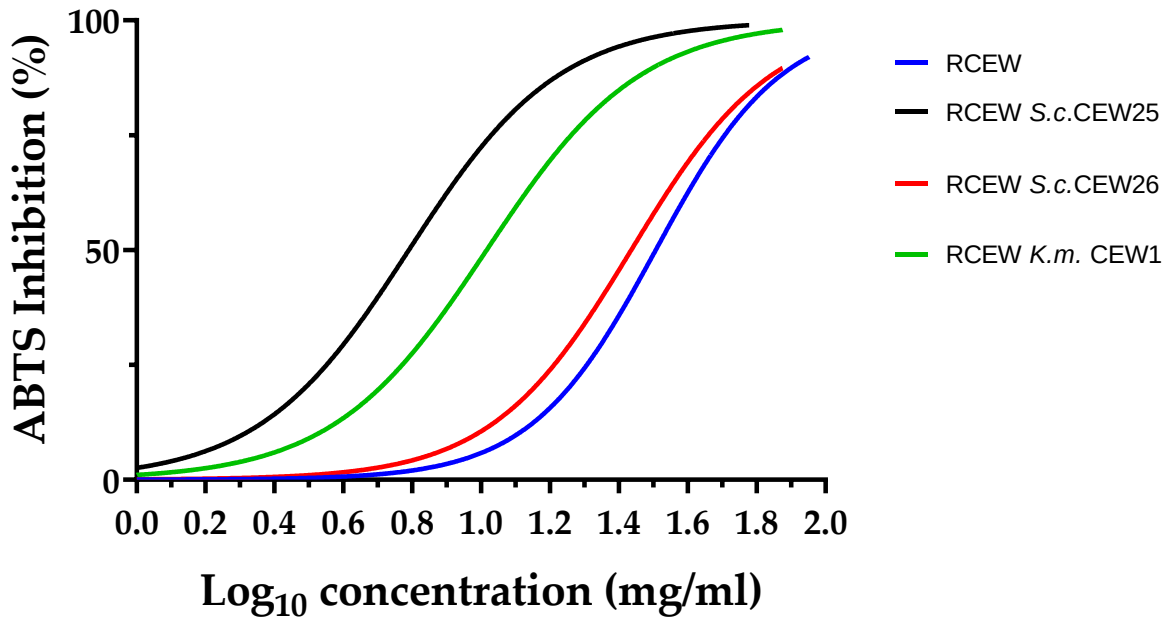


Figure 21 EC_{50} values of tested samples. Results were reported in Log Concentration (mg/mL)

Antioxidant activity	RCEW	RCEW fermented *S. cerevisiae* CEW25	RCEW fermented *S. cerevisiae* CEW26	RCEW fermented *K. marxianus* CEW1
% inhibition	20,17 ± 1,66	72,22 ± 0,054****	34,08 ± 0,061***	62,81 ± 2,96****
µM trolox/mL	0,094 ± 0,015	0,359 ± 0,0002****	0,165 ± 0,0031***	0,311 ± 0,015****
EC50 (mg/mL)	32,14 ± 1,047	6,17 ± 1,0095	27,28 ± 1,047	10,38 ± 1,0228

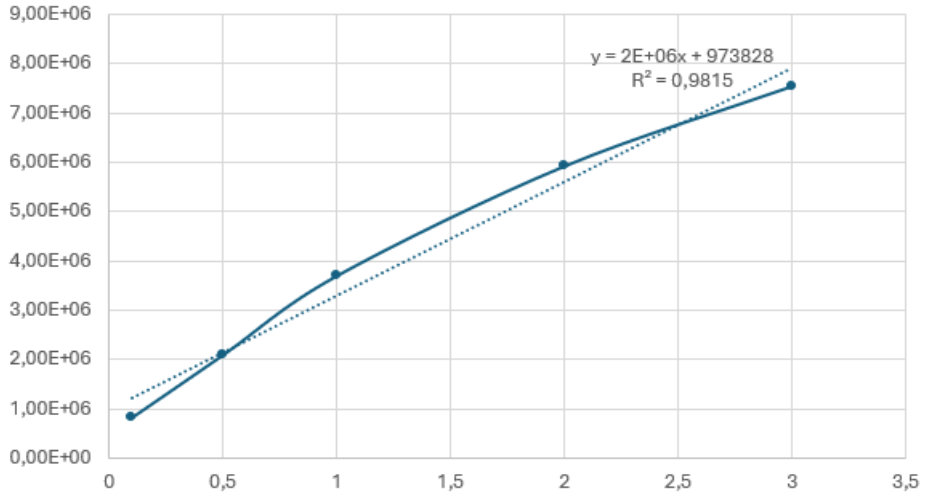
Table 9 Resume of antioxidant activity expressed as % inhibition, µM trolox/mL and EC50 (mg/mL). Results were expressed as means ± SD. Statistical analysis was performed using Tukey's multiple comparisons test. Significant differences are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs RCEW group).

DPP-IV inhibitory activity assay

The DPP-4 inhibitory activity assay was performed to measure the inhibitory activity of peptides on the activity of the DPP-4 enzyme involved in the regulation of blood sugar levels. DPP-4 plays a crucial role in the degradation of incretin hormones, which are involved in glucose homeostasis. Inhibiting DPP-4 has become a therapeutic strategy for type 2 diabetes, as it prolongs the activity of GLP-1 and GIP, enhancing insulin secretion. The findings may contribute to the development of novel hypoglycemic agents (Deacon, 2019). Standard curve was previously made by testing known concentrations of AMC std and subsequently the equation of the std curve was used to calculate the inhibition of the DPP-IV enzyme expressed as % inhibition. All strains showed inhibition of DPP-IV activity, RCEW had an activity of 20,86 %, while fermented RCEW with *S. cerevisiae* CEW25 and CEW26 showed respectively an

inhibition of 29,73 and 33,23 %, while RCEW fermented with *K. marxianus* CEW1 showed a slight but not significant increase of inhibition around 23,47 %. DPP-IV synthetic inhibitor P32/98 was used as control with 84,19 % inhibition (**Figure 22**).

AMC Std Concentration µM	Means fluorescence
0,1	8,26E+05
0,5	2,10E+06
1	3,70E+06
2	5,93E+06
3	7,55E+06



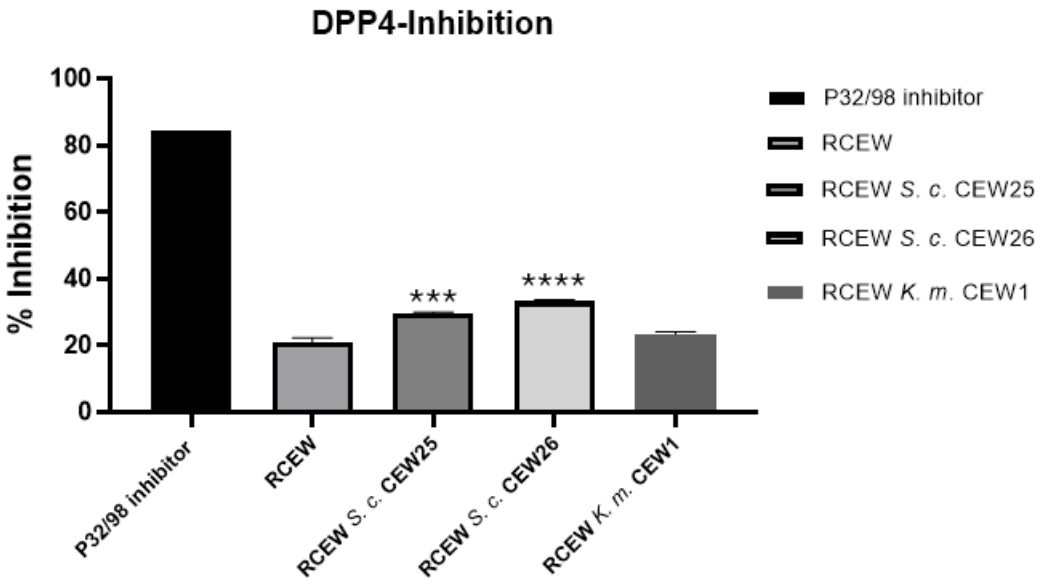
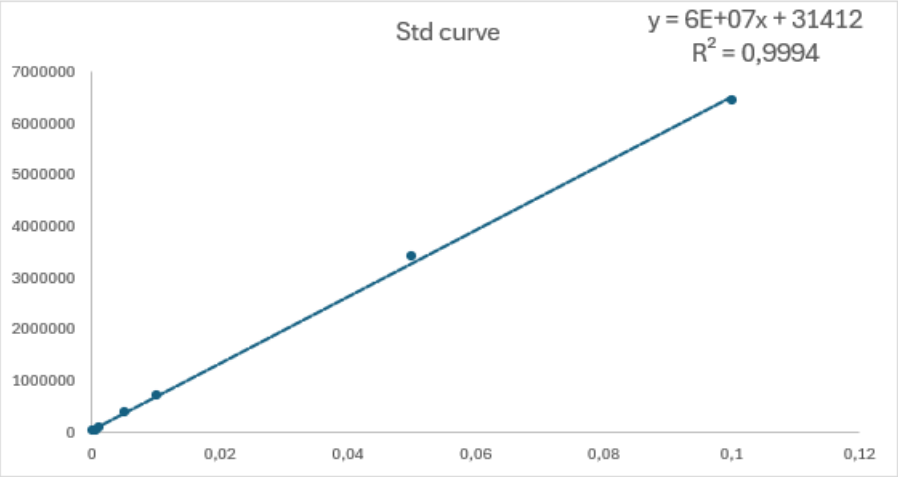


Figure 22 DPP-IV inhibitory assay of fermented RCEW. Results were reported as % inhibition. Values are presented as the mean ± SD of 3 replicates; Statistical analysis was performed using Tukey's multiple comparisons test. Significant differences are indicated by asterisks (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 vs RCEW group).

ACE – inhibitory activity assay

The ACE inhibitory activity assay was performed to measure the inhibitory activity of peptides on ACE enzyme (Angiotensin-Converting Enzyme) that is a crucial enzyme in the renin-angiotensin-aldosterone system (RAAS), which helps regulate blood pressure, fluid balance, and overall cardiovascular function. ACE plays a key role in converting angiotensin I into angiotensin II, a powerful vasoconstrictor that increases blood pressure (Wei et al., 2024). Standard curve was previously made by testing known concentrations of Hippuric acid and subsequently the equation of the std curve was used to calculate the inhibition of the ACE enzyme expressed as % inhibition. The samples were tested at 500 mg/mL and 250 mg/mL concentration. % inhibition of RCEW 500 mg/mL was 16,59 while RCEW 250 mg/mL and RCEW fermented with *S. cerevisiae* CEW25 at both tested concentrations did not show any inhibition activity. RCEW fermented with *S. cerevisiae* CEW26 showed % inhibition of 48,99 at a concentration of 500 mg/mL without any activity at the concentration of 250 mg/mL. RCEW fermented with *K. marxianus* CEW1 showed the best inhibition value (92,85 %) at 500 mg/mL and a slight inhibitory activity (11,83 %) at 250 mg/mL (**Figure 23**).

Retta acido ippurico (CH5, 228 nm, RT 4.7)	Media
0,0001	12534
0,0005	30364
0,001	71130
0,005	368960
0,01	689707
0,05	3393122
0,1	6447880



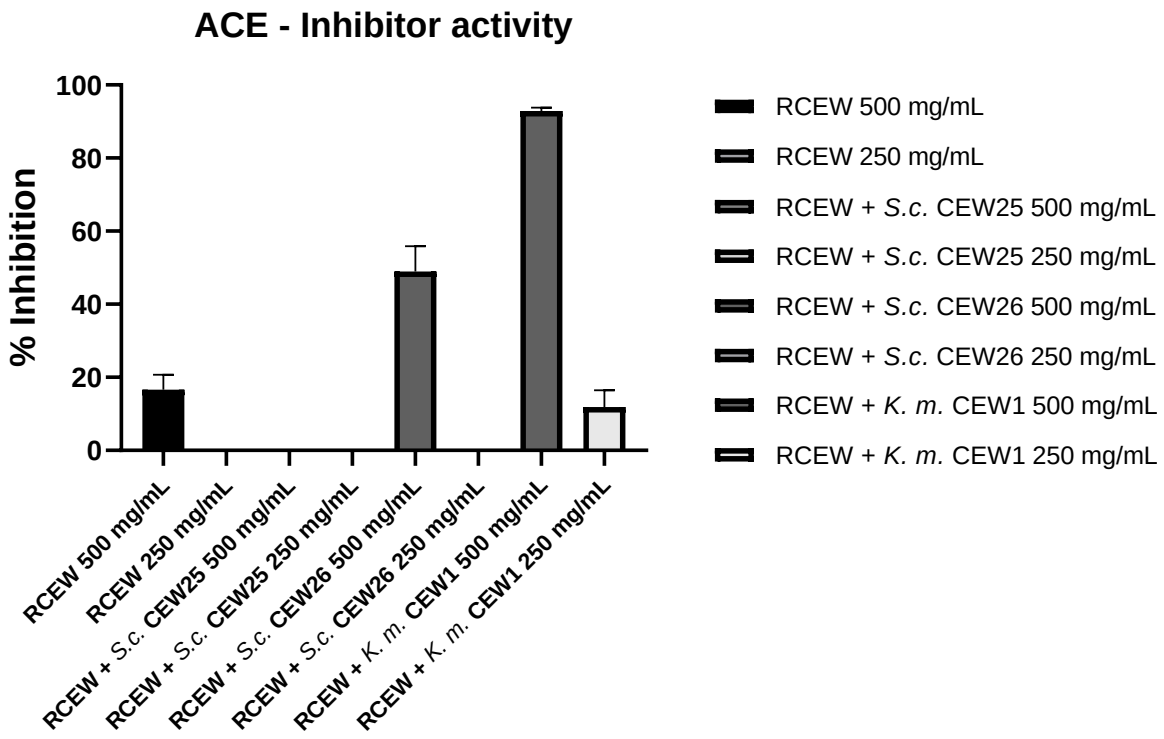


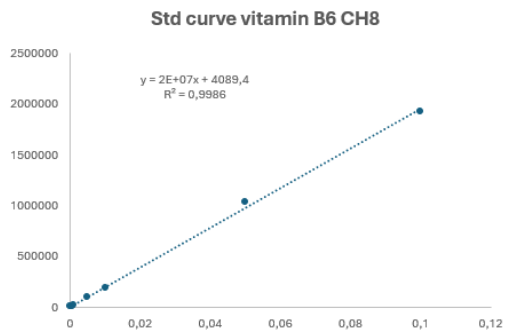
Figure 23 ACE-inhibitory assay of fermented RCEW. Results were reported as % inhibition. Values are presented as the mean ± SD of 3 replicates. Results report the name of the sample indicated as RCEW with the name of the strain used for fermentation Statistical analysis was performed using Tukey's multiple comparisons test. Significant differences are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs RCEW group).

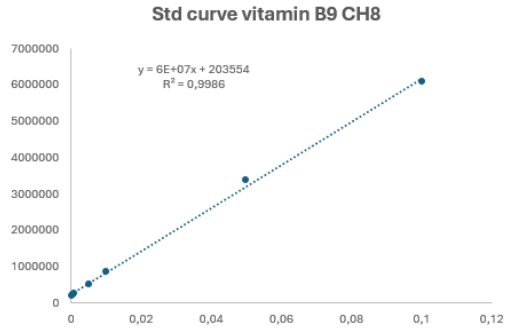
Determination of Vitamins of group B content

RCEW naturally contains a scarce quantity of pyridoxine (B6) and folic acid (B9) and the fermentation with isolated yeast strains has shown an increase in the concentration of these molecules (Magan et al., 2020). Vitamin B6 content in RCEW was 3.219 ± 0.160 mg/g RCEW and fermentation caused a significant increase to values of 6.632 ± 0.080 and 5.977 ± 0.055 for RCEW fermented with *S. cerevisiae* CEW25 and *K. marxianus* CEW1, respectively. Fermentation with *S. cerevisiae* CEW26 did not cause a significant increase in vitamin B6 content (3.132 ± 0.030). Vitamin B9 content in RCEW is very low but every tested strain, during fermentation, caused a significant increase in its concentration. Initially, the concentration in RCEW was 0,039 ± 0,003, *S. cerevisiae* CEW25 and CEW26 increased the concentration to 0,192 ± 0,004 and 0,114 ± 0,01 respectively, while *K. marxianus* CEW1 was the strain that increased most vitamin B9 content at a concentration of 0,348 ± 0,003 (**Figure 24, Table 10**).

Std curve vitamin B6 (CH8, 290 nm, RT 4.22)	Media
0,0001	6508
0,0005	7634,5
0,001	16404
0,005	91790,5
0,01	189263
0,05	1036367
0,1	1919723

Std curve vitamin B9 (CH8, 290 nm, RT 5.26)	Media
0,0001	170095
0,0005	195497
0,001	229410,5
0,005	497860,5
0,01	831630
0,05	3356418
0,1	6078849





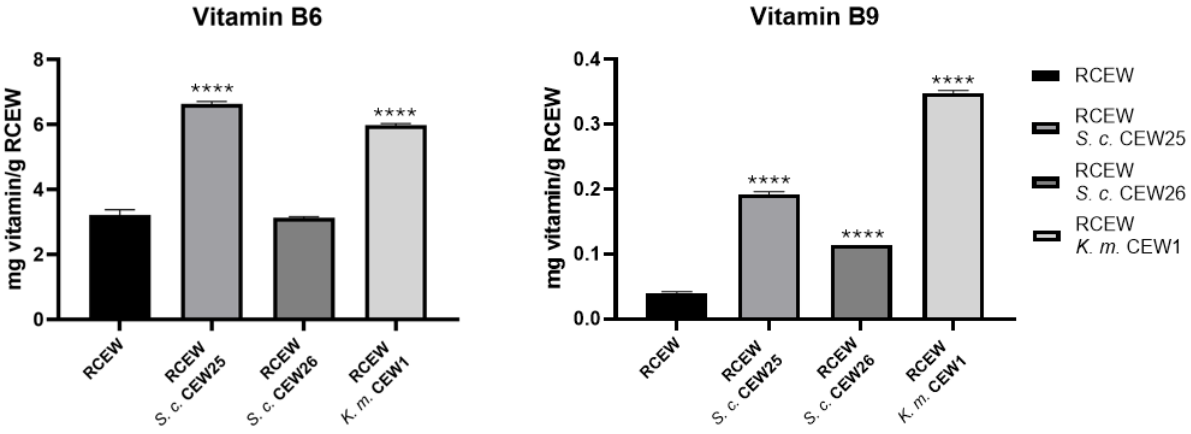


Figure 24 Vitamin B6 and B9 content in RCEW and fermented RCEW. Values are presented as the mean ± SD of 3 replicates and results were expressed as mg vitamin/g RCEW; Statistical analysis was performed using Tukey's multiple comparisons test. Significant differences are indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs RCEW group).

	Vitamin B6	Vitamin B9
RCEW	3,219 ± 0,160	0,039 ± 0,003
RCEW fermented *S. cerevisiae* CW25	6,632 ± 0,080	0,192 ± 0,004
RCEW fermented *S. cerevisiae* CW26	3,132 ± 0,030	0,114 ± 0,0
RCEW fermented *K. marxianus* CW1	5,977 ± 0,055	0,348 ± 0,003

Table 10 Vitamin content in tested samples. Values are presented as the mean ± SD of 3 replicates and results were expressed as mg vitamin/g RCEW.

Discussion

During the fermentation of RCEW carried out by isolated yeast strains, the metabolic activity of the involved microbial species leads to nutrient consumption and the production of new molecules, resulting in changes to the product's characteristics and qualities (Gänzle, 2015). It is well established that, in substrates containing proteins or their derivatives, microbial metabolism, along with chemical or physical treatments, can cause the cleavage of peptide bonds. This process releases small molecules, such as bioactive peptides, which may exhibit various health benefits, including antimicrobial, antioxidant, antihypertensive, antidiabetic, and immunomodulatory effects. These peptides can confer nutraceutical properties to the product (Korhonen & Pihlanto, 2006). A food or food component is defined as a nutraceutical when it provides health benefits that go beyond its basic nutritional value, including the prevention and treatment of diseases. The term combines "nutrition" and "pharmaceutical" and is commonly used to describe products derived from natural sources that have biological effects on the body. During the fermentation of cheese whey, each strain tested improved the nutraceutical characteristics of fermented RCEW compared to fresh RCEW, such as antioxidant activity, ACE enzyme inhibition and DPP-IV enzyme inhibition.

The content of B-group vitamins was also evaluated to determine whether the strains could utilize the precursors present in cheese whey. The breakdown of cheese whey proteins releases amino acids that serve as precursors for the synthesis of these vitamins, while minerals and trace elements act as cofactors in the enzymatic processes involved in vitamin biosynthesis.

In general, as resumed in **table 11** the fermentation process improved the nutraceutical properties of non-fermented RCEW, with varying effects on different tested characteristics. Specifically, *S. cerevisiae* CEW25 and *Kluyveromyces marxianus* CEW1 were most effective at improving antioxidant activity and vitamin B6 content, while *S. cerevisiae* strain 26 was most effective at improving DPP-IV inhibitory activity. As regards the inhibitory activity of the ACE enzyme, responsible for vasoconstriction, but also involved in the stimulation of aldosterone production and the release of antidiuretic hormones (ADH), non-fermented RCEW showed a very low inhibitory activity while high values was shown in fermented RCEW with *S. cerevisiae* CEW26 and *K. marxianus* CEW1. These strains represent strong candidates for the development of functional foods with potential health benefits, especially in the context of blood pressure regulation, glycemic control, and antioxidative protection.

	RCEW fermented *S.c.* CEW25	RCEW fermented *S.c.* CEW26	RCEW fermented *K.m.* CEW1
Antioxidant activity	+++	+	+++
Antihypertensive activity	-	++	+++
Antidiabetic activity	+	++	-
Vitamin B6	+++	-	+++
Vitamin B9	++	+	+++

Table 11 Resume of the effect of the fermentation on RCEW. "+" indicated slight increase, "++" indicated good increase and "+++" indicated a great increase of the tested parameter. (-) indicated no increase.

An important factor influencing the presence and quality of peptides in RCEW is the entire processing chain. For fermentation, RCEW was autoclaved to ensure sterility and to allow the yeast inoculated to dominate the fermentation. Fresh RCEW is a secondary raw material that is easily degradable and subject to rapid deterioration; for this reason, it must be treated in the shortest time. The fermented RCEW is then centrifuged, and the supernatant is frozen for freeze-drying. This step can be stressful and may affect the stability of the molecules present. Freeze-drying, however, is the optimal preservation method for RCEW, as it removes water and significantly extends the product's shelf life. The resulting product can be stored at low temperatures and used in various concentrations for the aforementioned analyses.

For the development of a nutraceutical product, it is essential to adopt a comprehensive approach that considers the origin of the raw material, the biotechnological transformation process, the microbial strains and their optimal conditions, the desired product characteristics, and the best preservation methods to maintain its properties over time.

6. Material and methods

Samples collection

Two Buffalo cheese whey samples, collected from the local producers of Campania Buffalo mozzarella cheese were provided from "NGN New Generation Nutraceutical" as Cheese exhausted whey (CEW), the by-product of Mozzarella cheese manufacturing process, and Ricotta Cheese exhausted whey (RCEW), the by-product of Ricotta cheese manufacturing process; samples were stored at 4 °C.

Metabarcoding analysis

- **DNA extraction**

One sample was collected for each type of Cheese whey, frozen and powdered by lyophilization and DNA was extracted with 3C-TAB method (Beccaccioli et al., 2021), based on the use of a three C-TAB buffer [C-Tab I (C-Tab 4%); C-Tab II (C-Tab 10%, NaCl 0.7 M); C-Tab III (C-Tab 1%, Tris–HCl 50 mM)] and Solution 2A (NaCl 2.8 M, Tris–HCL 200 mM at pH 8, EDTA 40 mM at pH 8). DNA was extracted from 2 mL of CEW and RCEW placed in sterile 2 ml tubes. Extracted DNA was treated with RNase (20 mg/mL, Merck-Sigma-Aldrich, Italy), quality/quantity was checked by agarose gel (1%) and spectrophotometric technology (NanoDrop, ThermoScientific, United States).

- **DNA amplification by PCR**

PCR amplification was performed on 25 ng of DNA of each sample. For recognized the bacterial community were used universal primers 27-For (5′-GAGATT TGATCCTGGCTCAG-3′) and 1495 Rev. (5′-CTACGGCTACC TTGTTACGA-3′) specific for bacteria 16S rDNA region (Mora et al., 1998). Primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) have been used to identify the fungal ITS rDNA region (Schoch et al., 2012). Amplification was performed by using Taq polymerase (Bioline, Meridian Bioscience United States), and the PCR products were verified through gel electrophoresis in agarose gel (1%).

- **Nanopore sequencing**

PCR amplicons of each sample were pooled using a ratio of 1:1 (amplicon 16S: amplicon ITS) and purified using AMPure beads. About 45 ng were used for library preparation using the Rapid Barcoding Kit 96 (SQK-RBK 110.96) following the manufacturer's instructions (Oxford Nanopore Technologies, United Kingdom). The run was performed by Flongle Flow Cell (R9.4.1) on a Mk1B device (Oxford Nanopore

Technologies, United Kingdom). Amplicon sequencing run was performed for one o.n. Basecalling and demultiplex was performed by MinKNOW software (v22.08.9) running Guppy software (v6.2.11 + e17754e) (Beccaccioli et al., 2023).

- **identification of microbial isolated strains**

For the isolation and identification of the microbial strains, the cheese whey samples were diluted with serial decimal dilution and plated on the Man, Rugosa, and Sharpe (MRS) agar for bacteria and on YPD (2% glucose) for yeasts, incubated aerobically for 24 h at 28 °C. The most abundant grown colonies were individually streaked on MRS agar and stored at – 80 °C in 20% (v/v) glycerol. DNA extraction of isolated microorganisms was carried out using microLYSIS Buffer (Labogen, Rho, Italy) following the manufacturer's instructions and 3 µl of genomic DNA were used for the amplification of 16S ribosomal RNA (rRNA) for bacteria and the Internal Transcribed Spacer (ITS) region for yeasts and fungi in a Mastermix containing: 5 µl of 10x AccuBuffer (Meridian, Bioscience Inc, Cincinnati, USA), 2 µl 50 mM MgCl2, 0,5 µl 100 mM dNTP MIX, 1 µl for each primers, 1 µl ACCUZYME DNA Polymerase 2,5 U/µL. For the amplification of specific 16S ribosomal RNA (rRNA), universal bacterial primer P0 and P6 were used and ITS4 and ITS5 primers were used to amplify the yeast-specific ITS4-5 region, sequences are reported in **table 13**. The PCR protocol used was: 95 °C x 3' + (95°C x 15'' + 55 C° x 15'' + 72 °C x 2') x30 + 72 °C x 10'. PCR amplification was verified by agarose gel electrophoresis. The band of about 1500 bp for 16S rRNA and 400-800 bp for ITS region were purified (Gel/PCR DNA fragments Extraction kit Geneaid cat. DF 100) and sequenced with both primers by a sequencing service (http://www.biofabresearch.it/index2.html). For the yeast strains the D1/D2 region of the 26S rDNA encoding gene was also amplified using the primers NL1 and NL4 as indicated in **table 12** following the protocol proposed by Lõoke et al., 2011. The resulting sequences were analyzed using the online BLAST nucleotide tool, available at https://blast.ncbi.nlm.nih.gov. Identified bacteria were tested for their potential probiotic activity.

Primer name	Oligonucleotide Sequence	REFERNCES
P0	5'-GAGAGTTTGATCCTGGCT	Younis et al., 2016
P6	5'-CTACGGCTACCTTGTTAC	Younis et al., 2016
ITS4	5'-TCCTCCGCTTATTGATATGC	McBreen et al., 2003
ITS5	5'-GGAAGTAAAAGTCGTAACAAGG	McBreen et al., 2003
NL1	5′-GCATATCAATAAGCGGAGGAAAAG-3′	Leaw et al., 2006
NL4	5′-GGTCCGTGTTTCAAGACGG-3′	Leaw et al., 2006

Table 12 Primers used for amplification of yeast specific region and their nucleotide sequence

Phylogenetic analysis

16S rRNA sequence was used to analyze the phylogenetic relationships among wild yeasts, using MEGA software (Molecular Evolutionary Genetics Analysis) v.11 (https://www.megasoftware.net) for alignment, construction and visualization of the phylogenetic tree (Tamura et al., 2021). Sequence alignments were performed using ClustalW, which was also used to generate phylogenetic trees using the bootstrap method with 1000 replications through neighbor-joining statistical method.

Resistance to lysozyme

Resistance to lysozyme test was evaluated by the (L. Samedi and A.L. Charles., 2019) protocol: overnight cultures of tested strains were centrifuge, washed two times with phosphate-buffered saline solution (PBS) and suspended in 2 mL of Ringer's solution (8,5 g/L NaCl, 0,4 g/L KCl and 0,34 g/L hydrated $CaCl_2$ Sigma-Aldrich) (0.1 M pH 7.0). 10% of bacterial suspension (around 200 µl) were inoculated in SES (0,22 g/L $CaCl_2$, 6,2 g/L NaCl, 2,2 g/L KCl, 1,2 g/L $NaHCO_3$) in presence of 100 and 50 mg/L of lysozyme (Sigma-Aldrich). Bacterial suspension without lysozyme was inoculated as a control. Strains viability was estimated as percent of CFU/mL after 0, 30 and 180 mins of exposure to lysozyme at 28 °C and plated on Man, Rugosa, and Sharpe (MRS) agar at 28 °C for 48 h.

Tolerance to acid pH and bile salts

Resistance to low pH was evaluated in vitro, simulating stomach conditions in 3 h, estimated time for digestion, where the environmental pH reaches value around 1.5/2.0 (Fujimuri., 2020) with (Prasad et al., 1998) method. An overnight culture of tested strain in Man, Rugosa, and Sharpe (MRS) broth for bacteria or YPD (2% glucose) for yeasts were growth, and cells were harvested by centrifugation for 10 mins at 2500 g and washed once in Phosphate saline buffer (PBS). Cell suspension in PBS (0.5 mL) was diluted to 5 mL with PBS adjusted to pH 2 and 3 with HCl. The incubation mixtures were maintained stirred at 28 °C and viable microorganisms were enumerated after 1,2 and 3 h after plating on Man, Rugosa, and Sharpe (MRS) agar or YPD agar and incubated at 28 °C for 48 h. Results were expressed in % viability:

$$\% \text{ viability} = \frac{(\log(\text{CFUNt})}{(\log(CFUNi)} \text{ x } 100$$

Where CFU_{Ni} and CFU_{Nt} denote the log CFU mL^{-1} at time 0 and after different time intervals, respectively.

For resistance to bile salts the protocol of (Schifano et al., 2021) was used with PBS with 0.3 and 0.5 % of bile salts (Sigma-Aldrich) and results expressed as growth rate CFU/mL.

Tolerance test to antibiotics

The susceptibility of potentially probiotic strains was determined toward 15 antibiotics belonging to inhibitors of cell wall synthesis, inhibitors of protein synthesis and inhibitors of nucleic acid synthesis (**Table 13**). Bacterial cultures were grown in Man, Rugosa, and Sharpe (MRS) broth at 28 °c overnight to obtain a density of 10^8-10^9 cells/mL. MRS agar plate were inoculated with 200 µl of cellular suspension 10^6-10^7 cells/mL and kept at room temperature for 1 h; antibiotic discs were dispensed on to the solid media and incubated at 28 °C for 24 h in aerobic conditions. Inhibition zones are measured, and susceptibility is expressed in terms of resistance (R), moderate susceptibility (MS), and susceptibility (S) that depends on Interpretative zone diameters (mm) (Charteris et al., 1998).

	Antimicrobial		Interpretative zone diameters		
Group	Name	Disc concentration (µg)	R	MS	S
Inhibitors of cell wall synthesis	Cefuroxime	30	≤ 15	16-17	≥ 18
	Cephalothin	30	≤ 14	15-17	≥ 18
	Ampicillin	10	≤ 12	13-15	≥ 16
	Cefotaxime	30	≤ 14	15-22	≥ 23
	Azreonam	30	≤ 15	16-21	≥ 22
	Vancomycin	30	≤ 14	15-16	≥ 17
	Penicillin G	10	≤ 19	20-27	≥ 28
Inhibitors of protein synthesis	Gentamicin	10	≤ 12	-	≥ 13
	Chloramphenicol	30	≤ 13	14-17	≥ 18
	Amikacin	30	≤ 15	16-17	≥ 18
	Streptomycin	25	≤ 11	12-14	≥ 15
	Tetracycline	30	≤ 14	15-18	≥ 19
	Clindamycin	2	≤ 8	9-11	≥ 12
	Erythromycin	15	≤ 13	14-17	≥ 18
Inhibitors of nucleic acid synthesis	Rifampicin	30	≤ 14	15-17	≥ 18

Table 13 Antimicrobial agents and associated interpretative zone diameters for disc diffusion antibiotic susceptibility testing. Susceptibility expressed as R (resistant), MS (moderately susceptible), or S (susceptible).

Proteolytic activity

Protease activity test was performed on skimmed milk agar (SMA) prepared following the (Abdelmoteleb et al., 2017) protocol: a 10 % (w/v) stock of skimmed milk powder and agar solution 2,5 % was prepared and autoclave separately at 121 °C for 20 min; the two solutions were mixed to a final concentration of 1 % skimmed milk. Tested strain were spotted on SMA plates and incubated 3 days at 28, 37 and 42 °C, the formation of clear zone around the colony was considered as protease positive.

Antimicrobial activity

Evaluation of antimicrobial activity was carried out using indicator strains *Pseudomonas aeruginosa* PA01, *Escherichia coli* NCIMB11943, *Bacillus cereus* NCIMB9373, *Staphylococcus aureus* NCDO949 belonging to the culture collection of Department of Biology and Biotechnology "C. Darwin" (Sapienza University of Rome, Italy) and routinely propagated in Luria Bertani (LB) broth at 37 °C. Two-hundreds microliters of an overnight culture of the indicator strains were plated onto surface of MRS plates and kept at room temperature for 1 h. Ten microliters of the LAB strains cultures were spotted on the plate. Also, the supernatants of the LAB 24h and 48h cultures, sterilized through filtration (Millipore, 0.22 μm pore size) were tested. The diameter (mm) of the inhibition zones was measured after 48 hours of incubation at 37 °C.

Early adhesion and biofilm formation in potential probiotic bacteria

The adhesion capacity of the tested strains was assessed using the Clinical BioFilm Ring Test (cBRT) protocol, following the methodology described by Di Domenico et al. (2016). *Lactobacillus paracasei* I 1688, a strain with minimal adhesion capacity at 5 hours, was employed as a negative control (Truglio et al., 2024). Conversely, *Staphylococcus aureus* ATCC 6538, a well-established biofilm producer at 5 hours, was used as positive control.

Potential prebiotic activity of isolated yeast strains

To assess prebiotic activity, the tested strains were grown in co-culture with a known probiotic bacterium as *Lactobacillus rhamnosus* ICIM03 isolated from BIONIKE AKNET PRO-SKIN, to determine their ability to enhance the growth and activity of these strains. Yeast strains were initially growth in a pre-culture for 16h at 37 °C and then yeast cell concentration was adjusted to 2 x 10^6 Cell/ml. Co-cultures were established at yeast:bacteria ratios of 1:1 and 1:10. Cells count were performed using a Thoma chamber after 0, 3, 6 and 24 hours.

Influence of temperature and lactic acid on the use of different sugars on Yeast growth

Yeast strains were grown overnight in YPD broth (Bacto Peptone 2%, Bacto Yeast Extract 1%, Glucose 2%) at 30 °C in a 15 ml culture tube containing 5 ml of medium. The OD_{600nm} was measured, and the cells were diluted to value of 0.5 in 5 ml of fresh medium and incubated at 30 °C for 3 hours, when the OD_{600nm} of the samples were roughly 1-2. Cultures were centrifuged at 5000 rpm for 5 min and supernatant was discarded. Pellets were resuspended in 5 ml of sterile water and were washed as above two more times. Each sample was diluted 1:10 and OD_{600nm} was measured again and normalized with the lowest OD_{600} value. Subsequently, 200 µl of each pure sample was added to a sterile 96-well microtiter plate and serial dilution were prepared in subsequent columns. A 3 µl drop of each dilution was deposited on the appropriate agar medium and incubated at 30 °C and 37 °C for 48 hours. Dilution spot assay was performed on different S - Minimal Media agar plate (BactoAgar 2%, Carbon source 2%) with different carbon source (Glucose, Lactose and Galactose) supplemented with a 10% Mineral solution (Ammonium sulfate 5%, Potassium phosphate 3% and Magnesium sulfate h epta hydrate 0,5%) and trace elements at 0.1%. To test resistance to lactic acid, Minimal media agar plates were prepared with a final lactic acid concentration of 0, 50, 75, 100 and 200 mM with pH adjusted to 3 with HCl (Kuanyshev et al., 2021).

Checking for the presence of gene LAC12 and LAC4 for lactose consumption

Yeast Genomic DNA Extraction

One yeast colony was picked from the plate and suspended in 100 µl of 200 mM LiOAc, 1% SDS solution. Yeast suspension was incubated for 5 minutes at 70 °C and then 300 µl of 96-100 % ethanol was added before vortex. DNA and cellular debris were spinned down at 15000 g for 3 minutes. Pellet was washed with 300 µl of 70 % ethanol and spinned at 15000 g for 3 minutes; then supernatant was removed by pouring, was centrifuged again and the remainder was removed with pipette. The pellets were dissolved in 100 µl of H2O or TE and cellular debris were spinned down for 15 seconds at 15000 g. 1 µl of supernatant was used for PCR.

For the identification of the 4 isoforms of LAC12 for lactose permease and LAC4 for beta-galactosidase were used primers kmLAC12, LAC12-2, LAC12-3, LAC12-4 and LAC4 (sequences in **Table 14**) PCR master mix was composed by: 6 µl of water, 1 µl for each primer, 10 µl of OneTaq 2X MM w/Standard Buffer (New England Biolabs,

Ipswich, Massachusetts, USA) and 2 µl of template. PCR cycle was set as follow: 94 °C x 6′ + (94 °C x 30″ + 52 °C x 45″ + 68 °C x 3′) x30 + 68 °C x 5′.

Primer name	Oligonucleotide Sequence
kmLAC12-1-F	5'- ATGGCAGATCATTCGAGCAGCTCA
kmLAC12-1-R	5'- TAACAGATTCTGCCTCTGAAAGCTT
LAC12-2-ORF-F	5'-ATGACAGAAAAGTGGAGTGAATCATC
LAC12-2-ORF-R	5'-CAAACTGATAGTGAATCAACCTCTTC
LAC12-3-ORF-F	5'-ATGGCAGACACTTCTAGCTTG
LAC12-3-ORF-R	5'-CAAACGTACTCCTTTATTTTGATTAATTTCTC
LAC12-4-ORF-F	5'-ATGACAGAAAAGTGGAGTGAATCA
LAC12-4-ORF-R	5'-TTAAACTGATTCCAAATCGGAGACT
LAC4-ORF-F	5'-ATGTCTTGCCTTATTCCTGAGAA
LAC4-ORF-R	5'-TTATTCAAAAGCGAGATCAAACTCA

Table 14 Primers used for amplification of yeast specific region and their nucleotide sequence (Varela et al., 2017)

Kinetics growth and lactose consumption of yeast strains on minimal media and spray-dried cheese whey

Yeast strains were cultivated overnight in YPD medium in 250 ml shake flasks at 30 °C and OD_{600} was adjusted to a value of 0.1 in 50 mL of S – Minimal Media with 2% lactose as carbon source. Culture was incubated at the same condition and OD's culture was measured every 2 h for 48-72 h. Spray-dried cheese whey was resuspended in distilled water and used as a medium for the inoculation of the strains and treated in the same conditions. For each measurement a sample was collected for the quantification of lactose using HPLC.

Cheese exhausted whey fermentation

The RCEW was supplied by a local dairy in the Caserta area belonging to the Consortium for the Protection of Mozzarella di Bufala Campana DOP and stored at -20 °C. RCEW was initially autoclaved at 121°C for 20 min for sterilization and following inoculated with a 24 h pre-inoculum of yeast strain in single culture, growth in YPD (2% glucose) and inoculated with OD_{600}= 0.5 into a 2 L bioreactor with a working volume of 1.5 L. The fermentation conditions were set for 24 h fermentation under conditions of aeration and continuous stirring at 28 °C. pH and O_2 concentration were monitored. Fermented RCEW was centrifuged at 4600 rpm for 10 min, the supernatant was collected and freezed for lyophilization. Freeze-dried RCEW was used for all in-vitro tests to verify its nutraceutical properties.

Antioxidant activity test: ABTS Radical Scavenging Assay

The ABTS assay is an antioxidant protocol based on the ability of the molecules to react with $ABTS^{\bullet+}$ radicals (2, 20-azinobis (3-ethylbenzotiazoline-6-sulfonate)). ABTS solution was obtained by shaking 2.5 mL of an ethanolic solution of ABTS 7.0 mM with 44 μL of an aqueous solution of potassium persulfate 140 mM, which was stored for at least 7 h at 5 ◦C in darkness. After this time, the working solution was prepared by diluting the obtained mixture with an ethanol-water solution until an absorbance value of 0.700 ± 0.05 was acquired at 754 nm (Jasco Inc., Easton, MD, USA). The assay was performed by mixing 1000 μL ABTS working solution with 100 μL of the sample previously diluted in the extraction solvent. The mixture was incubated for 2.5 min in the darkness. After this time, the sample absorbances were read at 734 nm. Autozero was made with ethanol while the blank was obtained by replacing the samples with 100 μL of solvent used for the resuspension of the freeze-dried fermented RCEW. The radical inhibition was calculated according to the formula: $[1 – (A_S/A_B)] \times 100$, where A_S is the absorbance of the sample after 2.5 min, and A_B is the absorbance of the blank at time zero. The antioxidant standard used as a positive control was Trolox. The results are expressed both as μmol of TE and EC_{50}, which is the amount of antioxidants necessary to decrease the initial ABTS•+ concentration by 50% (Maisto et al., 2023).

DPP-IV inhibitory activity assay

DPP-IV inhibitory activity assay was performed using DPPIV Drug Discovery Kit, Enzo Life Sciences following the protocol provided by the kit. P32/98 inhibitor was diluted 1:10, fluorogenic substrate (H-Gly-Pro-AMC) was diluted 1:50 and the DPPIV enzyme was diluted to obtain a stock concentration of 17.3 μU/μL, using the assay buffer for all dilutions. Assay buffer was added to each desired well of microplate so that the total assay volume was 100 μL, see **Table 15** for examples. Microplate and diluted assay components were equilibrated to assay temperature of 37°C. DPPIV enzyme previously diluted was added to the "Control", "Inhibitor" and "Test Sample" wells; the final amount of DPPIV per well was to be 0.26 mU. 10 μL P32/98 inhibitor was added to the "Inhibitor" well, to obtain a final inhibitor concentration of 10 μM. 50 μL of test samples were added to appropriate wells. Plate was incubated for 10 min at reaction temperature to allow inhibitor/enzyme interaction. Reaction was initiated by adding 50 μL of the diluted fluorogenic substrate, pre-equilibrated to reaction temperature, to each well. Plate was read continuously at Ex:380 nm/Em:460 nm and data were recorded at 1 min. interval from 0 to 15 minutes. Data analysis was performed to calculate % DPP-IV inhibition and evaluate inhibitor activity based on fluorescence intensity changes.

Sample	Assay Buffer	DPPIV (17.3 µU/µl)	Inhibitor	Substrate
Blank	50 µL	0	0	50 µL
Control	35 µL	15 µL	0	50 µL
Inhibitor‡	25 µL	15 µL	10 µL	50 µL
Test sample*	X µL	15 µL	Y µL	50 µL

Table 15 * Test sample is the experimental inhibitor. Experimental inhibitor was dissolved into assay buffer and added to appropriate wells at desired volume "Y". Volume "X" was adjusted to bring the total volume to 100 µL (X + Y= 35 µL)

ACE – inhibitory activity assay

The assay was performed according to the procedure described by Wu et al. 2002 with slight modification, testing fermented RCEW with three isolated yeasts in single culture. Freeze-dried fermented RCEW was resuspended in sodium borate buffer 0,1 M pH 8,3 and 25 µl of each sample was added to 50 µl 5 mM Hippuryl-L-histidyl-L-leucine (HHL) and incubated at 37 °C for 10 min. Then 20 µl of ACE enzyme 100 mU/ml was added, following by incubation for 1 h at 37 °C. The reaction was terminated by the addition of 100 µl of 1 M HCl and then centrifuged at 14,000 g for 10 min. Finally, the solution was filtered through a 0.45-µm nylon syringe filter for HPLC analysis. Negative control was prepared using the same procedure replacing inhibitor extract with sodium borate buffer 0.1 M, pH 8.3. Blank negative control was prepared using the same procedure replacing inhibitor extract with sodium borate buffer (0.1 M, pH 8.3) and ACE enzyme with Trizma® hydrochloride and blank sample was prepared using the same procedure replacing ACE enzyme with Trizma® hydrochloride for each sample. HPLC analysis was performed on Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD) equipped with photo diodearray detector and autosampler. All data were collected and processed with JASCO ChromNAV (version 2.02.04). The chromatographic separation was tested on analytical column Kinetex C18 (250 × 4.6 mm, 5 µm, 100 Å) and security guard column both supplied by Phenomenex (Torrance, CA, USA). The analysis, according to Wu et al., 2002, was performed at a flow rate of 1 ml/min with solvent A (0.05% TFA in water) and B (0.05% TFA in acetonitrile). Elution was performed according to the following linear gradient: 5–60% B for the first 10 min, maintained for 2 min at 60% B and then returned to 5% B in 1 min. This was followed by isocratic elution for 4 min at a constant flow rate of 0.5 ml/min. Calibration curve of HA, detected at 228 nm, was performed with standard samples prepared freshly daily over a concentration range of 0.1–1.0 µg/µl with six

different concentration levels (Schisano et al., 2019). The ACE inhibitory activity was calculated as percentage inhibition:

$$\% \text{ inhibition} = (1 - (Abs_{control}/Abs_{extract})) \times 100$$

Where $Abs_{control}$ is the absorbance of control prepared with distilled water while $Abs_{extract}$ is the absorbance of the samples.

Determination of Vitamin B6 and B9 content

To extract the vitamins of group B, 1 g of the freeze-dried fermented RCEW was weighed and resuspended in 5 mL of distilled water with 1% (*v*/*v*) formic acid. The mixture was vortexed for 1 minute to ensure initial homogenization. The sample was then subjected to ultrasonic treatment for 10 minutes and agitated in a thermomixer at 25°C and 600 rpm for 10 minutes to enhance solubilization. Samples were centrifuged at 13,000 × g for 10 minutes at room temperature. The resulting supernatant was carefully collected and filtered through a 0.45 μm syringe filter and transferred to a clean Falcon tube. The pellet obtained after centrifugation was resuspended in an additional 5 mL of distilled water with 1% (*v*/*v*) formic acid. The extraction process, including vortexing, sonication, agitation, centrifugation, and filtration, was repeated as described above. HPLC analysis was performed on Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD) equipped with photo diodearray detector and autosampler. All data were collected and processed with JASCO ChromNAV (version 2.02.04). The chromatographic separation was tested on analytical column Kinetex C18 (250 × 4.6 mm, 5 μm, 100 Å) and security guard column both supplied by Phenomenex (Torrance, CA, USA). The analysis was performed at a flow rate of 1 ml/min with solvent A (0.01% TFA in water) and B (acetonitrile). Elution was performed according to the following linear gradient: 20–70% B for the first 30 min, maintained for 4 min at 70% B and then returned to 2% B in 1 min. This condition was maintained for a further 5 min. The calibration curve of vitamins, detected at 290 nm, was performed with standard samples prepared freshly daily over a concentration range of 0.1–1.0 μg/μl with six different concentration levels.

7. General conclusions and perspectives

The management of waste from the food industry has become a challenge of growing interest today due to the impact it can have on the health of planet Earth, which has repercussions on the health of human beings. A constantly growing sector such as the dairy industry, due to the continuous increase in the world population, inevitably causes the production and release of a vast variety and quantity of waste. Due to the high nutritional and economic value of this waste, wasting it would mean an enormous loss with significant impacts and therefore require increasingly cutting-edge strategies that also meet market needs. Today, there are several differently classified products on the market, which derive from the recovery of waste from the dairy industry which produces cheese whey as its main by-product. Specifically, in the regions of southern Italy, where the production of Mozzarella di Bufala Campana DOP takes place, there are products with unique characteristics due to the origin of the milk; this leads to having a unique cheese whey for compositional characteristics and rich in bioactive molecules such as peptides that have a beneficial action on the human body. However, these compounds are present in the form of inactive molecules within the long amino acid sequences but once released they can exert a multitude of beneficial effects ranging from an antioxidant, antihypertensive, antidiabetic, antitumor or immune system regulation activity. It must also be considered that in the processing environments a microflora typical of that place is established which characterizes the final product in its organoleptic, aromatic and sensorial characteristics. These microorganisms, from in-depth studies, have been found to possess beneficial characteristics for the human body as well as for their application in biotechnological processes. Therefore, combining the uniqueness of the product from a molecular and microbiological point of view, it is essential to find new innovative processes for its recovery. One of these has been the application of biotechnological processes such as fermentation which, thanks to the activity of microorganisms, has improved the quality of the product, giving us a so-called value-added product. These products defined as nutraceuticals combine nutritional qualities with pharmaceutical qualities and are attracting increasing interest for having the characteristic of being of natural origin but with beneficial effects of various types on the human body. The in vitro results obtained from the fermentation of cheese whey using different isolated yeast strains in monoculture are promising. These findings support the potential for further, more detailed characterization of the fermented cheese whey, and open the possibility of evaluating these bioactive compounds in vivo. Nevertheless, despite the positive in vitro outcomes, the specific identity and quantity of the active molecules responsible for the observed effects remain unclear. To address this, a comprehensive and detailed molecular analysis will be essential. From a microbiological point of view, however, it is interesting to note the uniqueness of the microflora that inhabits the Campana

Buffalo cheese whey, strongly characterized by the environment and the origin of the milk that comes from buffaloes raised according to stringent rules imposed by very rigid production specifications. Another perspective is that obtaining products with low lactose content thanks to the ability to consume this rich carbon source in milk, offering the market a product accessible to a wider range of consumers. Specifically, regarding the microorganisms isolated and tested for their characteristics, it is known that yeasts possess excellent biotechnological and phenotypic characteristics making them suitable for some different application. Bacteria, on the other hand, are known to influence the organoleptic component of the processed product to a greater extent, while with regard to their possible use as probiotic strains, some species, such as those belonging to the Enterococcus genus, require a more in-depth genotypic characterization to reveal the possible presence of virulence genes, being known to be strains potentially pathogenic for humans.

Future research should focus on optimizing fermentation conditions to maximize bioactive compound production and improve the stability of functional ingredients derived from cheese whey. Moreover, in-depth studies on the safety and regulatory aspects of newly identified microbial strains are necessary to facilitate their commercial application. Additionally, integrating cheese whey valorization into a circular economy framework can contribute to sustainable waste management strategies, reducing the environmental impact of the dairy industry while promoting the development of innovative bioproducts.

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