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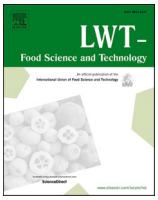

Evaluation of shelf life and technological properties of bread elaborated with lactic acid bacteria fermented whey as a bio-preservation ingredient

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ABSTRACT

Nowadays, food lost by fungal contamination is one of the main safety problems for the bakery industry. As the consumer is rejecting the use of regular food additives new methods are being investigated. One of the more promising alternatives is the use of microorganism in the preservation of food, bio-preservation. In this article the ability of a lactic acid bacteria fermented whey was assessed as bio-preservative ingredient to inhibit the growth of *Aspergillus* sp. and *Penicillium* sp. in bread. The rheological, mechanical and antifungal properties of 3 different concentrations of this ingredient were studied. Results evidence that the addition lactic acid bacteria fermented whey in a of a 5% concentration did not modify drastically the quality of the bread, increased the occurrence of antifungal metabolites such as lactic acid and phenyllactic acid and managed to extend the shelf life from bread contaminated with *Aspergillus flavus* and *Penicillium verrucosum* and significantly reduced the fungal population of *Penicillium verrucosum* in the bread after 7 days in comparation to a regular bread.

1. Introduction

Approximately around a 25% of the food worldwide is contaminated with filamentous fungi and the toxins produced by this microorganism (H. Cao, Jiang, et al., 2021). As a result of this fact, fungal and mycotoxigenic contamination have become a severe problem which cost food industry million euros per year and threatens the life of the consumers with their toxigenic effects (Russo et al., 2017; Zain, 2011). Among all the food-contaminant species of moulds, bakery products are especially susceptible to contamination from the *Aspergillus*, *Penicillium*, *Fusarium* and *Rhizopus* (Debonne et al., 2021). *Aspergillus* and *Penicillium* genera are considered the most harmful to human health. This fungal species are the main producers of aflatoxins (AF) and ochratoxin (OTA) in cereal and different cereal-based foods (Bryła et al., 2021). AF ingestion is related to hepatotoxic, mutagenic and carcinogenic activities in animals, and is classified as Group 2B by the International Agency for Research on Cancer (IARC) (Bennett & Klich, 2003; Gupta, 1994). OTA is also related to nephrotoxic, teratogenic, embryotoxic, genotoxic, neurotoxic suppression of the immune system effects (Belkacem-Hanfi et al., 2014).

In the production of bread, the temperatures reached during the baking stage destroy the fungal vegetative forms, but some of the spores are resistant to this treatment and grow in the final product. In addition, post preparation practices lead to an increasing contamination of the bread (Cizeikiene et al., 2013; Jideani & Vogt, 2016). It is interesting to find effective fungicides resistant to high temperatures to prevent the growth of moulds and mycotoxin production after the bakery stage. Chemical preservatives, such as calcium propionateand calcium acetate are commonly added to bread in order to prevent fungal and further toxin contamination. (Perincherry et al., 2019). Nevertheless, there is an increasing demand from the consumer for "natural" and "healthier" food by the reduction of the concentration from these chemical preservatives present in food (Sadiq et al., 2019). As a result, novel technological approaches are being studied to reduce fungal contamination with safer techniques. Use of lactic acid bacteria (LAB) as bio-protective agents postulates as a great alternative to the synthetic origin preservatives.

Many reports in the bibliography show evidence of the antifungal effect of this microorganism (Chen et al., 2021; Illueca et al., 2021). The use of these bacteria as bio-preservative agents has already being proved. Álvarez et al. (2021) used edible coatings incorporating LAB to prolong the shelf life of cherry tomatoes against *Fusarium* sp. and *Rhizopus* sp. Also Omedi et al. (2021) evidence the bio-preservation potential of bread contaminated by *Cladosporium*, *Aspergillus* and

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Penicillium species using a LAB fermented ingredient. Among all the different uses of the LAB as bio-preservatives the application of a fermented matrix as the antifungal agent is one of the most studied techniques (Bulgasem et al., 2016; Li et al., 2020). In these studies, a nutrient-rich medium is used as a substrate for the bacterial metabolization of different antifungal compounds.

Whey from the cheese preparation is the principal residue produced by dairy industry (Verma & Subudhi, 2021). For each 10 kg of cheese up to 9 kg of whey are produced (Prazeres et al., 2012). This bio-residue is usually used for fertilization, livestock feed and human consumption. Nevertheless, most of this dairy by-product must be processed as residual water (Verma & Subudhi, 2021). When this by-product is not correctly treated is a mayor contaminant agent and regulations over this waste have become more rigorous during last years (Koutinas et al., 2009; Smithers, 2008). This cheese effluent contains a diverse pool of proteins, sugars, salts and fatty acids, which postulates this matrix as an ideal medium for LAB growth (Gregg et al., 2020). The goals agreed by the World Health Organization (WHO) set for 2030 for sustainable development indicate that new methods of waste reduction should be studied (United Nations, 2015). In addition, the ideal method for food conservation ought to be economical and non-complicated to use (Leroy & De Vuyst, 2004). The preparation of a food ingredient which only needs a LAB fermentation of a residue and a future addition to a food preparation accomplishes this description of ideal method.

The objective of this study was to produce an ingredient made of LAB fermented whey for the bio-preservation of bread against toxigenic fungal contamination. To accomplish this goal a) the modifications of the technological properties from the dough and bread were assessed; b) different antifungal compounds present in the dough and bread were quantified; c) the antifungal potential of the LAB fermented whey ingredient was studied in bread contaminated with *Penicillium* spp. and *Aspergillus* spp.

2. Materials and methods

2.1. Materials and microorganism

Media cultures used for the assays: Man Rogosa and Sharpe broth (MRS-B), plate count agar (PCA), Rose Bengal Chloramphenicol agar (RBA) and potato dextrose agar (PDA) were purchased to Liofilchem (Teramo, Italy). The double deionized water from a water purification system (Millipore Corp., Massachusetts, United States). Goat whey was provided by the Quesería Dehesa Dos Hermanas company, S.L (Santa Barbara de Casa, Spain).

Acetic acid glacial (99%), acetonitrile (99.9%), ethyl acetate (99.9%), formic acid (99%), D-glucose anhydrous and methanol (99.9%) were obtained from VWR Chemicals (Radnor, United States). C18, magnesium sulphate, sodium chloride, DL-3-phenyllactic acid (97%), potassium phosphate dibasic, ammonium citrate dibasic, sodium acetate, manganese (II), sulphate monohydrate magnesium sulphate and DL-lactic acid (90%) were acquired from Sigma–Aldrich (St Louis, United States). Tryptone from Liofilchem (Teramo, Italy). Yeast extract from Oxoid Holdings ltd. (Hampshire, United Kingdom).

The wheat flour, salt and sugar were Hacendado, bottled water was from Fuente primavera, and yeast was from Levitan, used in the bread preparation were all brought in Mercadona (Valencia, Spain).

The *Lactiplantibacillus plantarum* 5L1 studied in this paper was previously isolated and identified in previous investigations by the Colección Española de Cultivos Tipo (CECT) in Valencia, Spain. Fungi used were *Aspergillus flavus* ISPA 8111, from Istituto di Scienze delle Produzioni Alimentari (Bari, Italy) and *Penicillium verrucosum* VTT 01847, from the VTT Culture Collection (Espoo, Finland).

2.2. Preparation and fermentation of the cell free supernatant (CFS)

The studied culture medium was whey complemented with 10 g/L of glucose, 10 g/L of tryptone, 5 g/L of sodium acetate, 4 g/L of yeast extract, 2 g/L of dipotassium phosphate, 2 g/L of ammonium ferric citrate, 0.2 g/L of magnesium sulphate and 0.05 g/L of manganese sulphate.

To produce the cell free supernatant (CFS) first the LAB was incubated in MRS-B for 24 h at 37 °C. Then the pre-inoculum was added to the whey culture medium with a 5% (v/v) proportion and incubated for 72 h at 37 °C. Afterwards, the medium was centrifuged at 3000×*g*, 4 °C for 15 min. Finally, the CFS was frozen at −20 °C and lyophilised for further uses as ingredient. This CFS was proved in previous investigations pendent to be published to have antifungal activity against the tested fungi.

2.3. Bread baking with whey as ingredients

Dough was prepared as it follows: 300 g of wheat flour was mixed with 6.5 g of salt and a suspension of 165 mL bottled water at 37 °C with 20 g of yeast and 10 g of sugar. Then the dough was kneaded in a breadmaking machine SilverCrest SBB 850 A1 (SilverCrest, Bochum, Germany), for 10 min. Split in parts of 100 g and fermented for 1 h at room temperature. Finally, loaves were baked in a Memmert UNB 100 Oven (Gemini b.v., Apeldoorn, Netherlands) at 200 °C for 45 min and left at room temperature for 20 min. A total of three controls were prepared: a bread replacing salt with flour (non-salt control), a bread (salt control) and a bread with 3000 mg/kg of calcium propionate (salt propionate control). The studied ingredient, the fermented whey, was added at different concentrations: 5 g (1% fermented whey), 25 g (5% fermented whey), and 50 g (10% fermented whey). Flour was replaced with whey for the treatment addition. Salt and sodium propionate concentrations used were the highest permitted by the European Union (EFSA, 2016; European Commission, 2012).

2.4. Effect of the fermented whey on the dough

2.4.1. Effect on the expansion volume

The expansion volume was studied following the steps described in Sang et al. (2020) with a few modifications. Five grams of dough were placed at the bottom of a sterilized graduated cylinder (50 mL). After a fermentation of 1 h at room temperature, the increase in dough volume (cm) was calculated as dough expansion. All the measurements were performed in triplicates.

2.4.1. Effect on the pH and titratable acidity

Before the fermentation of the dough and after the fermentation of the dough the subsequent parameters were measured. The pH from the samples was measured before the fermentation and after the fermentation using a pH-meter XS pH 7 Vio (XS Instruments, Carpi, Italy) equipped with an electrochemical sensor 2 Pore Steel T (XS Instruments, Carpi, Italy). The titratable acidity was determined as mL of NaOH 0.1 M needed to raise the pH of a 10 g sample to pH 8.5 (Hugo et al., 2003). The result were expressed as equivalents of grams of lactic acid per 100 g dough. The equation used to quantify the titratable acidity was:

$$g/100g = (M_{NaOH} * V_{NaOH})/Mw_{lactic\ acid})*10$$

Where: g/100 mL are the g of lactic acid for each 100 g of dough, M_{NaOH} is the molar concentration of the NaOH used as titrant, V_{NaOH} the volume of NaOH used as titrant and $Mw_{lactic\ acid}$ the molecular weight of lactic acid (90).

2.4.3. Effect on the microbial population

The microbial population of the samples was measured as described in Lan et al. (2012) with a few modifications. Serial dilutions of 10 g from the sample were homogenised with a stomacher Masticator Classic 400 mL 220/240 V 50/60 Hz (IUL S.A., Barcelona, Spain) saw in PCA (to determine the total microbial population) and RBA (to determine the

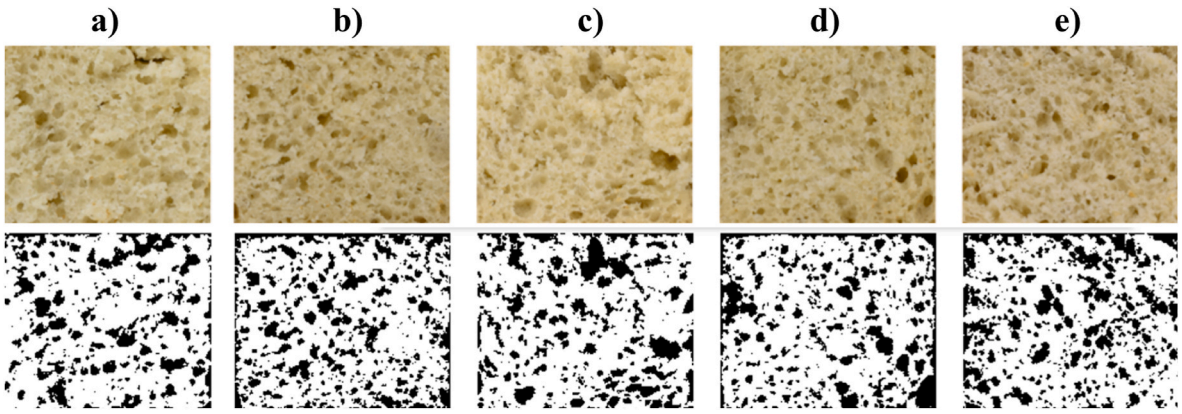

Fig. 1. Alveolar structure from the crumb before and after processed with the software ImageJ V1.52. Non-salt control (a), salt control (b), salt propionate control (c), 1% fermented whey (d) and 5% fermented whey (e).

total fungal population) then incubated at 25 °C 48 h.

2.5. Impact of the fermented whey on the bread

2.5.1. Impact on the pH and titratable acidity and microbial population

The measure of the pH, titratable acidity, total microbial population and total microbial population were performed on the bread following the steep performed to the dough.

2.5.2. Impact on the specific volume

The specific volume of the bread was studied as described in Mannuramath et al. (2015). Bread were weight then the volume was measured using the millet seed displacement method. A beaker with a calibrated mark was filled with millet grains until it reached the mark, then the bread was put into the baker and filled the millet grains. The displaced volume of seeds by the bread was measured. This measure was performed by triplicate. Specific volume was calculated as the ratio between volume and weight (cm^3/g).

2.5.3. Impact on the water activity

The water activity (Aw) was measured using a Humimeter RH2 water activity meter (Max-Schaller-Straße, Styria, Austria) from 3 bread slices (Sadeghian Motahar et al., 2021).

2.5.4. Impact on the objective color values from the crust

Colour changes were measured using a ColorQuest® XE (HunterLab, Leicestershire, United kingdom) to measure various locations from the crust of the bread (Aldughpassi et al., 2021). Results were expressed as luminosity (L*), the range from 0 (black) to 100 (white), redness (a*) the range from + a* (red) to -a* (green), and yellowness (b*), the range from + b* (yellow) to -b* (blue). Each sample was measured nine times. With these values the browning index (BI) was calculated as described in Kowalski et al. (2022), where:

$$BI = (100*(X-0.31)/ \quad 0.17)$$

where:

$$X = (a^* + 1.75\ L^*)/(5.645\ L^* + a^* - 3.012\ b^*)$$

2.5.5. Impact on the alveolar structure from the crumb

The study of the alveolar structure from the crumb was performed using the steep reported in Genevois and de Escalada Pla (2021) with a few modifications. Bread slices of 10 mm thickness were photographed, then with the software ImageJ V1.52 (NIH, Maryland, United States) the central alveolar characterization was measured (Schneider et al., 2012). Results were expressed as the alveolar percentage on the total area of the bread. Each measure was performed per triplicate. The processes can be observed in Fig. 1.

2.6. Antifungal metabolites present in bread and dough

2.6.1. Study of the occurrence of organic acids in bread and dough

The identification and quantification of the main present organic acids and phenolic compounds from the dough and the bread were performed as described in Dopazo et al. (2021) with a few modifications. The organic acid identification used 5 g of the samples which were diluted in 20 mL doble deionized water and homogenised with an Ultra Ika T18 basic Ultraturrax (IKA®-Werke GmbH & Co., Staufen, Germany) for 1 min. Then centrifuged at 14000×*g* for 5 min and filtered thought a 0.22 μm pore. For the analysis, a high-performance liquid chromatography (HPLC) Agilent 1100 Series (Agilent, California, United States) equipped with a quaternary pump, a 20 μL sample injection loop, a Rezex ROA-Organic Acid (150 × 7.8 mm) reverse phase column (Phenomenex Inc, California, United States) and a MD 4015 PDA diode array detector was used. Mobile phase was a solution of water with sulfuric acid at 0.1% at a flow rate of 0.8 mL/min. The assays were replicated 3 times per sample.

2.6.2. Study of the occurrence of phenolic compounds in bread and dough

For the study of the phenolic compounds present in the samples 5 g were diluted in 20 mL doble deionized water and homogenised Ultraturrax for 1 min. Then a QuEChERS extraction was performed of 10 mL of the homogenised mix (Brosnan et al., 2014). In this extraction 10 mL the sample were mixed with 10 mL of ethyl acetate, 4 g de magnesium sulphate and 1 g sodium chloride and vortexed for 1 min. Subsequently, samples were centrifuged at 3000×*g* for 10 min at 4 °C. The ethyl acetate supernatant was extracted and mixed with 0.15 g C18, 0.9 g magnesium sulphate and vortexed for 1 min. The extract was centrifuged under same conditions. The ethyl acetate phase was transferred to 15 mL tubes and dried under nitrogen flow with a TurboVap (Caliper Life Sciences, California, United States). Before the injection, the dry samples were suspended in 1 mL of a water-acetonitrile 90:10 (v/v) solution. An Agilent 1200 (Agilent, California, United States) was used as chromatograph for the assay. The chromatograph was equipped with a vacuum degasser, an autosampler, a binary pump and a column Gemini C18 (Phenomenex Inc., California, United States). The mobile phase was formic acid 0.1% (v/v) in water as solvent A and 0.1% formic acid in acetonitrile as solvent B. The elution rate was: 0 min, 5% B; 30 min, 95% B; 35 min, 5% B. Volume injected was 20 μL per sample. The run time selected was 37 min and a flow rate of 0.3 mL/min. The mass spectrophotometry (MS) assay was performed with a Q-TOF-MS 6540 Agilent Ultra High-Definition Accurate Mass (Agilent Technologies, California, United States) with an Agilent Dual Jet Stream electrospray ionisation

Table 1
Parameters of the doughs before the fermentation: volume expansion (cm), pH, titratable acidity (lactic acid g/100 g), total microbial population (Log_{10} CFU/g) and total fungal population (Log_{10} CFU/g)..

Bread	Volume expansion	pH	Titratable acidity	Total microbial population	Total fungal population
Non-salt control	1.63 ± 0.06^{ab}	0.4 ± 0.0^{c}	4.2 ± 0.2^{c}	8.6 ± 0.2^{a}	8.7 ± 0.1^{a}
Salt Control	1.37 ± 0.12^{b}	0.4 ± 0.0^{c}	4.8 ± 0.4^{c}	8.5 ± 0.1^{a}	8.5 ± 0.1^{a}
Salt propionate control	1.47 ± 0.12^{b}	0.4 ± 0.0^{c}	4.7 ± 0.3^{c}	8.6 ± 0.1^{a}	8.5 ± 0.1^{a}
1% Fermented whey	1.80 ± 0.10^{a}	0.7 ± 0.0^{b}	7.9 ± 0.1^{b}	8.6 ± 0.1^{a}	8.5 ± 0.1^{a}
5% Fermented whey	1.47 ± 0.06^{b}	0.7 ± 0.0^{b}	8.2 ± 0.1^{b}	8.7 ± 0.1^{a}	8.6 ± 0.1^{a}
10% fermented whey	nd	1.8 ± 0.1^{a}	20.3 ± 0.6^{a}	8.6 ± 0.1^{a}	8.5 ± 0.1^{a}

Data were presented in mean ± SD. Significatived differences among samples (colums) were maked different letters ($p < 0.01$). Data under the limit of detection was marked as nd.

Table 2
Parameters of the doughs after the fermentation: pH, titratable acidity (lactic acid g/100 g), total microbial population (Log_{10} CFU/g) and total fungal population (Log_{10} CFU/g).

Bread	pH	Titratable acidity	Total microbial population	Total fungal population
Non-salt control	5.3 ± 0.1^{a}	0.3 ± 0.0^{c}	8.4 ± 0.1^{a}	8.5 ± 0.3^{a}
Salt Control	5.2 ± 0.1^{a}	0.4 ± 0.0^{c}	8.7 ± 0.1^{a}	8.6 ± 0.1^{a}
Salt propionate control	5.7 ± 0.1^{a}	0.3 ± 0.0^{c}	8.6 ± 0.1^{a}	8.6 ± 0.1^{a}
1% Fermented whey	5.3 ± 0.1^{a}	0.7 ± 0.0^{b}	8.7 ± 0.1^{a}	8.6 ± 0.1^{a}
5% Fermented whey	5.3 ± 0.1^{a}	0.8 ± 0.0^{b}	8.4 ± 0.1^{a}	8.4 ± 0.1^{a}
10% fermented whey	4.5 ± 0.4^{b}	1.8 ± 0.1^{a}	6.2 ± 0.3^{b}	6.5 ± 0.2^{b}

Data were presented in mean ± SD. Significatived differences among samples (colums) were maked different letters ($p < 0.01$).

(Dual AJS ESI) operating in the negative ion mode. The analysis was set at: capillary voltage 3.5 kV, drying gas flow (N2) 8.0 L/min, nebuliser pressure 30 psig, temperature of 350 °C and fragmentor voltage of 175 V. Targeted MS/MS study was performed using collision energy of 10, 20 and 40 eV. Calibration curves were conducted using concentrations ranging from of 0.01–1 mg/L. Software used for the data integration was MassHunter Qualitative Analysis Software B.08.00 (Agilent, California, United States). Each sample was injected 3 times (Dopazo et al., 2021).

2.7. Protection of bread from fungal spoilage with fermented whey as ingredient

Adapting the steps from Luz et al. (2020) with a few modifications after the bread preparation under sterile conditions in a Telstar MH 100 laminar flow hood (Telstar, Terrassa, Spain) 4 loafs of bread were contaminated in 4 spots with 10 μL of a fungal suspension 2.5 * 10^6 spores/mL, reaching a final concentration of 10^4 spores/g of bread. Then the bread were incubated at room temperature for 7 days. Each day fungal growth was studied by observation on each spot. Finally, 3 replicas of each bread were mixed at random with distilled peptone 0.1% (w/v) water in a 1/10 ratio (w/v). Afterwards, serial dilutions were performed of the samples and sow in PDA plates. After an incubation of 48 h at 25 °C the colony forming unis (CFU) were studied. Results were expressed in spores per gram of bread and the presence or absence of fungal growth per day.

2.8. Statistical analysis

To evaluate significant differences among each sample a multiple comparation analysis was performed with the software InfoStat 2019 using a one-way analysis of variance (ANOVA) test followed by Tukey's HSD post-test for the identification of significant differences (Universidad Nacional de Córdoba, Córdoba, Argentina). Differences between means were determined at $p < 0.01$.

3. Results and discussion

3.1. Effect of the fermented whey on the dough

The values of the pH and titratable acidity of the dough before fermentation and after fermentation can be seen in Table 1 and Table 2. The result of the pH measure evidenced that an addition of 1%, 5% and 10% of LAB fermented whey before fermentation significantly decreased the pH of the samples in comparation to the control. Nevertheless, After fermentation only the addition of 10% LAB fermented whey managed to reach a similar reduction. The titratable acidity increased significantly in all doughs that included the fermented whey as ingredient before fermentation and after fermentation in comparation to the control. The reduction of the pH and rise of titratable acidity is generally related to a higher resistance to microbial contamination (Pitt & Hocking, 2009). Besides, low concentrations of this acidic compounds increase the acceptability of the product as they reproduce the flavour of sourdough bread (Bartkiene et al., 2018; Robert et al., 2009).

The study of the dough expansion during the 1 h fermentation is shown in Table 2. The addition of fermented whey at 1% to the bread showed a significant increase of the expansion volume compared to the control bread. The addition of the nutrients from the fermented whey may increase CO_2 yeast production. Moreover the decrease of the pH helps to strength gluten networks which rises the alveolar CO_2 retention (Correa et al., 2021; Schober et al., 2003). The 5% addition of fermented whey to the dough gave statistically similar results of the expansion volume compared to the control. Finally, there was no volume increase in the dough with a 10% fermented whey. In this two samples the nutrient and pH phenomena described before is not happening probably due to the substitution of wheat flout with the fermented whey, as the concentration of wheat flour decreases the quantity of gluten and then the alveolar CO_2 retention also drops (Tomar et al., 2022). To increase the expansion volume surfactants are usually added as ingredient: egg yolk, soy lecithin and glucose oxidase (Y. Cao, Jiang, et al., 2021; Sang et al., 2020). Some other studies that substitute flour in the breadmaking with different residues from the food industry did not show this great results. This evidence can be shown in da Cunha et al. (2021) were this replacement of wheat flour by flour and pulp of *Caryocar brasiliense* lead to a reduction of the dough expansion during the fermentation.

The study of the total microbial population (TMP) and total fungal population (TFP) did not show any significant changes between samples and control before fermentation, as it can be appreciated in Tables 1 and 2. Dough before fermentation reached concentrations among 8.5 to 8.7 Log_{10} of colony forming units (CFU)/gram of TMP and TFP. After the 1 h fermentation the TMP and TFP was steady around a similar concentration of Log_{10} of CFU/gram in the 1% bread, 5% bread and all 3 controls. Nevertheless, the dough with a 10% LAB fermented whey showed a significative a reduction of 2.0 Log_{10} of CFU/gram of dough compared to the other doughs. Probably as a result of the antimicrobial activity from the compounds present in the fermented whey such as lactic acid (Cizeikiene et al., 2013). This reduction of the microbial population in

Table 3
Parameters of the bread were presented in two tables. Table 3a (a): pH, titratable acidity (lactic acid g/100 g), total microbial population (Log_{10} CFU/g), total fungal population (Log_{10} CFU/g), specific volume (cm^3/g). Table 3b (b) luminosity (L*), redness (a*), yellowness (b*), BI (browning index) and porosity (% of alveolar surface in a slice of bread).

a)

Bread	pH	Titratable acidity	Total microbial population	Total fungal population	Specific volume	Aw
Non-salt control	5.5 ± 0.1^{ab}	0.3 ± 0.0^{d}	nd	nd	3.25 ± 0.08^{a}	0.72 ± 0.01^{a}
Salt Control	5.6 ± 0.1^{a}	0.3 ± 0.0^{d}	nd	nd	2.64 ± 0.09^{b}	0.72 ± 0.01^{a}
Salt propionate control	5.4 ± 0.2^{ab}	0.3 ± 0.0^{d}	nd	nd	2.57 ± 0.13^{b}	0.75 ± 0.02^{a}
1% Fermented whey	5.1 ± 0.1^{bc}	0.5 ± 0.0^{c}	nd	nd	2.86 ± 0.23^{ab}	0.77 ± 0.01^{a}
5% Fermented whey	4.8 ± 0.1^{c}	0.6 ± 0.0^{b}	nd	nd	2.61 ± 0.22^{b}	0.78 ± 0.02^{a}
10% fermented whey	4.3 ± 0.1^{d}	1.4 ± 0.1^{a}	nd	nd	1.59 ± 0.07^{c}	nd

b)

Bread	L*	a*	b*	BI	Porosity
Non-salt control	84.85 ± 0.30^{a}	0.35 ± 0.14^{a}	1.40 ± 0.33^{a}	1.93 ± 0.49^{a}	25.9 ± 1.2^{ab}
Salt Control	84.75 ± 0.42^{a}	0.40 ± 0.22^{a}	1.31 ± 0.41^{a}	1.86 ± 0.58^{a}	23.7 ± 0.7^{ab}
Salt propionate control	85.11 ± 0.23^{a}	0.57 ± 0.11^{a}	1.64 ± 0.21^{a}	2.38 ± 0.26^{a}	22.7 ± 0.6^{b}
1% Fermented whey	84.56 ± 0.43^{a}	0.46 ± 0.22^{a}	0.84 ± 0.44^{a}	1.36 ± 0.68^{a}	26.1 ± 1.0^{ab}
5% Fermented whey	84.56 ± 1.20^{a}	0.46 ± 0.20^{a}	0.84 ± 1.16^{a}	1.36 ± 1.30^{a}	26.4 ± 1.2^{a}
10% fermented whey	84.44 ± 0.32^{a}	0.41 ± 0.17^{a}	0.95 ± 0.36^{a}	1.46 ± 0.52^{a}	Nd

Data were presented in mean ± SD. Significatived differences among samples (colums) were maked different letters ($p < 0.01$). Data under the limit of detection was marked as nd.

the 10% bread might be another of the reasons leading to the decrease on the dough expansion, as the yeast are the CO_2 producers in the dough (Tomar et al., 2022). Finally, by the results it can be appreciated that yeast were the main microorganisms found in the doughs and bread. Results from the TMP and TFP evidence similar microbial concentrations in all the samples. This is because Rose-Bengal agar is a selective medium with chloramphenicol, a compound that inhibits bacteria growth (Pilote-Fortin et al., 2021). Non-sourdough bread tent to have microbial population primarily consisting of yeast from *Saccharomyces* spp. (Madden et al., 2022).

3.2. Effect of the fermented whey on the bread

The pH and titratable acidity of the bread can be seen in Table 3. The pH was significative lower in the samples with a 5% and 10% fermented whey compared to the control. Similar results can be observed for the titratable acidity where samples also evidenced higher titratable acidity compared to the control bread. In the study of the TMP and TFP no CFU/

Table 4
Results of the quantification of organic acids (g/kg), lactic acid (a) and phenolic acids (mg/kg), DL-3-phenyllactic acid (b), benzoic acid (c) and ferulic acid (d) present in the dough and bread.

a) Lactic acid

Samples	Non-salt control	Salt Control	Salt propionate control	1% Fermented whey	5% Fermented whey	10% fermented whey
Before fermentation	nd	nd	nd	1.40 ± 0.04^{cB}	6.56 ± 0.1^{bA}	27.16 ± 0.04^{aA}
After fermentation	nd	nd	nd	1.68 ± 0.03^{cA}	5.52 ± 0.04^{bB}	24.44 ± 0.04^{aB}
After bake	nd	nd	nd	1.04 ± 0.03^{cC}	5.64 ± 0.11^{bB}	23.96 ± 0.18^{aC}

b) DL-3-Phenyllactic acid

Samples	**Non-salt control**	**Salt Control**	**Salt propionate control**	**1% Fermented whey**	**5% Fermented whey**	**10% fermented whey**
Before fermentation	0.14 ± 0.00^{cC}	0.24 ± 0.02^{cA}	0.50 ± 0.26^{cA}	1.54 ± 0.15^{cA}	7.86 ± 0.74^{bA}	14.78 ± 1.35^{aA}
After fermentation	0.67 ± 0.00^{cA}	0.25 ± 0.02^{cA}	0.43 ± 0.04^{cA}	1.21 ± 0.08^{cA}	3.49 ± 0.50^{bB}	7.28 ± 0.05^{aB}
After bake	0.24 ± 0.02^{cdB}	0.18 ± 0.02^{cA}	0.15 ± 0.01^{cA}	1.84 ± 0.14^{cA}	3.65 ± 0.49^{bB}	6.24 ± 0.02^{aB}

c) Benzoic acid

Samples	**Non-salt control**	**Salt Control**	**Salt propionate control**	**1% Fermented whey**	**5% Fermented whey**	**10% fermented whey**
Before fermentation	0.19 ± 0.01^{aC}	0.31 ± 0.01^{aC}	0.27 ± 0.06^{aB}	nd	0.75 ± 0.05^{aA}	2.13 ± 0.17^{aA}
After fermentation	0.50 ± 0.01^{bB}	0.51 ± 0.00^{bB}	0.37 ± 0.02^{cB}	0.70 ± 0.02^{aB}	nd	0.40 ± 0.02^{cC}
After bake	1.94 ± 0.01^{aA}	1.61 ± 0.01^{bA}	1.33 ± 0.08^{bcA}	1.22 ± 0.07^{cA}	0.80 ± 0.02^{dA}	1.32 ± 0.12^{bcB}

d) Ferulic acid

Samples	**Non-salt control**	**Salt Control**	**Salt propionate control**	**1% Fermented whey**	**5% Fermented whey**	**10% fermented whey**
Before fermentation	0.24 ± 0.04^{c}	0.70 ± 0.03^{aA}	nd	nd	0.38 ± 0.00^{bA}	nd
After fermentation	nd	0.42 ± 0.02^{bB}	0.75 ± 0.12^{a}	0.28 ± 0.04^{bB}	0.30 ± 0.07^{bA}	0.46 ± 0.11^{bA}
After bake	nd	nd	nd	1.03 ± 0.02^{aA}	0.14 ± 0.01^{cB}	0.22 ± 0.05^{bA}

Data were presented in mean ± SD. Values that were significantly different among the stage of the bread preparation between samples (rows) were marked with lower case letters, significative differences during the bread preparation in the same sample (columns) were marked with different capital letters ($p < 0.01$). Data under the limit of detection was marked as nd.

gram of bread were detected in the in any of the samples (Table 3). As is described by Cauvain and Young (2007) at high temperatures like the ones used for the bake (200 °C for 45 min) most the microorganism present in the bread are inactivated.

Specific volume is one of the results (Table 3) described a similar tendency to the evidenced in the in the expansion volume assay. Non-salt control evidenced a significant increase of the cm^3/g of bread in comparation to the other bread, except the bread 1%. The specific volume of bread 1% was slightly greater to the salt-control and the salt-propionate-control. Nevertheless, no significative differences were observed among the bread 1%, the bread 5%, the salt-control and the salt-propionate-control. The only a reduction of the specific volume was observed in the bread 10% in comparation to the control bread. Usually treatments like the one used in this study lead to a reduction of the wheat flour content of bread with no addition of proteases decrease the specific volume of bread (Honda et al., 2021; Sadeghian Motahar et al., 2022). The addition of other ingredients with high sugar concentrations, like red beetroot powder and raw fermented maize, in quantities around 5–10% usually leads to this reduction (Cui et al., 2022; Sun et al., 2022). Water activity values of all bread (Table 3) did not evidence any relevant differences. They average a 0.72 to a 0.78 Aw witch is below the ideal Aw for the fungal growth (Pitt & Hocking, 2009).

Regarding the colorimetric parameters of the LAB fermented whey bread the luminosity did not show any significative differences compared to the samples. The redness and the yellowness evidenced a similar trend (Table 3). Usually, the addition of new ingredients to the bread caused changes in the color. Among many other authors, Cirlincione et al. (2021) reports a reduction in the L*, a* and b* parameter with the *Pleurotus eryngii* powder to the recipe (Angioloni & Collar, 2012; Cirlincione et al., 2021). No significative differences were manifested browning index among the bread. The addition of reductor sugars and proteins increase Maillard reaction during the bakery of the product (Kowalski et al., 2022; Sadeghian Motahar et al., 2022). Nevertheless, the lactose and proteins present in the whey did not alter the color of the bread.

Finally, the study of the alveolar surface evidenced a significative increase of the bread with a 5% fermented whey in comparation to the bread control with propionate and salt (Table 3). No significative differences were observed in comparation with the other samples. This alveolar surface is related to the yeast CO_2 production. Higher concentrations of sugars like the lactose present in the whey help to improve this CO_2 production (Zhou et al., 2021). Specific volume, color and porosity of the bread are the attributes that most affect consumer opinion. Higher similarities of these parameters between a modified bread to a regular bread are related to an increase of the acceptance of these products by the consumer (Morais et al., 2014).

3.3. Antifungal metabolites present in bread and dough

After the determination and quantification of the different antifungal metabolites occurrence four different compounds were present in detectable quantities. This observed compounds in the dough and bread are shown in Table 4. This phenolic and organic acids were lactic acid, DL-3-phenyllactic acid (PLA), benzoic acid and ferulic acid. Lactic acid was only found in the dough and bread containing the LAB fermented whey. The concentration of this metabolite slightly decreased during process. This compound is the mayor metabolite produced by the LAB fermentation (Carr et al., 2002). Therefore, as it was expected the quantity of this compound was significantly higher when more fermented whey was added to the bread recipe. The concentration of this acid during the bread production. reached concentrations of 1.04–1.40 g/kg in the dough and bread with 1% fermented whey, from 5.52 to 6.56 g/kg in the ones with 5% and from 23.96 to 27.16 g/kg in the with a 10%. Among the phenolic compounds only the PLA was present in significative greater concentrations in the 5% and 10% fermented whey bread compared to the control. During the fermentation steep this acid significantly decreased its concentrations in all dough. Nevertheless, PLA concentrations kept stable during the bake steep. The concentration of this compound before the fermentation was 7.86 and 12.78 mg/kg in the 5% fermented whey sample and 10% fermented whey samples, respectively. Then after the fermentation and in the bread the concentration of PLA decreased to values between 3.49 and 3.65 mg/kg in the 5% fermented whey bread and from 7.28 to 6.24 in the 10% fermented whey sample. Benzoic acid and ferulic acid were found at lower concentrations.

Many authors report the wide antifungal spectrum of the detected metabolites (Axel et al., 2016; Corsetti et al., 1998; Illueca et al., 2021; Magnusson et al., 2003). Lactic acid is a commonly used by the industry as a food preservative. Use of this compound is even approved by Asia and the European Union as it is known to be safe for the consumer and have antimicrobial properties (ANNEX 1: ASEAN MAXIMUM USE LEVELS OF FOOD ADDITIVES, 2021; European Parliment, 2010). PLA is another naturally occurring compounds produced during LAB fermentations (Xu et al., 2021). This compound is known to have antifungal activity against many fungal species by targeting and disaggregating fungal cell membranes (Dieuleveux et al., 1998). Therefore, presence of this compound have been linked to the growth inhibition of many fungal species (Lipinska-Zubrycka et al., 2020; Russo et al., 2017; Schnürer & Magnusson, 2005).

Table 5
Shelf life of the bread loaves contaminated with *A. flavus* (a) and *P. verrucosum* (b) monitored in days. Presence of fungal growth in any of the sports among the 4 replica was indicated as "+".

a) *Aspergillus flavus*

Day	Non-salt control	Salt Control	Salt propionate control	1% Fermented whey	5% Fermented whey
1	–	–	–	–	–
2	–	–	–	–	–
3	–	–	–	–	–
4	+	+	+	–	–
5	+	+	+	–	–
6	+	+	+	+	–
7	+	+	+	+	+

b) *Penicillium verrucosum*

Day	Non-salt control	Salt Control	Salt propionate control	1% Fermented whey	5% Fermented whey
1	–	–	–	–	–
2	–	–	–	–	–
3	–	–	–	–	–
4	+	+	+	–	–
5	+	+	+	+	–
6	+	+	+	+	–
7	+	+	+	+	+

3.4. Bio-preservation of bread

The results of the shelf life of bread monotonized though the 7-day incubation are displayed in Table 5. As it can be appreciated the bread with a 10% concentration of fermented whey was not included in this study as a result of not reaching the ideal properties expected from a bread. Focusing on shelf live, both studied bread (the 1% and 5% fermented whey) extended the shelf life of this product compared to the controls. In the bread contaminated with *A. flavus* the shelf life was extended to the fifth day of incubation, while on the control bread the growth of mold was observable after only 3 days. In the bread inoculated with *P. verrucosum* the fungi growth after 4 days in all 3 control bread, after 5 days in the bread with a 1% fermented whey and finally, the bread with a 5% fermented whey the mold only was visible after 7 days incubation. Therefore, the addition of a 5% fermented whey managed to extend the shelf life of the bread 2 days against *A. flavus* and for 3 days

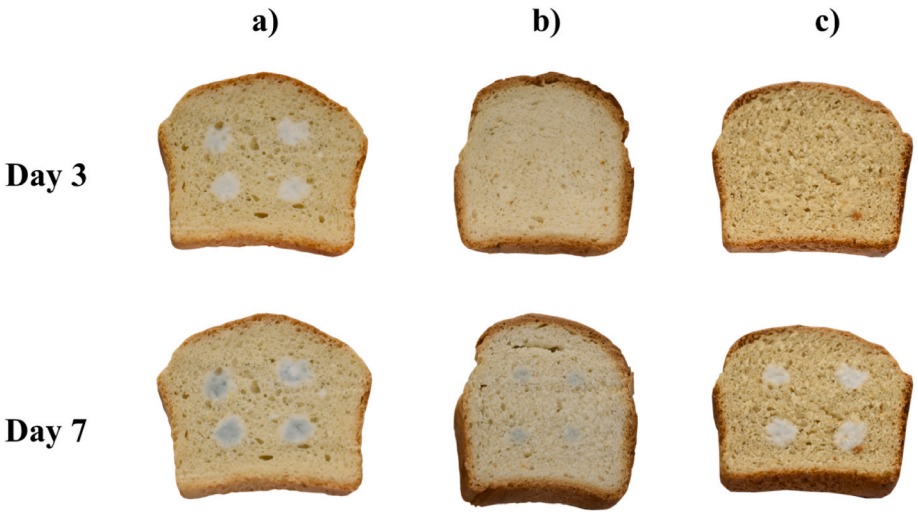

Fig. 2. Loaves of bread inoculated with *P. verrucosum*. Salt Control bread (a) and 1% fermented whey bread (b) and 5% fermented whey bread (c) after 3 and 7 day incubation.

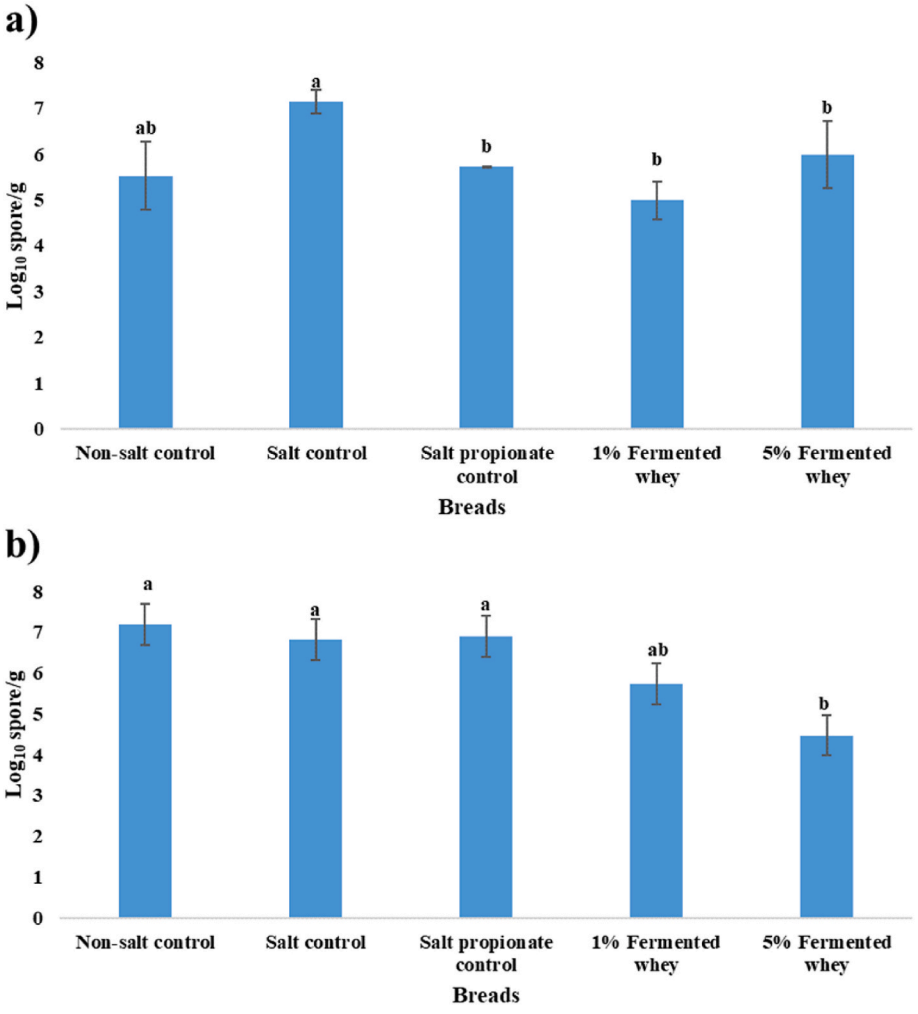

Fig. 3. Results of determination of the fungal colony forming units in bread contaminated with *A. flavus* (a) and *P. verrucosum* (b) after a 7 day incubation. The difference of letters indicates significant differences ($p < 0.01$).

against *P. verrucosum* in comparation to the control bread. In Fig. 2 it can be appreciated the state of the bread after the third and seven days of incubation.

The treatment proved to increase the shelf life of the bread. The microbiological study of the bread (Fig. 3) proved the previous results. Bread with a 5% reduced the presence of fungi in a 2.4 LOG_{10} spores/g of

bread compared to the control bread. Other bread contaminated by these fungi did not show any significative differences between them. It should be pointed that the control bread with calcium propionate, the most common antifungal product used in the bakery industry, did not managed to extend the shelf life of the bread more than the studied treatment (EFSA, 2016). On the other hand, the treatment against *A. flavus* did not evidence a reduction of the fungal population after the 7 days incubation.

The use from the LAB fermented whey as a bio-preservative agent of bread is really interesting for many reasons, especially two. First, in Spain and other countries the addition of milk derivates to bread is legal and allowed by the legislation (Real Decreto 308/2019). Secondly, lactic acid bacteria have the consideration as Qualified Presumption of Safety (QPS) by the European Union and Generally-regarded-as-safe (GRAS) by the Food and Drug Administration of the United States of America, this means that the addition of this microorganisms in permitted in foods among these regions. In addition, the other advantages exposed in the introduction postulates the use of this antifungal agent as an interesting alternative or complementary product to the commonly used in the industry, like the calcium propionate, propionic acids, or sodium benzoate (Mandal et al., 2013).

The use of LAB fermented whey ingredient is a novel application for the bread bio-preservation. There are very few applications of for a LAB fermented whey in the bibliography. Luz et al. (2021) studies evidence the possible use of fermented whey by *L. plantarum* and *L. ghanensis* species as a substitute of water in the bread production to extend the shelf life of bread for 2 days compared to a control bread with sodium propionate against a *Penicillium expansum* contamination. Some other possible applications of LAB to preserve bread can be found. Gerez et al. (2009) report the use of sourdough starters with LAB with antifungal properties is *Lactobacillus plantarum, Lactobacillus reuteri, and Lactobacillus brevis* for the preservation of bread against *A. niger* obtains similar result that a calcium propionate control. Lavermicocca et al. (2000) study evidence the potential in *in vitro* test of many species of *Lactobacillus alimentarius, Lactococcus lactis, Lactobacillus brevis and Lactobacillus plantarum* to inhibit the growth of fungi from the *Aspergillus* and *Penicillium* genera that typically contaminate bread.

4. Conclusions

This ingredient (LAB fermented whey) has the potential to reducing the use of the chemical additives added by the industry to bread to prevent fungal contamination. The addition of this ingredient to a bread preparation did not greatly altered the physical parameters of the bread. In addition, an increase of the shelf life of the bread was achieved in comparation to a regular bread and a bread with calcium propionate as an additive. This reduction of the fungal contamination was achieved with fungal concentrations far superior to the ones detected naturally. Further studies will be focused on the scalable production and sensory evaluation of the bakery product with the ingredient. Moreover, the production of fermented whey by lactic acid bacteria is really cheap. This subproduct is the principal residue from the cheese making industry and is a great matrix for lactic acid bacterial fermentation. Finally, in addition, the revalorization from the whey is among the 2030 objectives for sustainable development proposed by the WHO.

CRediT authorship contribution statement

Victor Dopazo: Data curation, Writing – original draft, Investigation, Writing – review & editing. **Fran Illueca:** Data curation, Writing – original draft, Investigation. **Carlos Luz:** Conceptualization, Methodology, Visualization, Writing – review & editing. **Leo Musto:** Investigation, Writing – review & editing. **Ana Moreno:** Investigation, Writing – review & editing. **Jorge Calpe:** Software, Writing – review & editing. **Giuseppe Meca:** Conceptualization, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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