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Functional benefits of probiotic fermented dairy drink elaborated with sheep milk processed by ohmic heating

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Abstract:	This study examined the effects of ohmic heating pasteurization on probiotic fermented sheep milk compared to conventional heating by analyzing microbial viability, volatile compounds, riboflavin content, and bioactive peptides in the resulting dairy product. Four batches of milk were processed using conventional heating (CH) and three different ohmic heating (OH). Microbiological analysis demonstrated the safety of OH-processed milk. Probiotic viability remained consistent during fermentation and storage, and the simultaneous inoculation of bacteria reduced fermentation time, indicating cooperation interplays. OH-processed probiotic fermented sheep milk showed a substantial increase in antioxidant, antidiabetic, antihypertensive activities, and riboflavin content. Moreover, the flavor profile was characterized by specific volatile compounds. Multiple-factor analysis was performed to identify relationships between samples and various parameters, offering insights into the impact of processing methods. In conclusion, OH enhanced the health-promoting and flavor-related compounds of probiotic fermented sheep milk, suggesting its application to produce functional dairy products.
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November 28th, 2023

To: Dr. Jian Chen

Editor-in-Chief, Food Bioscience

Dear Editor,

I have to inform you that, I am corresponding author to the original research manuscript entitled "**Functional benefits of probiotic fermented dairy drink elaborated with sheep milk processed by ohmic heating"** and I hereby request to consider it for publication in **Food Bioscience**.

This study compared ohmic heating to conventional heating pasteurization to elaborate a probiotic fermented sheep milk. Briefly, microbial analysis ensured the safety of ohmic processing; probiotic viability remained consistent, reducing fermentation time; and ohmic processing increased antioxidant, antidiabetic, and antihypertensive activities, suggesting its potential for enhancing functional dairy products.

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Respectfully,

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Functional benefits of probiotic fermented dairy drink elaborated with sheep milk processed by ohmic heating

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Abstract

This study examined the effects of ohmic heating pasteurization on probiotic fermented sheep milk compared to conventional heating by analyzing microbial viability, volatile compounds, riboflavin content, and bioactive peptides in the resulting dairy product. Four batches of milk were processed using conventional heating (CH) and three different ohmic heating (OH). Microbiological analysis demonstrated the safety of OH-processed milk. Probiotic viability remained consistent during fermentation and storage, and the simultaneous inoculation of bacteria reduced fermentation time, indicating cooperation interplays. OH-processed probiotic fermented sheep milk showed a substantial increase in antioxidant, antidiabetic, antihypertensive activities, and riboflavin content. Moreover, the flavor profile was characterized by specific volatile compounds. Multiple-factor analysis was performed to identify relationships between samples and various parameters, offering insights into the impact of processing methods. In conclusion, OH enhanced the health-promoting and flavor-related compounds of probiotic fermented sheep milk, suggesting its application to produce functional dairy products.

***Keywords*:** electric field, fermented sheep milk, ohmic heating, bioactive peptides, probiotic, riboflavin.

Highlights

- Peptide variation due to heating methods (CH vs. OH);
- OH processing impacted positively on bioactive peptides;
- 12 bioactive peptides were identified;
- Fifty-two volatile compounds were profiled.
- Outstanding riboflavin increases on probiotic fermented sheep milk processed by OH.

1 Introduction

The rising demand for probiotic dairy products is driven by consumers looking for flavorful and healthful options (Kabir et al., 2023). While bovine milk products are prominent, small ruminant milk, such as sheep milk, is emerging as a viable source for fermented dairy with potential metabolic and health benefits. Sheep milk has higher total solids compared to cow milk and similar hypoallergenic claim of goat milk, being recognized as an excellent source of nutrients and functional compounds to promote human well-being (Balthazar et al., 2017; De Devitiis et al., 2023).

Despite the advantages of functional foods, these products face challenges related to sensory characteristics and practicality. Conventional heat treatment methods applied to food products can impact the sensory attributes and reduce the content of bioactive compounds. In this sense, emerging food processing technologies have recently been explored to overcome health and sensory pleasure challenges. Within the domain of food processing, these technologies aim to employ non-thermal or optimized thermal treatments to minimize nutrient loss, preserve bioactive compounds, and ensure food safety (Bento et al., 2023; De et al., 2023; Krishnamoorthy et al., 2023; Nath et al., 2023). In recent years, ohmic heating (OH) has shown promise in enhancing bioactive properties and is an economically efficient alternative for processing sheep milk (Balthazar et al., 2022, 2024).

OH relies on electrical conductivity for heating and offers rapid and uniform heating of food materials. It involves the passage of alternating current through a given food, generating internal heat due to electrical resistance. OH consumes less energy than conventional heating, making it an environmentally

79 friendly option. OH is suitable for various food processing applications, including
80 blanching, evaporation, dehydration, fermentation, extraction, sterilization, and
81 pasteurization (Kaur et al., 2023; Kubo et al., 2023).

82 The high levels of proteins and fat in sheep milk allow the production of
83 yielder cheese and ice cream, which can protect probiotic bacteria during
84 digestion and commercial storage (Szopa et al., 2023). Moreover, the high
85 protein content provides essential nutrients for microbial growth, reducing
86 fermentation time without affecting the manufacturing process (Balthazar et al.,
87 2017). Probiotics, such as *Lactobacillus* and *Bifidobacterium*, are well-known
88 food-grade microorganisms that benefit the host. The characterization of
89 potential probiotics involves *in vitro* testing to assess their resistance to gastric
90 juice and enteric enzymes. Selecting suitable food matrices is crucial to
91 enhance probiotic survival through gastrointestinal barriers and to promote
92 efficient intestinal colonization (Coelho-Rocha et al., 2023; Yeung et al., 2023).

93 The species *Lactiplantibacillus plantarum* is commonly found in naturally
94 fermented foods, including, among others, dairy, meat, plant-based products,
95 and wine. A wide range of beneficial activities, such as regulating intestinal
96 microflora, reducing serum cholesterol levels, and stimulating the immune
97 system, has been reported for strains belonging to *L. plantarum* (Zhao et al.,
98 2023; Rocchetti et al., 2023). *Lactiplantibacillus plantarum* B2 is a spontaneous
99 roseoflavin-resistant riboflavin-overproducing food-grade strain showing
100 remarkable probiotic properties both in laboratory settings and in live organisms
101 (Arena et al., 2014; Russo et al., 2015). This strain has been extensively
102 investigated for applications in the food industry, either as a starter culture or as
103 a probiotic additive (Russo et al., 2015; Russo et al., 2016; Zhu et al., 2023).

Furthermore, *L. plantarum* B2 is noteworthy for its ability to produce significant amounts of riboflavin (vitamin B2), one of the key components in metabolic processes, serving as a precursor to flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) (Prieto-Parades et al., 2023; Ripa et al., 2022). Since humans cannot synthesize riboflavin alone, dietary supplementation is crucial. Nevertheless, certain microbes in the large intestine can also contribute to riboflavin production. Identifying microbial strains with both probiotic qualities and the capacity for riboflavin overproduction opens the potential for formulating functional foods that offer additional health benefits to the host (Tang et al., 2023).

This study aimed to assess the effects of ohmic heating pasteurization on probiotic fermented sheep milk previously processed through ohmic heating with varying electric field strengths or traditional heating methods. The study primarily evaluated the dairy product's microbiological aspects, volatile compounds profile, and health-related parameters such as riboflavin and bioactive peptides.

2 Material and Methods

2.1 Sampling and sheep milk processing

The experiment was carried out with raw sheep milk (4.81% fat w/v, 5.10% protein w/v, 4.63% lactose w/v, 14.97% solids nonfat w/v, 0.85% ashes w/v 1.035 g/mL density, 0.693 °H freezing point, pH 6.71 and 26 °D) collected from bulk tanks of Lacaune sheep herd located in northeast São Paulo region, Brazil. The milk was divided into four batches of 1 L to be processed by conventional heating (CH) or three different ohmic heating (OH) electric field strengths (EFS)

129 to produce the fermented milk samples, as described below. Samples were
130 coded as CH fermented sheep milk (CHFM) or OH processed fermented sheep
131 milk by applying 4 V/cm EFS (OH4FM), 7 V/cm EFS (OH7FM), and 10 V/cm
132 EFS (OH10FM).

133 For CH, 1 L of raw sheep milk was heated in a double jacket stainless
134 steel container until it reached 73±1 °C and held for 15 seconds. Then, it was
135 immediately cooled in an ice bath (0±2 °C) to reach 32±1 °C prior to bacteria
136 inoculation for fermentation. In contrast, OH processing was carried out in an
137 OH cell composed of a 5 L food-grade polycarbonate container (220 x 252 x
138 104 mm) and titanium electrodes (200 x 250 mm with 1.6 mm thickness) with a
139 100 mm gap. The system was composed of a voltage variator (TDGC2-2kVA,
140 JNG®, Shenyang, China) from 0 to 220 V, which was performed in experimental
141 voltages (40, 70 and 100 V) at 60 Hz frequency with respectively electric field
142 strength: 4, 7 and 10 V/cm. OH processing data were collected by digital
143 multimeters (ET-2042E, Minipa, Brazil) measuring the voltage (V), temperature
144 (°C) profiles, and electrical current (A) in the system to monitor the processing.
145
146 *2.2 Bacterial strains activation and fermented sheep milk production*

147 *Streptococcus thermophilus* TH-4® (Chr Hansen, Valinhos, Brazil) was
148 used as a microbial starter. The probiotic *Lactiplantibacillus plantarum* B2,
149 deposited at the Spanish Type Culture Collection (CECT, Paterna, Spain) under
150 the Budapest Treaty conditions with the code CECT 8328, was kindly provided
151 from the microbial collection of the Industrial Microbiology group of the
152 University of Foggia. The starter and the probiotic were activated according to
153 Zacarchenco et al. (2006) and Balthazar et al. (2019), respectively, achieving

10 $\log_{10}$ CFU/mL. Then, 15 mL of each strain were inoculated in sheep milk samples, gently mixed, and incubated at 35 °C (± 1) for 8 h until pH reached 4.3 (±1) and stored for 30 days under refrigerated conditions (4 ±1 °C).

2.3 In vitro gastrointestinal simulation assay

The digestion assay was performed in triplicate on days 1, 15, and 30, following the procedure described by Minekus et al. (2014) and Soares et al. (2019). Briefly, 5 g of each probiotic fermented sheep milk sample was put in a 3.5 mL salivary fluid solution containing 50 µL $CaCl_2$. NaOH 1M was used to adjust pH to 7 and purified water to complete 10 mL solution. Samples were exposed to this solution for 2 min at 37 °C to mimic the oral phase of digestion. As the probiotic fermented sheep milk did not contain some other carbohydrates besides the intrinsic lactose of sheep milk itself, the amylase enzyme did not need to be included in this first step of *the in vitro* digestion protocol.

Then, 7.5 mL of a gastric fluid solution containing 10 mg porcine pepsin and 3.08 mg lipase enzymes (Sigma-Aldrich Brasil Ltda, São Paulo, Brazil), and 10 µL $CaCl_2$ was added to the solution, and pH was adjusted to 3 and completed with purified water to 20 mL. Samples were exposed to the gastric solution under 200 rpm agitation for 2 h at 37 °C to simulate the gastric phase. Afterward, 11 mL of an intestinal fluid solution containing 66 mg bile and 4.5 mL of a pancreatin enzyme solution (Sigma-Aldrich Brasil Ltda) were added to mimic the intestinal phase. Then, the pH was adjusted to 5, and purified water was added to obtain 40 mL of intestinal solution. Samples were incubated at 37 °C for 1 h under agitation (200 rpm), followed by pH alkalinization up to 7 and further 1 h under agitation. Finally, digested samples were employed for

179 probiotic bacteria counting on MRS agar. Samples were immediately subjected
180 to microbiological analysis.
181
182 *2.4 Microbiological analyses*
183 Raw, CH, and OH sheep milk or probiotic fermented sheep milk samples
184 were subjected to serial dilutions (1:10) in sterile saline solution (0.9% NaCl
185 w/v) and counted by pour plate technique. For raw and processed sheep milk,
186 total aerobic mesophilic and psychrotrophic bacteria were analyzed using Plate
187 Count Agar (CM0325, Oxoid, Hampshire, England), incubated for 48 h at 36±1
188 ºC and 7±1 ºC for 7-10 days in aerobic condition, respectively.
189 Enterobacteriaceae count was carried out in Violet Red Bile Glucose Agar
190 (CM0485, Oxoid) incubated for 48 h at 36±1 ºC in microaerophilic conditions.
191 Lactic acid bacteria (LAB) and *Streptococci* were enumerated using MRS agar
192 (CM0359, Oxoid) and M17 agar (CM0785, Oxoid), respectively, in
193 microaerophilic conditions, after incubation for 72 h at 36±1 ºC. Molds and
194 yeasts were determined in aerobic conditions after 7 days of incubation at 25±1
195 ºC in DRBC agar base (K25-610237, KASVI, Porto Alegre, Brazil), according to
196 APHA (2015). Counts of probiotic fermented sheep samples were performed
197 following Codex Standard 243–2003 for fermented milk (Codex Alimentarius,
198 2010). Microbiological counts were carried out in triplicate either in raw sheep
199 milk and after CH or OH processed milk, as well as in probiotic fermented
200 sheep milk at days 1, 15, and 30.
201
202 *2.5 Physico-chemical analyses*

Sheep milk composition was obtained by infrared spectrometry device (MilkoScanTM FT3, Foss North America, Eden Prairie, USA). pH and titratable acidity were measured in sheep milk and probiotic fermented sheep milk, according to AOAC (2016). Titratable acidity was expressed as Dornic acidity (°D). Probiotic fermented sheep milk analyses were carried out in triplicate and performed on days 1, 15, and 30.

2.6 Riboflavin analysis

In order to determine the riboflavin content in probiotic fermented sheep milk, the method of Abranches et al. (2008) was adopted. Analysis was carried out in triplicate on day 1, and riboflavin was expressed in mg/100 g.

2.6 Bioactive peptides and volatile compounds identification and properties

Bioactive peptides and volatile compounds were identified in probiotic fermented sheep milk, according to Balthazar et al. (2023). Briefly, an Autoflex® maX MALDI mass spectrometer (Bruker Daltonics, Billerica, USA) equipped with a 355 nm Nd:YAG laser with 1 kHz frequency and accelerating voltage of 19 kV was used to acquire mass spectrum data in the ion detection range between *m/*z 0.7 to 3.5 kDa for bioactive peptides identification.

The volatile compounds were identified by gas chromatography-mass spectrometry (GC-MS gas chromatograph, Varian 3800, Sunnyvale, CA, with direct interface with the Varian 2000, Varian Spa Mass Spectrometer) with a CP-Wax 52 CB column (60 m, 0.25 mm id, film thickness of 0.25 mm) and compared with the linear retention index standards of the C8–C40 alkanes (Supelco, Sigma-Aldrich Brasil Ltda).

The bioactive properties of sheep milk were carried out according to Ayyash et al. (2018) spectrophotometric assays, which determined the antioxidant activity by 2,2-diphenyl1-picrylhydrazyl (DPPH) absorption at 517 nm, antihypertensive activity by angiotensin-converting enzyme (ACE) absorption at 228 nm, and antidiabetic activity by α-amylase and α-glucosidase enzymes absorption at 540 and 400 nm, respectively. Results were expressed as % of inhibition calculated by the equations proposed by the same authors. Analyses were carried out in triplicate on days 1, 15, and 30.

2.7 Statistical analyses

The study was completely randomized with three experimental replications in four conditions (CHFM, OH4FM, OH7FM, and OH10FM). The data obtained in the study were subjected to analysis of variance (ANOVA) accessed by GLM procedure using the method of least squares to fit general linear models ($p \leq 0.05$) to compute means and standard error (SE), performed in the software SAS Studio (SAS Institute Inc., Cary, NC). Multiple factor analysis (MFA) was used to analyze data obtained in probiotic fermented sheep milk analyses, according to Cruz et al. (2013). It involved the establishment of a matrix where the rows were the probiotic fermented sheep milk (4 lines), and the columns were the parameters: peptides mass-to-charge ratio (m/z), volatile compounds areas, and bioactivities measured by enzyme inhibition and riboflavin content. The matrix was plotted in the software XLSTAT for Windows version 2012.5 (Adinsoft, Paris, France).

3 Results and Discussion

3.1 Microbiological analysis of sheep milk and probiotic fermented sheep milk

The counts of total mesophilic, lactic acid, psychrotrophic, coliform bacteria, molds, and yeasts in raw bulk sheep milk were 3.4, 2.3, 1.6, 2.5, and 1.0 $\log_{10}$ CFU/mL, respectively. After processing by CH or OH, bacteria and fungi counts were under the detection limit for the pour plate method, showing that OH could be used to bring safety to processed milk adequately, as previously reported (Shin et al., 2020).

Concerning probiotic fermented sheep milk, no effect on lactic acid bacteria inoculated as a starter and probiotic after fermentation or during storage was observed ($p > 0.05$, Fig. 1A-B). *Streptococcus thermophilus* TH-4® reached a concentration of about 9.5 $\log_{10}$ CFU/mL after 8 h of fermentation in all the samples analyzed ($p > 0.05$), while *L. plantarum* B2 achieved a concentration around 9.3 $\log_{10}$ CFU/mL. Moreover, it was noted that the simultaneous inoculation of *S. thermophilus* TH-4® and *L. plantarum* B2 strongly reduced sheep milk fermentation time, compared to what was previously reported by using *L. plantarum* B2 as a single starter culture (Balthazar et al., 2018). This result may suggest a proto-cooperative behavior between *S. thermophilus* TH-4® and *L. plantarum* B2. For instance, *S. thermophilus* produces formate, a well-known cofactor in yogurt fermentation that accelerates the growth of LAB and plays a key role in determining the acidification rate of *S. thermophilus* as well (Mahony et al., 2023;).

A decrease from day 1 to day 30 around 0.2 $\log_{10}$ CFU/mL and 0.4 $\log_{10}$ CFU/mL ($p < 0.05$) was observed from refrigerated storage samples for *S. thermophilus* and *L. plantarum* B2, respectively. Moreover, no significant

277 difference was noted between the samples counting during storage ($p > 0.05$,
278 Fig. 1A-B). A reduction in LAB viability in fermented milk during storage is
279 expected due to temperature, O_2, and water activity over time (Jannah et al.,
280 2022). Nevertheless, a positive relationship was observed between those LAB
281 added to sheep milk since their concentration was higher than the minimum
282 expected counts for fermented milk according to the Codex Alimentarium (7
283 log_{10} CFU/mL 2010).

284 *L. plantarum* B2 was subjected to an *in vitro* digestion assay (Fig. 1C) to
285 investigate its ability to achieve the intestinal *loci* at an adequate concentration
286 to exert its beneficial effects (Ibrahim et al., 2023). This strain presented a
287 survivor rate of around 5.4 log_{10} CFU/mL in all samples at day 1, which
288 increased up to 6 log_{10} CFU/mL for CHFM and OH4FM and 6.8 log_{10} CFU/mL
289 for OH7FM and OH10FM ($p < 0.05$) in day 15, reaching at least 7 log_{10} CFU/mL
290 for OH4FM in day 30 ($p < 0.05$).

291 The acidity of probiotic fermented milk was also monitored, and an
292 increase during storage was observed (from pH 4.29 to pH 4.02 and titratable
293 acidity from 77.75 to 111.80 °D, $p < 0.05$; Fig. S1).

294 According to Zhao et al. (2023), *L. plantarum* develops extracellular
295 glycolipids and glycoproteins to surround the cell and protect itself from an acid
296 environment, an ability triggered when lactose content decreases.

297 According to the FDA (2023), a milk-based drink corresponds to 240 mL
298 per serving and at least 7 log_{10} CFU/mL probiotics per serving (Hill et al., 2014).
299 In the present study, all the analyzed samples contained a concentration of live
300 bacteria per serving in the dairy product above the minimum content required (6
301 log_{10} CFU/mL, Centurion et al., 2021), suggesting that *L. plantarum* B2

achieved alive the intestinal track even at day 1. Eckert et al. (2017) and Andrade et al. (2023) detected a similar trend in *the viability of L. plantarum* strain during the storage of milk and cream cheese, respectively. Darmastuti et al. (2021) also demonstrated using a murine model that 40% and 60% *of L. plantarum* ingested could attach to the intestine's surface.

3.2 Identification of bioactive peptides and volatile compounds in probiotic fermented sheep milk *processed by conventional heating or ohmic heating.*

A total of 12 peptides with some healthy activities were identified by the MALDI-TOF-MS tool, as summarised in Table 1. Most of these peptides (10) were derived from casein subunits and comprehended chains between 7 and 17 amino acids with 1100 to 2200 Da. Usually, peptides in the range of 2 to 20 amino acids present bioactivity as antioxidant, antithrombotic, antihypertensive, antidiabetic, immunomodulatory, and opioid (Du et al., 2023),

LNENLLRFFVAPFPEVFG and DKVGINYW peptides were found in the CHFM sample and derived from αS1-casein and α-lactalbumin, respectively (Table 1). The absence of these peptides in the OH samples may be related to how sheep milk was processed. OH involves direct and proportional heating, while CH relies on indirect heating. This difference in heating methods likely affected the denaturation of the parent proteins. Consequently, smaller peptides or amino acids may have been formed later, possibly through the action of proteases from added bacteria. The same trend could explain the presence of LAYFYPEL peptide in samples OH4FM and OH7FM (Table 1). These samples presented a longer heating duration step during sheep milk processing (Fig. S2).

The highest and fastest heating generation in food processed by OH is the reason why this technology is considered more efficient than CH processing (Balthazar et al., 2022; Kaur et al., 2023). Also, OH processing can tailor the charge state on the protein surface, enhancing the protein's interactions and modifying their functionality (Li et al., 2024). Further, these proteins could be targeted by bacteria proteases, releasing smaller peptides or amino acids (Shirkhan et al., 2023). Many studies showed that bioactive peptides are formed and stable at mild heat food processing temperatures of 60-80 °C (Ashaolu et al., 2023; Ren et al., 2023).

The wider peptide fragment variety found in CHFM does not mean that CH processing was responsible for producing more bioactive peptides. Indubitably, the average signal intensity in MALDI-TOF-MS for bioactive peptides identified in CHFM was lower than in OH samples (Fig. 2). The intensity of peptides identified by MALDI-TOF-MS analysis did not quantify peptides amount in a sample. However, it could indirectly indicate its concentration (Albalat et al., 2013). The average intensity of the peptides signal in CHMF was below $2 \cdot 10^4$ a.u., while in OH samples, it was between 2 and $4 \cdot 10^4$ a.u.

Volatile compounds play a crucial role in determining the aroma and flavor characteristics of fermented milk products, contributing to the pleasant and distinctive sensory attributes that make this type of dairy product appealing to consumers (Wang et al., 2024). Therefore, identifying the volatile compounds profile of probiotic fermented sheep milk previously processed by ohmic heating should provide exciting insights into the distinctive aromatic features of this product.

A total of 52 volatile compounds were identified in probiotic fermented sheep milk samples by GC-MS, whose chemical groups were divided into organic acids (20), hydrocarbons (8), esters (8), aldehydes (7), ketones (6), and alcohols (3). OH10FM was the sample containing the higher abundance of volatile compounds (51), while only 42 compounds were detected in OH4FM (Fig. 3), probably due to the shortest duration of sheep milk processing for OH10FM samples (Fig. S2). Most of the volatile compounds identified were also found in milk fermented by *S. thermophilus* and *L. plantarum* from other studies (Dan et al., 2017, 2019; Pan et al., 2014; Zhang et al., 2020; Zhao et al., 2023).

Furanone compounds are esters produced by microbial starters and present a sweet, coumarin, and nutty taste (Zhang et al., 2020). 5-heptyldihydro-2(3H)-furanone was identified in all probiotic sheep milk samples, whereas (Z)-dihydro-5-(2-octenyl)-2(3H)-furanone was not detected in OH4FM, as other compounds such as 1-dodecanol, nonanal and 2-heptanone (Table 2). The following compounds appeared in OH10FM: myristoleic acid, heptanal, 2,6,11,15-tetramethyl-hexadecane, and octadecane.

Due to this discrepancy, it was hypothesized that volatile compounds could be degraded by extended heating processing time of sheep milk or metabolized to further compounds during its fermentation since its appearance in some samples was residual.

The occurrence or absence of some volatile compounds in samples submitted to different OH treatments is strictly related to certain flavor compounds formed by covalent bonds with proteins, such as aldehydes and ketones. The protein-flavor binding is dependent on the conformational state of the proteins. Therefore, protein denaturation can decrease the flavor binding by

modifying the protein-specific binding sites. For instance, the formation of flavor compounds decreased due to whey protein denaturation over extended heating processing (Kühn et al., 2008).

In this respect, heptanal, an aromatic compound with a "green and sweet" flavor (Dan et al., 2017), was found only in OH10FM, probably due to the shortest time required for sheep milk pasteurization (ca. 9 min). Heating a sample with nonanal does not lead to the formation of heptanal, emphasizing the importance of the double bond in this process. Additionally, whey protein is essential for heptanal production, indicating a requirement for whey protein in this reaction (Kühn et al., 2008). Thus, a more extended heating period in other samples could denature the whey protein necessary for the reaction. Moreover, nonanal has a low threshold value and provides citrus and fatty aromas to fermented milk (Dan et al., 2019).

Dihydro-5-(2-octenyl)-2(3H)-furanone, also known as δ-octalactone, is formed by interacting with various compounds during milk fermentation. δ-octalactone can be sensitive to heat and may undergo degradation when exposed to prolonged high-temperature conditions (Chen et al., 2024). Also, the precursors of 2-heptanone, such as certain fatty acids, can be affected by heat treatment, which could negatively impact the formation or presence of 2-heptanone and related compounds (Gao et al., 2023).

The production of volatile compounds during bacterial fermentation of milk is a complex process involving various metabolic pathways. Some organic acids, including hexanoic, octanoic, and nonanoic acids, are related to the typic odor of fermented milk, such as spicy, rancid, and floral (Zhang et al., 2020). In addition, organic acids composed of nine to fourteen carbons are linked to fatty,

sweet, floral, and waxy aromas (Nguyen et al., 2023). According to Mirsalami and Alihosseini (2023), esters were detected at low levels in *L. plantarum* B2 fermented milk. However, esthers contribute for a floral and fruity flavor, which counterbalance the bitterness and sharpness of tastes derived from amines and fatty acids (Tylewicz et al., 2022). For instance, unsaturated fatty acids can be metabolized during the fermentation of γ- or δ-lactones, producing methyl ketones. Indeed, it was verified that *Lacticaseibacillus rhamnosus* GG may contribute to volatile compound formation besides free amino acids (Zhao et al., 2023).

Moreover, no change in bioactive peptides or volatile profiles was verified during 30 days of refrigeration. However, the volatile content is dynamic and could increase in fermented milk stored cold (Zhao et al., 2023). In addition, acetic and heptanoic acids are commonly found in milk fermented by *S. thermophilus*, and their concentrations increase during cold storage (Dan et al., 2017).

3.3 Improvement of health compounds in probiotic fermented sheep milk *processed by ohmic heating*

The results of bioactivity and riboflavin content in probiotic fermented sheep milk during refrigerated storage of 30 days are shown in Figure 3. A significant difference in bioactivities and riboflavin content ($p < 0.05$) between samples is believed to be related to the previous sheep milk processing. It was indeed observed that increasing EFS applied to sheep milk enhanced its bioactivities and riboflavin content ($p < 0.05$). In addition, it was reported in the literature that EFS application to food matrix had been demonstrated to improve

426 the production of secondary metabolites due to cellular-induced stress. Those
427 secondary metabolites have also shown beneficial health properties as
428 antioxidants, among others (Demir et al., 2023). This behavior was maintained
429 during refrigerated storage, but no significant variation was observed between
430 storage measurements in the same sample ($p > 0.05$, data not shown),
431 meaning that bacterial fermentation was responsible for bioactivity and
432 riboflavin increase in probiotic fermented sheep milk, been favored by those
433 secondary metabolites.

434 In particular, the antioxidant activity doubled from CHFM (23.33%) to
435 OH10FM (46.93%), while antihypertension triplicated from 11.60% in CHFM to
436 35.76% in OH10FM, as revealed by DPPH and ACE inhibitions respectively.
437 The α-amylase and α-glucosidase inhibition increase from 26.67% and 20.60%
438 (CHFM) to 42.03% and 37.20% (OH10FM), respectively, representing the
439 antidiabetic activity. Interestingly, riboflavin increased from 0.12 mg/100 g in
440 CHFM to 0.20 mg/100 g in OH10FM, considering that a serving of 240 mL
441 might contribute to cover between 20% and 35% of the daily recommended
442 intake for this vitamin, respectively. These results are in line with what was
443 previously reported from B2-overproducing *L. plantarum* strains in bakery
444 products and cereal-based fermented beverages (Capozzi et al., 2011; Russo
445 et al., 2016; Yépez et al., 2019), confirming the suitability of this species for bio-
446 fortification approaches also in the dairy sector. Until now, only a few studies
447 have reported on the employment of food-grade riboflavin-overproducing
448 bacteria to enhance the content of dairy products in mixed fermentation with *S.*
449 *thermophilus* as a starter. In particular, *Propionibacterium freudenreichii* was
450 proposed for the biofortification of yogurt (Burgess et al., 2006), while recently,

a strain of *Limosilactobacillus reuteri* was able to increase the level of riboflavin (about 1.5-fold higher than the control) during the fermentation of buttermilk but not of whole or semi-skilled cow milk (Spacova et al., 2022). Indeed, these authors demonstrated how the food matrix can strongly impact riboflavin production during fermentation.

Accordingly, in this study, we found that the riboflavin increase was more pronounced in OH samples, suggesting that applying EFS to milk induces the release of compounds that could encourage riboflavin biosynthesis by *L. plantarum* B2. The pulsed electric field is an emerging technology based on the same principle already used to extract compounds such as proteins, minerals, lipids, and others from the intracellular content using EFS. This electric-assisted extraction obtains high-value compounds from microorganisms as secondary metabolites (Demir et al., 2023; Martínez et al., 2020).

The electric current passing through the food matrix probably unfolded milk protein during processing. As EFS increased for treatments, sheep milk was exposed to higher electrical currents. Consequently, more amino acid sites in proteins were probably exposed and easier targets for bacteria enzyme action during fermentation, generating peptides with antioxidant, antihypertensive, and antidiabetic activities. The proteolytic potential of lactic acid bacteria for bioactive production in fermented food is known (Chourasia et al., 2023). Indeed, Balthazar et al. (2024) verified increased bioactivity and proteolysis in sheep milk processed with increasing EFS. The EFS can enhance antioxidant activity in liquid food by altering a protein's secondary and tertiary structure, displacing an active hydrogen (Zhang et al., 2019).

476 *3.4 Multiple Factor Analysis*

477 Multiple Factor Analysis (MFA) is a statistical technique used in Food
478 Science to analyze data from multiple variables or factors simultaneously. It is
479 an extension of Principal Component Analysis that is particularly useful when
480 data from different sources or categories must be analyzed together. MFA
481 provides a way to visualize the relationships between different variables,
482 factors, and categories. This result helps researchers and food scientists gain
483 insights into the complex interactions within food-related data (Cruz et al., 2013;
484 Shrestha, 2021).

485 MFA allowed the project of the parameters evaluated (volatile compounds,
486 peptides identified by MADI-TOF-MS, enzyme inhibition expressing the
487 bioactivities, and riboflavin) together to verify their relationship with the probiotic
488 fermented sheep milk samples. In this way, a bi-dimensional map was plotted,
489 explaining 87.71% of the data, of which F1 contributed 60.23% and F2 with
490 27.48%. As shown in Fig. 5, MFA separated the sample into quadrants (Q).
491 Samples OH4FM and OH7FM were located near each other in Q3, while
492 OH10FM was in Q2 and CHFM in Q1. The distance between samples is related
493 to the impact of sheep milk processing type and time.

494 The peptides identified in all samples were allocated in the center of the
495 map. In contrast, some peptides were near the presented samples, LAYFYPEL
496 in OH4FM and OH7FM and LNENLLRFFVAPFPEVFG and DKVGINYW in
497 CHFM. As expected, the bioactivities were near OH10FM because their content
498 was higher in that sample. The volatile compounds followed the same trend and
499 were dispersed in the map due to the relationship of the plotted area of each
500 one found in GC-MS analysis in each sample. For example, myristoleic acid,

2,6,11,15-tetramethyl-hexadecane, and heptanal were found only in OH10FM. Thus, MFA discriminated the parameters studied in probiotic fermented sheep milk by their relationship with each sample.

4 Conclusions

In this study, the effect of different electric field strengths applied to sheep milk was evaluated for the elaboration of potential probiotic fermented milk. Moreover, a comparison with conventional heating was performed. Even though a similar probiotic viability and survival to *in vitro* digestion assay was detected, a significant increase in antioxidant, antidiabetic, antihypertensive activities, and riboflavin content ($p < 0.05$). Furthermore, the most volatile compounds responsible for the fermented milk flavor were identified in the probiotic fermented sheep milk profiles produced by conventional and ohmic heating with different electric field strengths. Finally, MFA was an excellent statistical tool for quickly visualizing the relationship among samples, strongly corroborating the analytical results. Overall, our results suggest a positive impact of ohmic heating milk processing on health compounds produced in fermented sheep milk.

Declaration of interest

The authors declare no conflicts of interest.

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794

Figures captions

Figure 1. *Streptococcus thermophilus* TH-4® (A) and *Lactiplantibacillus plantarum* B2 (B) viability during refrigerated storage (4°C) and *L. plantarum* B2 survival through *in vitro* digestion (C), whose milk was treated by conventional or three different ohmic heating. Different letters mean significant differences ($p < 0.05$). Standard error bars are indicated. CHFM: conventional heating fermented milk; OH4FM: ohmic heating 4 V/cm fermented milk; OH7FM: ohmic heating 7 V/cm fermented milk; and OH10FM: ohmic heating 10 V/cm fermented milk.

Figure 2. Mass spectra schematic chart of bioactive peptides obtained by MALDI-TOF-MS in probiotic fermented sheep milk. CHFM: conventional heating fermented milk; OH4FM: ohmic heating 4 V/cm fermented milk; OH7FM: ohmic heating 7 V/cm fermented milk; and OH10FM: ohmic heating 10 V/cm fermented milk.

Figure 3. Antioxidant, antihypertensive and antidiabetic activities in probiotic fermented sheep milk processed by conventional or ohmic heating are represented as 2,2-Diphenyl-1-picrylhydrazyl inhibition (DPPHI, %), angiotensin-converting enzyme inhibition (ACEI, %), α-amylase and α-glucosidase inhibition (%), respectively; as well riboflavin content (mg/100 g). Different letters (a-d) mean significant difference ($p < 0.05$). Standard error bars are indicated. CHFM: conventional heating fermented milk; OH4FM: 4V/cm electric field strength ohmic heating fermented milk; OH7FM: 7V/cm electric

819 field strength ohmic heating fermented milk; OH10FM: 10V/cm electric field
820 strength ohmic heating fermented milk.
821
822 **Figure 4.** Number of volatile compounds found in probiotic fermented sheep
823 milk storage at 4 °C for 30 days divided by chemical groups. CHFM:
824 conventional heating fermented milk; OH4FM: 4V/cm electric field strength
825 ohmic heating fermented milk; OH7FM: 7V/cm electric field strength ohmic
826 heating fermented milk; OH10FM: 10V/cm electric field strength ohmic heating
827 fermented milk.
828
829 **Figure 5.** Representation of the parameters evaluated (volatile compounds,
830 peptides identified by MADI-TOF-MS, enzyme inhibition expressing the
831 bioactivities, and riboflavin) (A) and the four probiotic fermented sheep milk (B)
832 in two dimensions of Multiple Factor Analysis. CHFM: conventional heating
833 fermented milk; OH4FM: 4V/cm electric field strength ohmic heating fermented
834 milk; OH7FM: 7V/cm electric field strength ohmic heating fermented milk;
835 OH10FM: 10V/cm electric field strength ohmic heating fermented milk.
836
837 **Figure Supplementary 1.** Acidity evolution of probiotic fermented sheep milk
838 storage at 4 °C for 30 days, represented by pH (A) and titratable acidity (B).
839 Different letters mean significant differences ($p < 0.05$). CHFM: conventional
840 heating fermented milk; OH4FM: ohmic heating 4 V/cm fermented milk;
841 OH7FM: ohmic heating 7 V/cm fermented milk; and OH10FM: ohmic heating 10
842 V/cm fermented milk.

Figure Supplementary 2. The duration of sheep milk processing is in minutes to achieve the binomial 73 °C/15 s for pasteurization in different processing samples. Different letters mean significant differences ($p < 0.05$). Standard error bars are indicated. CHFM: conventional heating fermented milk; OH4FM: ohmic heating 4 V/cm fermented milk; OH7FM: ohmic heating 7 V/cm fermented milk; and OH10FM: ohmic heating 10 V/cm fermented milk.

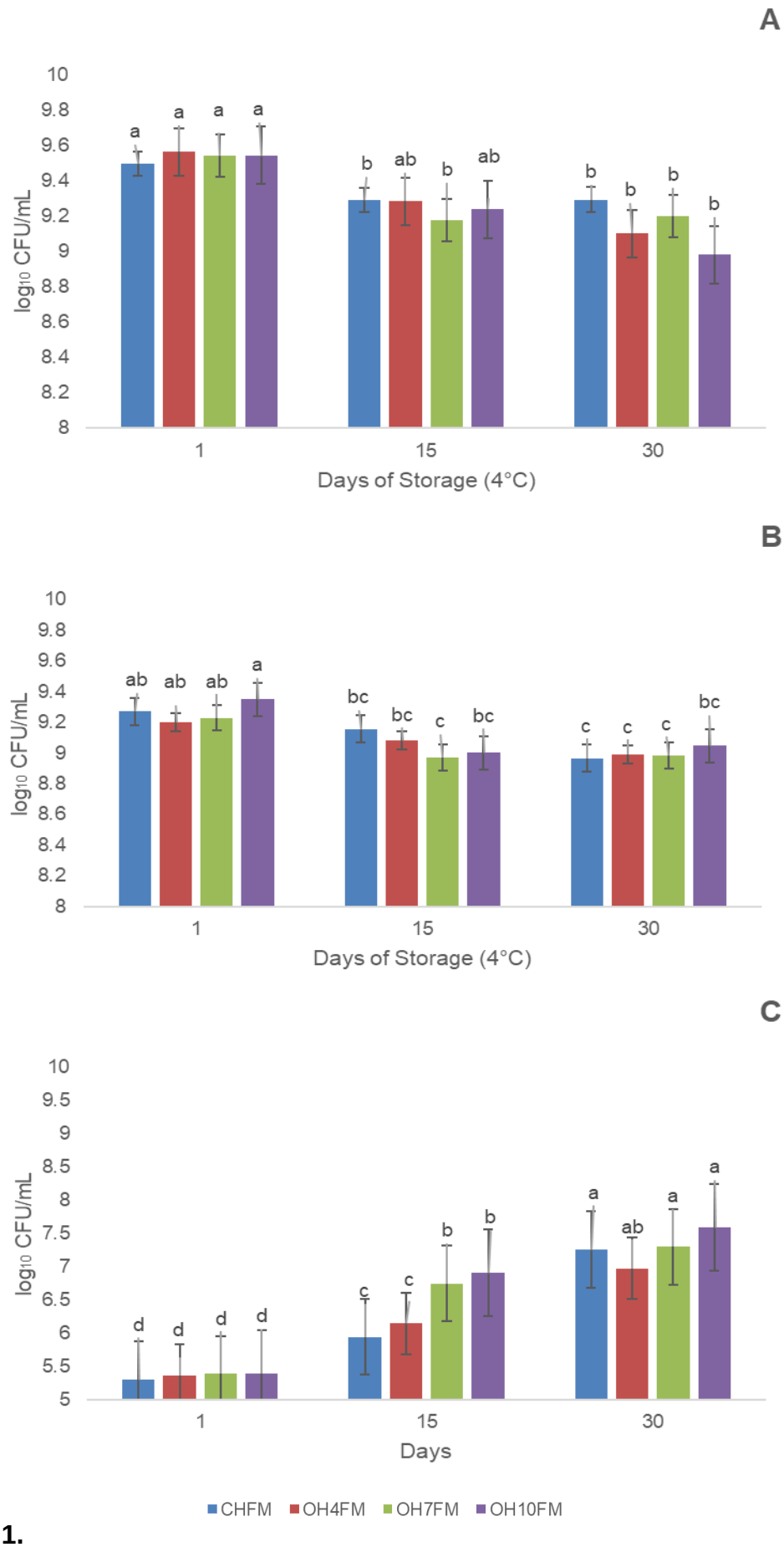

A
log10 CFU/mL
10
9.8
9.6
9.4
9.2
9
8.8
8.6
8.4
8.2
8
a a a a
b ab b ab
b b b b
1
15
30
Days of Storage (4°C)
B
log10 CFU/mL
ab ab ab a
bc bc c bc
c c c bc
Days of Storage (4°C)
C
log10 CFU/mL
10
9.5
9
8.5
8
7.5
7
6.5
6
5.5
5
d d d d
c c b b
a ab a a
Days
CHFM
OH4FM
OH7FM
OH10FM

Figure 1.

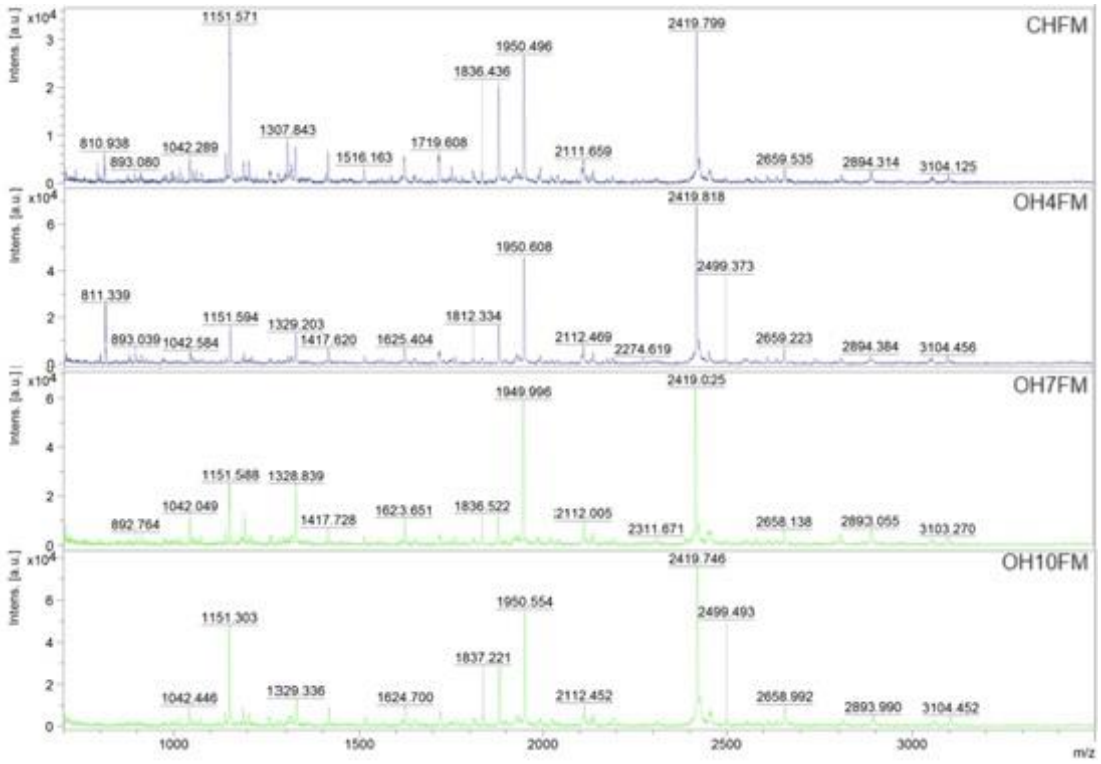
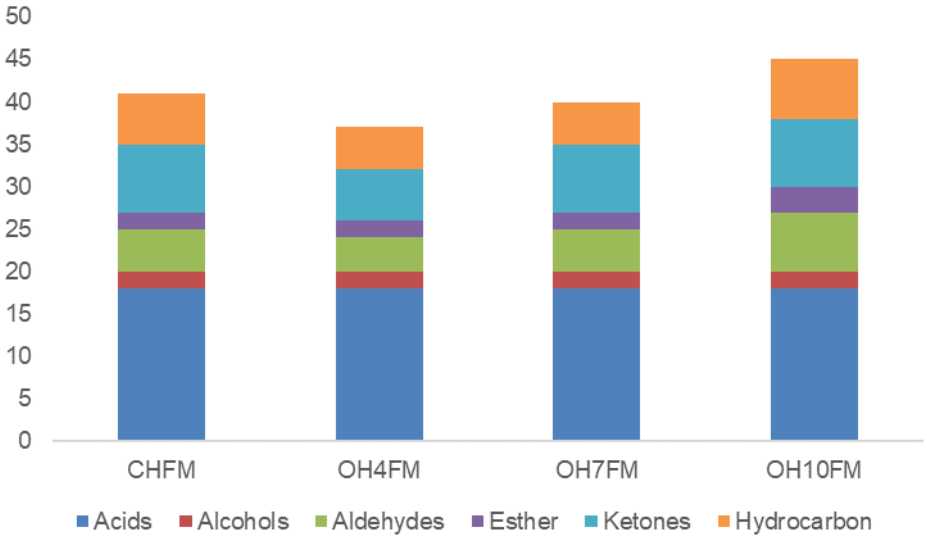


Figure 2.



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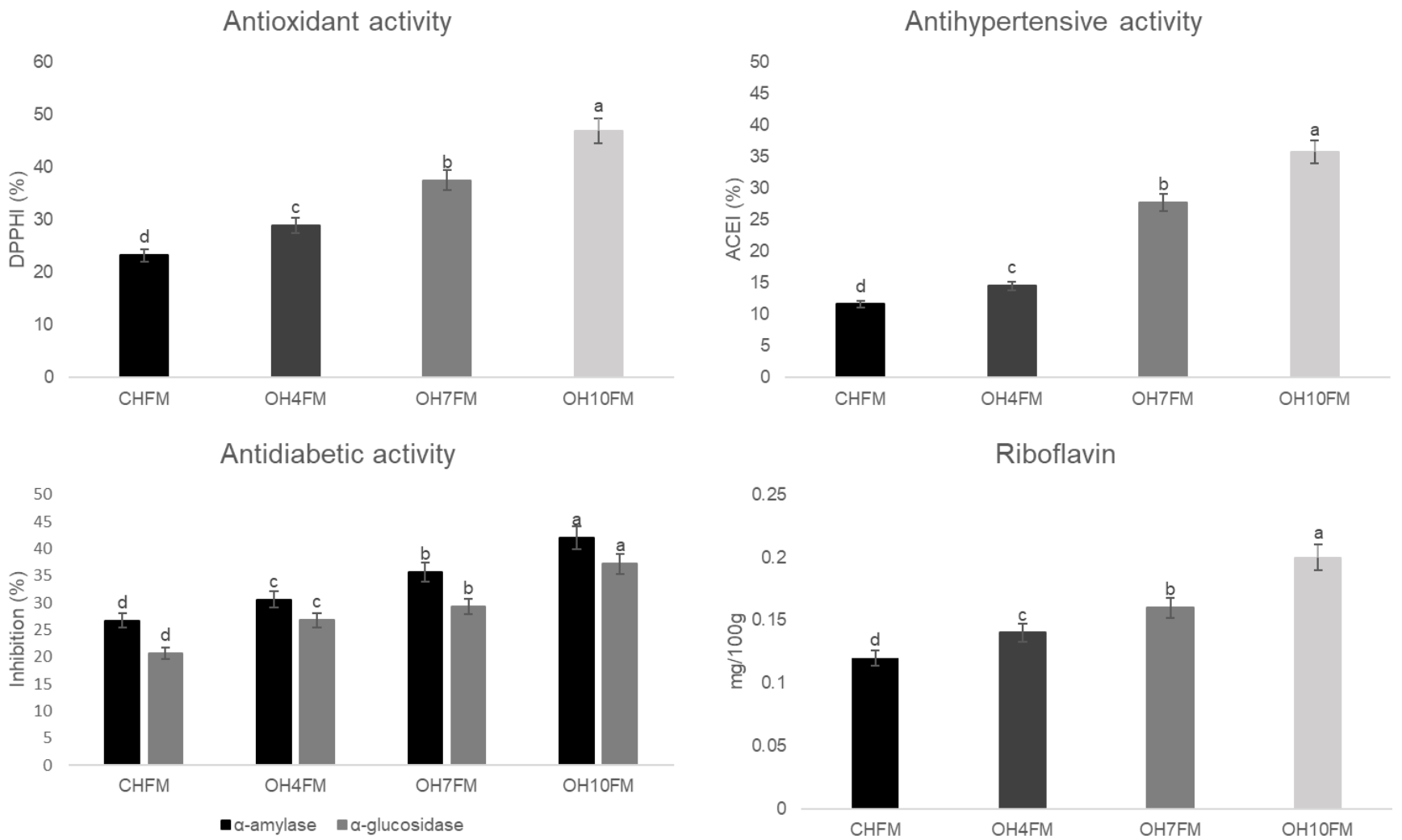
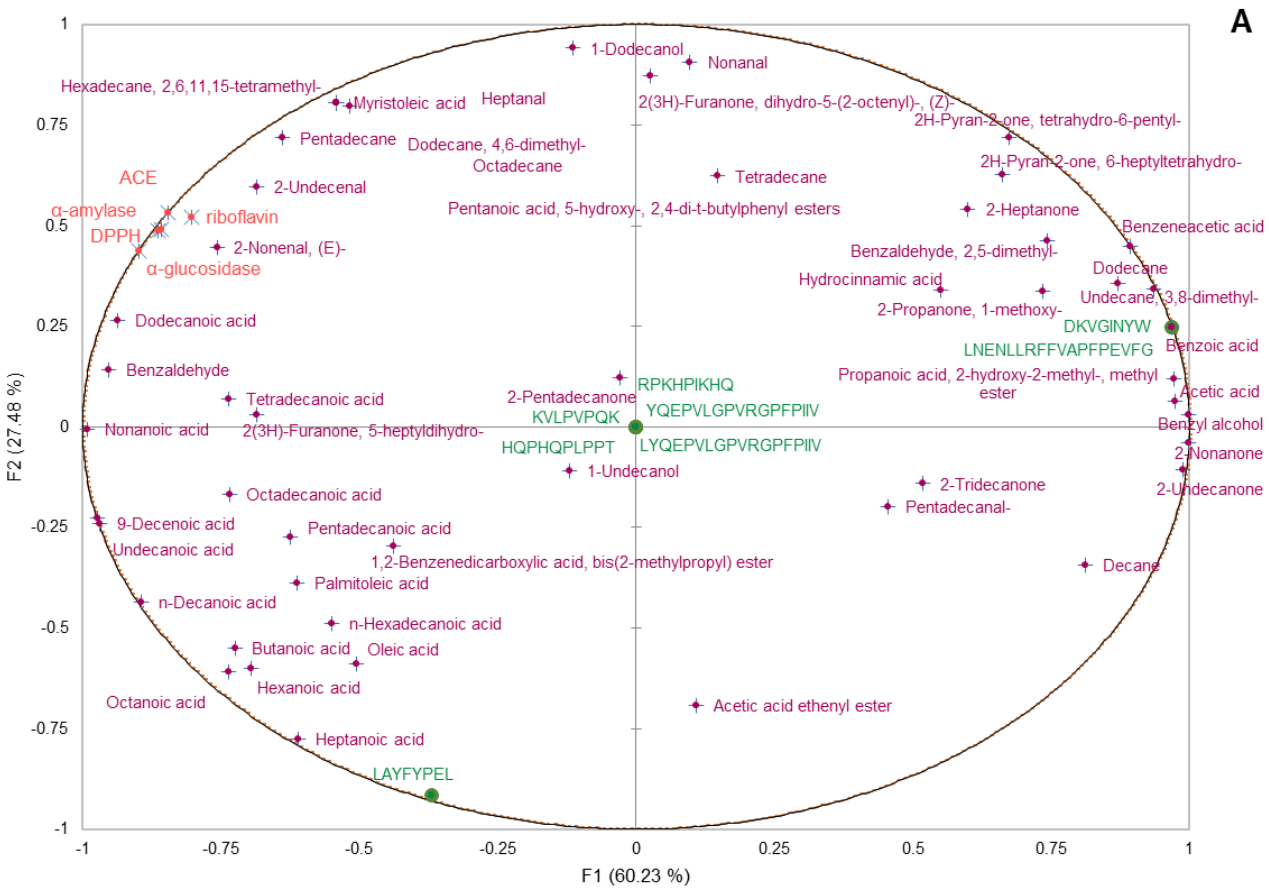


865
866 **Figure 4.**



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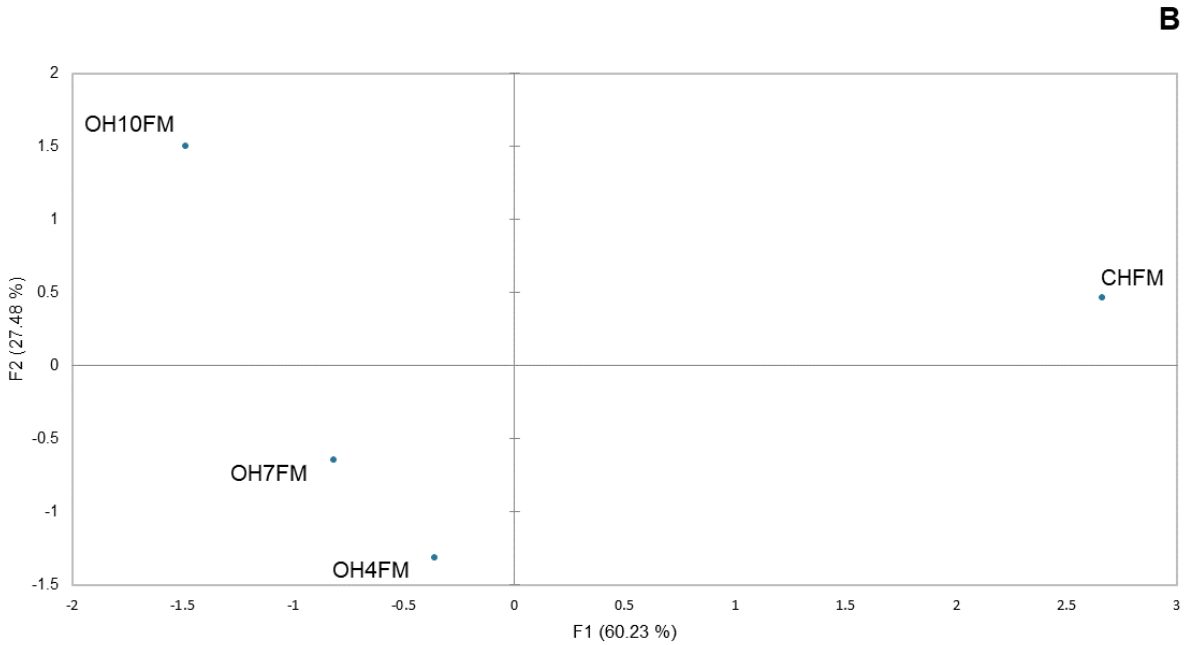


868

869 **Figure 5.**

870 **Table 1.** Peptides with biological functionality identified in probiotic fermented sheep milk samples by MALDI-TOF-MS analysis,

871 showing the respective protein's origin, mass-to-charge ratio (m/z), and biological activity.

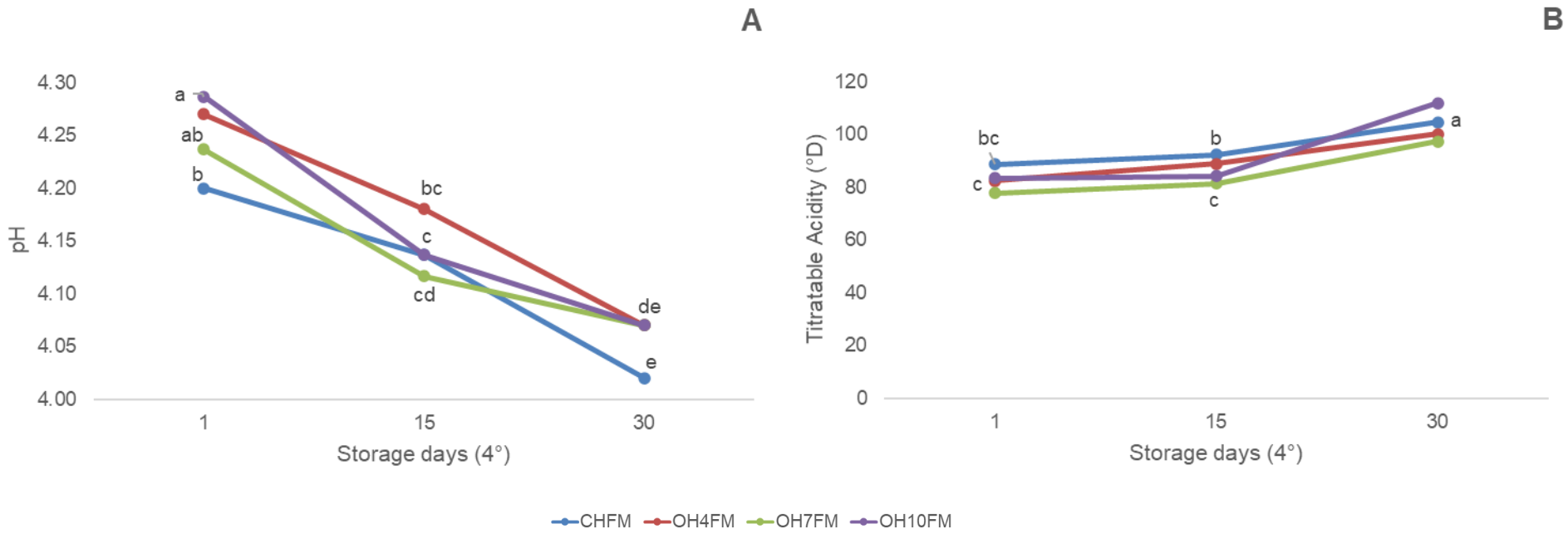
Sample	Protein origin	Peptide	m/z	Biological activity	Reference
OH4FM, OH7FM	αS1-casein	LAYFYPEL	1015	Immunomodulatory	Totsuka et al. (1998)
All		RPKHPIKHQ	1140	ACE inhibition	Balthazar et al (2024)
CHFM		LNENLLRFFVAPFPEVFG	2109	ACE inhibition	Robert et al. (2004)
All	β-casein	KVLPVPQK	908	Antioxidant	Tonolo et al. (2020)
All		HQPHQPLPPT	1151	ACE inhibition	Adams et al. (2020)
All		YQEPVLGPVRGPFPIIV	1881	Antibacterial, anti-coagulant, Immunomodulatory	Balthazar et al. (2024)
All		LYQEPVLGPVRGPFPIIV	1994	Immunomodulatory	Balthazar et al. (2024)
CHFM	α-lactalbumin	DKVGINYW	994	ACE inhibition	Tavares et al. (2012)

872 * CHFM: conventional heating fermented milk; OH4FM: ohmic heating 4 V/cm fermented milk; OH7FM: ohmic heating 7 V/cm fermented milk; and OH10FM: ohmic heating 10 V/cm fermented milk.

873 **Table 2.** Volatile compounds identified in probiotic fermented sheep milk.

SAMPLE	CHFM	OH4FM	OH7FM	OH10FM
Acids				
Acetic acid	X	X	X	X
Butanoic acid	X	X	X	X
Hexanoic acid	X	X	X	X
Heptanoic acid	X	X	X	X
Octanoic acid	X	X	X	X
Nonanoic acid	X	X	X	X
n-Decanoic acid	X	X	X	X
9-Decenoic acid	X	X	X	X
Undecanoic acid		X	X	X
Benzoic acid	X	X	X	X
Dodecanoic acid	X	X	X	X
Benzeneacetic acid	X	X	X	X
Hydrocinnamic acid	X	X	X	X
Tetradecanoic acid	X	X	X	X
Myristoleic acid				X
Pentadecanoic acid	X	X	X	X
n-Hexadecanoic acid	X	X	X	X
Palmitoleic acid	X	X	X	X
Octadecanoic acid	X	X	X	X
Oleic acid	X	X	X	X
Alcohols				
Benzyl alcohol	X	X	X	X
1-Dodecanol	X		X	X
1-Undecanol	X	X	X	X
Aldehides				
Heptanal				X
Nonanal	X		X	X
Benzaldehyde	X	X	X	X
2-Nonenal, (E)-	X	X	X	X
2-Undecenal	X	X	X	X
Benzaldehyde, 2,5-dimethyl-	X	X	X	X
Pentadecanal-	X	X	X	X
Esther				
Acetic acid ethenyl ester	X	X	X	X
Propanoic acid, 2-hydroxy-2-methyl-, methyl ester	X	X	X	X
2H-Pyran-2-one, tetrahydro-6-pentyl-	X	X	X	X
Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters				X
2(3H)-Furanone, 5-heptyldihydro-	X	X	X	X
2(3H)-Furanone, dihydro-5-(2-octenyl)-, (Z)-	X		X	X
2H-Pyran-2-one, 6-heptyltetrahydro-	X	X	X	X
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) es	X	X	X	X
Ketones				
2-Heptanone	X		X	X
2-Propanone, 1-methoxy-	X	X	X	X
2-Nonanone	X	X	X	X
2-Undecanone	X	X	X	X
2-Tridecanone	X	X	X	X
2-Pentadecanone	X	X	X	X
Hydrocarbon				
Decane	X	X	X	X
Dodecane	X	X	X	X
Undecane, 3,8-dimethyl-	X			
Tetradecane	X	X	X	X
Dodecane, 4,6-dimethyl-	X	X	X	X
Pentadecane	X	X	X	X
Hexadecane, 2,6,11,15-tetramethyl-				X
Octadecane				X

874
875 * CHFM: conventional heating fermented milk; OH4FM: ohmic heating 4 V/cm fermented milk; OH7FM: ohmic
876 heating 7 V/cm fermented milk; and OH10FM: ohmic heating 10 V/cm fermented milk.



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879 **Figure Supplementary 1.**

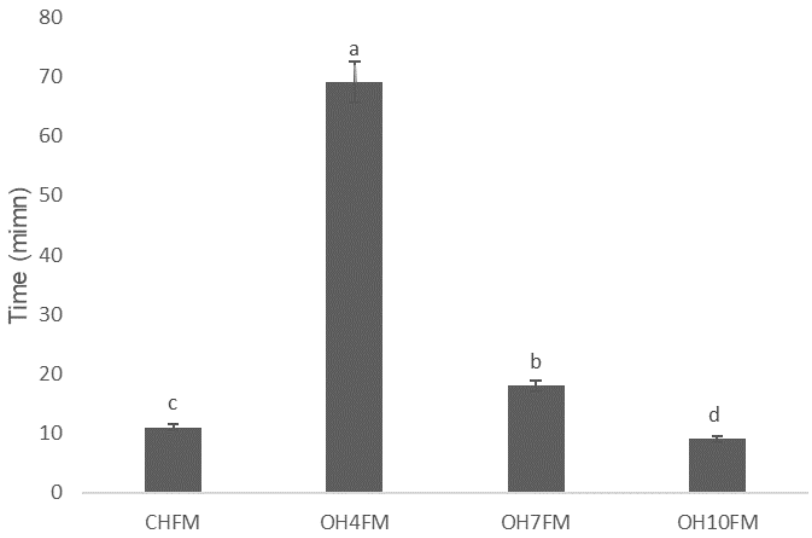

Figure Supplementary 2.

Declaration of interest

The authors declare no conflicts of interest.