

Original article

# Application of whey of Mozzarella di Bufala Campana fermented by lactic acid bacteria as a bread biopreservative agent

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**Summary** A total of nine isolated lactic acid bacteria (LAB) from tomato and sourdough with antifungal activity were employed to reevaluate the whey of Mozzarella di Bufala through the fermentation process for 72 h at 37 °C. Then, the fermented whey (BWF) was characterised and used as biopreservative in bread formulation. *L. plantarum* TR7 and *L. plantarum* TR2 strains showed average lactic acid concentration in BWF of 13.8 g L<sup>-1</sup>. Also, the bread volatile organic compounds (VOC) analysis showed an increase in hexanal, benzeneacetaldehyde, benzaldehyde and pyrazine tetramethyl when using BWF as ingredient. Moreover, the DPPH-inhibitory activity of bread with BWF extract also reflected a 33% rise in comparison with control bread. The application of BWF as a biopreservation agent in bread showed an increase in shelf life compared with bread with 0.3% calcium propionate and bread control for 2 and 15 days, respectively. BWF can be used as an interesting biopreservation strategy of bread.

**Keywords** Biopreservation, bread shelf life, lactic acid bacteria, whey.

## Introduction

Microbial spoilage of bakery products causes significant economic losses for the food industry and consumers. Fungi belonging to the genera *Aspergillus*, *Penicillium*, *Rhizopus*, *Cladosporium*, *Endomyces*, *Fusarium*, *Monilia* and *Mucor* are the most common microorganisms responsible for the spoilage of bread (Dal Bello *et al.*, 2007).

Currently, there is increased consumer interest in the relationship between food and health. In recent years, there has been an increase in the market for functional foods with health-promoting properties. In contrast, the widespread use of food additives for food preservation is causing discomfort in many consumers (Mohammadzadeh-Aghdash *et al.*, 2019). The food industry, pressured by market demands, is subjected to the development of new products and food transformation processes to obtain safe food.

Food biopreservation is an application which improves the food shelf life and increases food safety through the use of microorganisms or their metabolites (Crowley *et al.*, 2013). Recent studies have identified

various microbial species with antifungal properties. These have been isolated from different natural sources, such as fruits, vegetables, cereals, dairy and bread products, meat and other food-related products (Salas *et al.*, 2017). Research and development of natural antimicrobials derived from by-products of the food industry to increase the shelf life of food is on the rise (Ribes *et al.*, 2018).

Mozzarella di Bufala Campana (MBC) is listed as a protected designation of origin (POD) product of the European Council (Commission Regulation, 1996). Its production discipline identifies 96 municipalities in southern Italy as the only production sites for the so-called MBC DOP. The production of Mozzarella di Bufala increased by 26.1% from 2013 to 2017 (CLAL, 2019). Whey is the liquid fraction obtained after the coagulation of milk proteins during cheese production. This product represents around 85–90% of the volume of milk and retains approximately 55% of its nutrients (Smithers, 2008). The high content of organic matter is associated with a high biological oxygen demand (BOD), causing the disposal of this by-product to have a high environmental impact. From a microbiological point of view, whey fermentation by lactic acid bacteria (LAB) has been described by several authors.

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Furthermore, the fermentation of whey produces a reduction in BOD and allows for the procurement of compounds with a high added value for the food industry (Panchal *et al.*, 2020).

However, few studies on the revaluation of whey derived from the production of MBC are available. Thus, the aim of the present study was to increase the shelf life of loaf bread using MBC whey fermented by selected LAB as a biopreservative. To achieve this objective: (i) the production of antimicrobial compounds and total phenolic compounds and the antioxidant activity of MBC fermented whey; and (ii) the incorporation of MBC whey fermented in bread as a biopreservative were studied, and the antimicrobial compounds, total phenolic content, antioxidant activity and shelf life of bread were characterised.

## Materials and methods

### Bacterial isolation

From a preliminary study, a total of nine LAB isolated from sourdough and tomato for antifungal activity against toxigenic fungi belonging to the genera *Penicillium*, *Aspergillus*, *Fusarium* and *Alternaria* were selected. Bacterial identification was carried out by 16S rRNA gene sequencing and the full sequence of the 16S rRNA obtained and compared with the online tool BLAST confirmed the identity of isolates at the species level: *Leuconostoc pseudomesenteroides* IRK751, *Fructobacillus ficulneus* IRK81, *Lactobacillus brevis* IRK82, *Leuconostoc pseudomesenteroides* SMF76, *Lactobacillus brevis* POM, *Lactobacillus plantarum* TR7, *Lactobacillus plantarum* TR71, *Lactobacillus plantarum* TR14 and *Lactobacillus ghanensis* TR2 (Luz *et al.*, 2020a).

All strains were preserved in sterile 30% glycerol and stored at  $-80^{\circ}\text{C}$  before use. For the recovery of LAB, LAB were inoculated in MRS Broth culture medium obtained from Liofilchem (Roseto degli Abruzzi, Italy) at  $37^{\circ}\text{C}$  for 48 h under anaerobic conditions.

### MBC whey fermentation

Mozzarella di Bufala Campana whey was obtained in a local dairy 1 h after the curdling process. Then, the whey was pasteurised by heating in a water bath to  $70^{\circ}\text{C}$  for 30 min with continuous mixing, cooled rapidly using cool water and stored at  $-28^{\circ}\text{C}$ . MBC whey was obtained from Caseificio Visone (San Giorgio a Cremano, Italy) 'Mozzarella di Bufala Camapana DOP' producer.

For whey fermentation, LAB were cultivated in MRS Broth at  $37^{\circ}\text{C}$  until the exponential growth phase (12 h). Then, LAB were inoculated at a

concentration of  $10^6$  CFU  $\text{mL}^{-1}$  in 50 mL of MBC whey and incubated at  $37^{\circ}\text{C}$  for 72 h. After fermentation, LAB were separated by centrifugation at 3200 g for 10 min. Fermented MBC whey (BWF) was stored at  $-28^{\circ}\text{C}$  until analysis. Non-fermented MBC whey was processed the same way; this was considered the control sample. The BWF with the highest concentration of lactic acid was selected for characterisation and application as a biopreservative in bread.

### Bread making and determination of shelf life

Bread was prepared according to the method reported by Saladino *et al.* (2016), with slight modifications. Commercial wheat flour (250 g) was mixed with 2.5 g sugar, 20 mL of extra-virgin olive oil, 5 g salt, 15 g of instant yeast and 125 mL of tap water. The doughs were fermented at  $28^{\circ}\text{C}$  for 1 h in a Panasonic MIR-154 cooling incubator (Milan, Italy). In total, eight bread dough samples were baked at  $230^{\circ}\text{C}$  for 30 min in a BISTROT 665 VISION deck oven (Ferrara, Italy). After, the breads were cooled in cooling incubator at  $25^{\circ}\text{C}$  for 1 h. Eight different breads were formulated in triplicate: bread without additives (control), bread with calcium propionate 0.3% (control propionate), bread with lactic acid at a concentration of  $3\text{ g kg}^{-1}$  (control lactic), bread with non-fermented buffalo whey (control whey), bread with 50% BWF by *Lactobacillus plantarum* TR7 (bread TR7 BWF50), bread with 50% BWF by *Lactobacillus ghanensis* TR2 (bread TR2 BWF50), bread with 100% BWF by *Lactobacillus plantarum* TR7 (bread TR7 BWF100), bread with 100% BWF by *Lactobacillus ghanensis* TR2 (bread TR2 BWF100).

The impact of antifungal inhibition of BWF on bread was evaluated by following the method used by Ryan *et al.* (2011), with some modifications. After cooling the breads, three replicas for each bread were sliced, hermetically sealed in a low-density polyethylene bag using a Samic TS-150 thermo-sealer (Basarte, Spain) and incubated in a constant climate chamber from BINDER KBF 240 (Tuttlingen, Germany) at  $20^{\circ}\text{C}$  and 70% humidity for 20 days. Each day, the surface of the inoculated bread was examined visually for presence or absence of fungal growth and to establish the effect of the treatment on the shelf life. Finally, breads were crushed using an Oster Classic grinder (Madrid, Spain) and kept at  $-20^{\circ}\text{C}$  before characterisation analysis.

### BWF and bread characterisation

#### Total phenolic content

The total phenolic content (TPC) of bread was determined in 80% methanolic extracts. Two grams of bread were mixed continuously with 20 mL of 80%

methanol for 2 h at 37 °C. For the determination of TPC in BWF, no extraction was performed. Both were analysed following a laboratory procedure using the Folin–Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA). The final reaction mixture contained 1 mL of sample, 1 mL of Folin–Ciocalteu reagent, 6 mL of deionised water and 1.5 mL of saturated sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) solution. After 2 h of reaction, the absorbance at 765 nm was determined using a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer (Perkin-Elmer, Jügesheim, Germany). The results were derived from a calibration curve ( $y = 0.0154x - 0.0041$ ,  $R^2 = 0.999$ ) of gallic acid (0–50  $\mu\text{g mL}^{-1}$ ) and expressed in gallic acid equivalents (GAE) per gram of bread or millilitre of BWF (Michalska *et al.*, 2007).

#### DPPH radical-scavenging assay

The scavenging effects of BWF and 80% methanolic bread extract on DPPH free radical (Sigma-Aldrich) were measured using the method described by Zhang *et al.* (2013). A 1 mL sample was mixed with 1 mL of 0.2 mM DPPH solution in ethanol (extemporaneously prepared). The mixture was incubated at room temperature in the dark for 30 min. Then, the absorbance at 517 nm was determined using a spectrophotometer. Deionised water and 80% methanol were employed as a blank. The percentage inhibition of DPPH was determined considering the blank absorbance as the 0% inhibition value. Therefore, higher per cent inhibition values indicate higher free radical-scavenging ability.

#### Identification of organic acids

For the analysis of organic acids, samples were properly diluted in water and were injected into the UHPLC (Khosravi *et al.*, 2015). UHPLC analysis was performed on a Jasco LC-4000 UHPLC system (Hachioji, Japan) equipped with a pump (PU-4285) and a photometric diode array detector (MD-4010) using a 5  $\mu\text{L}$  sample injection loop. Analytical separation was performed using an isocratic mobile phase of acidified water (pH 2.1) at a flow rate of 0.6  $\text{mL min}^{-1}$  for 25 min using a Rezex ROA-Organic Acid H+ (8%) (150  $\times$  7.8 mm) ion exclusion column (Phenomenex, CA, USA). The chromatogram was monitored at 210 nm. Data acquisition was carried out with the HP-CORE ChemStation system Agilent Technologies (Santa Clara, CA, USA). Results were expressed as  $\text{g L}^{-1}$ .

#### Analysis of volatile organic compounds

Five grams BWF or 1 g of bread with 5 mL of water was placed in a 10 mL glass vial. VOCs were identified by gas chromatography coupled to single quadrupole mass spectrometry (GC/MS) analysis. Prior to

analysis, samples were incubated for 45 min at 55 °C in a water bath while being gently stirred with a rod. VOCs were extracted from the vial headspace by solid-phase microextraction (SPME). An SPME holder Supelco (PA, USA) containing a fused silica fibre coated with a 50/30  $\mu\text{m}$  layer of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) was used to trap VOCs in the vial headspace. The fibre was introduced into the splitless inlet of an Agilent 6890N GC system Agilent Technologies (Palo Alto, CA, USA), and thermal desorption of the analyses was performed at 250 °C for 5 min. The GC system was equipped with an HP-5MS (30 m  $\times$  0.25 mm, 0.25  $\mu\text{m}$  5% diphenyl/95% dimethylpolysiloxane) capillary column J&W Scientific (Folsom, CA, USA). The oven was programmed to start at 40 °C (held for 2 min) and to ramp up to 160 °C at 6 °C  $\text{min}^{-1}$ , then to ramp up to 260 °C at 10 °C  $\text{min}^{-1}$  (held for 4 min). Helium (99.999%) was used as the carrier gas, and the flow rate was 1  $\text{mL min}^{-1}$ . The flow was transferred from the column into an Agilent 5973 MS detector (Agilent Technologies). The ion source temperature was set at 230 °C, the ionising electron energy was 70 eV, and the mass range was 40–450 Da in full scan acquisition mode. Compounds were identified using the NIST Atomic Spectra Database Version 1.6 taking into consideration spectra with major similarities of 95% and quantified by internal calibration (Guarrasi *et al.*, 2017). The results were expressed in  $\text{mg kg}^{-1}$ . The analysis was carried out in triplicate.

#### Statistical analysis of data

The statistical analysis was performed using the software *InfoStat* version 2008 (Córdoba, Argentina). The differences between the groups were analysed with one-way ANOVA followed by the Tukey HSD *post hoc* test for multiple comparisons. The level of significance considered was  $P < 0.01$ .

## Results and discussion

#### Total phenolic content

The TPC in BWF and breads was evaluated by the Folin–Ciocalteu method, and the results are plotted in Table 1. No significant differences were observed between the TPC of the BWF and the non-fermented buffalo whey. However, significant differences were shown between the TPC of different types of bread. The TPC of breads ranged from 8.1 to 13.9  $\text{mg GAE/100 g}$ . Control propionate, control whey, TR7 BWF100 and TR2 BWF100 showed a significantly higher TPC than control breads, control lactic breads and breads treated by substitution of 50% of water with BWF. In particular, bread TR7 BWF100

**Table 1** Total polyphenol content (mg GAE/L) and % inhibitory activity DPPH of (a) MBC whey unfermented (control) and BWF by *L. plantarum* TR7 and *L. plantarum* TR2 and (b) eight typologies of bread. Different letters represent a significant difference among the treatments ( $P \leq 0.01$ ). The experiment was carried out in triplicate ( $n = 3$ ). Results are expressed as mean  $\pm$  standard deviation

(a) BWF	Total polyphenol content (mg GAE/L)	% Inhibitory activity DPPH
Control	21.2 $\pm$ 1.3 <sup>a</sup>	62.9 $\pm$ 0.8 <sup>a</sup>
<i>Lactobacillus plantarum</i> TR7	18.3 $\pm$ 0.4 <sup>a</sup>	76.4 $\pm$ 0.9 <sup>c</sup>
<i>Lactobacillus ghanensis</i> TR2	18.7 $\pm$ 0.7 <sup>a</sup>	73.2 $\pm$ 0.4 <sup>b</sup>
(b) Treatment	Total polyphenol content (mg GAE/100 g)	% Inhibitory activity DPPH
Control	8.1 $\pm$ 0.6 <sup>a</sup>	42.2 $\pm$ 0.9 <sup>a</sup>
Control propionate	11.2 $\pm$ 0.6 <sup>bc</sup>	61.4 $\pm$ 1.1 <sup>c</sup>
Control Lactic	9.7 $\pm$ 0.6 <sup>ab</sup>	42.3 $\pm$ 0.9 <sup>a</sup>
Control whey	10.4 $\pm$ 0.5 <sup>bc</sup>	55.8 $\pm$ 1.1 <sup>b</sup>
TR7 BWF50	9.6 $\pm$ 0.5 <sup>ab</sup>	56.8 $\pm$ 1.5 <sup>b</sup>
TR7 BWF100	13.9 $\pm$ 0.5 <sup>d</sup>	75.6 $\pm$ 1.1 <sup>e</sup>
TR2 BWF50	9.7 $\pm$ 0.7 <sup>ab</sup>	58.3 $\pm$ 1.9 <sup>bc</sup>
TR2 BWF100	12.3 $\pm$ 0.3 <sup>cd</sup>	70.3 $\pm$ 1.6 <sup>d</sup>

evidenced a TPC value greater than the control whey. In this sense, Skrajda-Brdak *et al.* (2019) reported an increase in the free phenolic acids and antioxidative capacity of bread fermented by strains *L. casei* and *L. brevis* compared with common fermentation with baker's yeast *Saccharomyces cerevisiae*. Moreover, Izzo *et al.* (2020) also reported production of twelve phenolic acids in cow's milk whey fermented by four strains of *L. plantarum*.

#### DPPH radical-scavenging assay

The antioxidant activity of BWF and breads was determined by the DPPH radical-scavenging assay, and the results are presented in Table 1. BWF by *L. plantarum* TR7 and *L. ghanensis* TR2 showed a significant increase in antioxidant activity ( $P < 0.01$ ). The percentages of inhibition of DPPH ranged from 63% to 76%. The inhibition of DPPH values of the BWF and non-fermented buffalo whey was, in an ascending order, non-fermented buffalo whey < BWF by *L. ghanensis* TR2 < BWF by *L. plantarum* TR7. Different bread treatments also influenced the antioxidant activity. Control bread and control lactic acid bread evidenced the lowest percentage inhibition of DPPH values (both 42%). The use of propionate and non-fermented whey showed greater activity than the

control bread. Also, breads in which 50% of the water was replaced with BWF by two LAB showed no significant differences from the use of twice the amount of non-fermented whey. However, the highest value of replacement with BWF (BWF100) in the dough formulation evidenced a significant increase of antioxidant activity (70%–76%) in comparison with use of non-fermented whey and 3 g kg<sup>-1</sup> of calcium propionate. The inhibition of DPPH values of each bread was, in ascending order, control < control lactic < control whey < TR7 BWF50 < TR2 BWF50 < control propionate < TR2 BWF100 < TR7 BWF100. Virtanen *et al.* (2007) indicated that milk fermentation by lactic acid bacteria produced an increment of radical scavengers. Also, they relate the development of radical scavengers with the proteolytic activity of lactic acid bacteria.

#### Identification of antifungal compounds

According to the literature, the antifungal activity of LAB is not due to a single compound but depends on the biocomplex of compounds present in the fermentation medium (Luz *et al.*, 2020a).

**Table 2** Concentration of lactic acid and pH values of (a) MBC whey unfermented (control) and BWF by nine LAB and (b) eight typologies of bread. Different letters represent a significant difference among the treatments ( $P \leq 0.01$ ). The experiment was carried out in triplicate ( $n = 3$ ). Results are expressed as mean  $\pm$  standard deviation

(a) BWF	pH	Lactic acid (g L <sup>-1</sup> )
Control	4.73 $\pm$ 0.06 <sup>h</sup>	6.51 $\pm$ 0.18 <sup>a</sup>
<i>Lactobacillus plantarum</i> CECT 220	3.75 $\pm$ 0.01 <sup>b</sup>	7.18 $\pm$ 0.23 <sup>b</sup>
<i>Lactobacillus plantarum</i> TR14	4.08 $\pm$ 0.01 <sup>e</sup>	11.78 $\pm$ 0.03 <sup>f</sup>
<i>Lactobacillus brevis</i> IRK82	3.99 $\pm$ 0.01 <sup>d</sup>	7.4 $\pm$ 0.00 <sup>b</sup>
<i>Leuconostoc pseudomesenteroides</i> IRK751	4.55 $\pm$ 0.01 <sup>fg</sup>	9.19 $\pm$ 0.16 <sup>d</sup>
<i>Leuconostoc pseudomesenteroides</i> SMF76	4.63 $\pm$ 0.01 <sup>g</sup>	8.58 $\pm$ 0.06 <sup>c</sup>
<i>Lactobacillus brevis</i> POM	4.51 $\pm$ 0.04 <sup>f</sup>	9.26 $\pm$ 0.03 <sup>d</sup>
<i>Lactobacillus plantarum</i> TR7	3.38 $\pm$ 0.01 <sup>a</sup>	15 $\pm$ 0.23 <sup>h</sup>
<i>Lactobacillus plantarum</i> TR71	3.96 $\pm$ 0.01 <sup>d</sup>	10.65 $\pm$ 0.43 <sup>e</sup>
<i>Lactobacillus ghanensis</i> TR2	3.87 $\pm$ 0.01 <sup>c</sup>	12.49 $\pm$ 0.01 <sup>g</sup>
(b) Treatment	pH	Lactic acid (g kg <sup>-1</sup> )
Control	5.42 $\pm$ 0.07 <sup>e</sup>	nd
Control propionate	5.33 $\pm$ 0.04 <sup>e</sup>	nd
Control Lactic	4.5 $\pm$ 0.05 <sup>a</sup>	3.42 $\pm$ 0.10 <sup>e</sup>
Control whey	5.15 $\pm$ 0.03 <sup>d</sup>	1.96 $\pm$ 0.08 <sup>c</sup>
TR7 BWF50	4.89 $\pm$ 0.06 <sup>bc</sup>	1.73 $\pm$ 0.04 <sup>b</sup>
TR7 BWF100	4.62 $\pm$ 0.00 <sup>a</sup>	3.97 $\pm$ 0.03 <sup>f</sup>
TR2 BWF50	5.01 $\pm$ 0.04 <sup>c</sup>	1.36 $\pm$ 0.04 <sup>a</sup>
TR2 BWF100	4.79 $\pm$ 0.01 <sup>b</sup>	2.85 $\pm$ 0.05 <sup>d</sup>

**Table 3** Identification and quantification of VOCs (mg kg<sup>-1</sup>) in a) MBC whey unfermented (control) and BWF by *L. plantarum* TR7 and *L. plantarum* TR2 and b) eight typologies of bread. Different letters represent a significant difference among the treatments ( $P \leq 0.01$ ). The experiment was carried out in triplicate ( $n = 3$ ). Results are expressed as mean  $\pm$  standard deviation

(a) VOCs	BWF							
	Control	TR7	TR2					
Alcohols								
Ethanol	61 $\pm$ 3 <sup>a</sup>	143 $\pm$ 9 <sup>c</sup>	87 $\pm$ 2 <sup>b</sup>					
1-Butanol, 2-methyl	7 $\pm$ 2 <sup>a</sup>	7 $\pm$ 2 <sup>a</sup>	10 $\pm$ 1 <sup>a</sup>					
1-Butanol, 3-methyl	nd	6 $\pm$ 1 <sup>a</sup>	8 $\pm$ 1 <sup>a</sup>					
1-Hexanol	nd	7 $\pm$ 1 <sup>a</sup>	13 $\pm$ 3 <sup>a</sup>					
1-Octanol	nd	21 $\pm$ 3 <sup>b</sup>	9 $\pm$ 1 <sup>a</sup>					
Acids								
Acetic acid	95 $\pm$ 4 <sup>a</sup>	314 $\pm$ 14 <sup>b</sup>	325 $\pm$ 7 <sup>b</sup>					
Hexanoic acid	179 $\pm$ 9 <sup>a</sup>	344 $\pm$ 9 <sup>b</sup>	386 $\pm$ 10 <sup>c</sup>					
Octanoic acid	311 $\pm$ 10 <sup>a</sup>	583 $\pm$ 23 <sup>b</sup>	464 $\pm$ 9 <sup>c</sup>					
Nonanoic acid	62 $\pm$ 3 <sup>b</sup>	77 $\pm$ 9 <sup>b</sup>	38 $\pm$ 5 <sup>a</sup>					
n-Decanoic acid	94 $\pm$ 2 <sup>a</sup>	78 $\pm$ 4 <sup>a</sup>	100 $\pm$ 8 <sup>a</sup>					
Benzoic acid, methylester	77 $\pm$ 5	nd	nd					
Butanoic acid	nd	20 $\pm$ 2 <sup>a</sup>	26 $\pm$ 5 <sup>a</sup>					
Butanoic acid, ethylester	nd	9 $\pm$ 2 <sup>a</sup>	11 $\pm$ 1 <sup>a</sup>					
Hexanedioic acid, dibutylester	nd	13 $\pm$ 3 <sup>a</sup>	13 $\pm$ 3 <sup>a</sup>					
Aldehydes								
Hexanal	8 $\pm$ 2 <sup>a</sup>	4 $\pm$ 1 <sup>a</sup>	4 $\pm$ 1 <sup>a</sup>					
Decanal	23 $\pm$ 2 <sup>a</sup>	19 $\pm$ 2 <sup>a</sup>	16 $\pm$ 2 <sup>a</sup>					
Benzaldehyde, 4-methyl	242 $\pm$ 7	nd	nd					
Hexadecanal	11 $\pm$ 2	nd	nd					
Benzaldehyde	nd	12 $\pm$ 2 <sup>a</sup>	86 $\pm$ 7 <sup>b</sup>					
Benzeneacetaldehyde	nd	12 $\pm$ 2 <sup>a</sup>	10 $\pm$ 3 <sup>a</sup>					
Nonanal	nd	30 $\pm$ 1 <sup>a</sup>	25 $\pm$ 3 <sup>a</sup>					
Benzaldehyde, 2, 4-dimethyl	nd	16 $\pm$ 2 <sup>a</sup>	9 $\pm$ 1 <sup>a</sup>					
Ketones								
2-Butanone, 3-hydroxy	26 $\pm$ 3 <sup>a</sup>	89 $\pm$ 3 <sup>b</sup>	83 $\pm$ 5 <sup>b</sup>					
2-Heptanone	14 $\pm$ 3 <sup>a</sup>	49 $\pm$ 5 <sup>b</sup>	55 $\pm$ 8 <sup>b</sup>					
2-Nonanone	8 $\pm$ 1 <sup>a</sup>	45 $\pm$ 3 <sup>v</sup>	26 $\pm$ 5 <sup>b</sup>					
2-Hexanone	4 $\pm$ 1	nd	nd					
2-Heptanone, 4-methyl	41 $\pm$ 2	nd	nd					
2-Undecanone	nd	34 $\pm$ 3 <sup>b</sup>	6 $\pm$ 1 <sup>a</sup>					
2-Tridecanone	nd	36 $\pm$ 6 <sup>b</sup>	7 $\pm$ 1 <sup>a</sup>					
Others								
Pyrazine tetramethyl	nd	1215 $\pm$ 58 <sup>b</sup>	689 $\pm$ 32 <sup>a</sup>					
Propane, 2-ethoxy-2-methyl	15 $\pm$ 2	nd	nd					
Pentane, 2, 3, 4-trimethyl	3 $\pm$ 1	nd	nd					
Heptane, 4-methyl	6 $\pm$ 1	nd	nd					
Heptane, 4-methyl	12 $\pm$ 2	nd	nd					
Octane	2 $\pm$ 1	nd	nd					
2, 4-Dimethyl-1-heptene	34 $\pm$ 4	nd	nd					
Heptane, 2, 2, 4-trimethyl	4 $\pm$ 1	nd	nd					
Benzene, 1, 3-bis(1, 1-dimethyl)	65 $\pm$ 4	nd	nd					
Phenol,2,4-bis(1, 1-dimethyl)	55 $\pm$ 3	nd	nd					
<b>Bread</b>								
(b) VOCs	Control		Control Lactic	Control whey	TR7 BWF50	TR7 BWF100	TR2 BWF50	TR2 BWF100
	Control	propionate						
Alcohols								
Ethanol	881 $\pm$ 17 <sup>f</sup>	719 $\pm$ 22 <sup>d</sup>	846 $\pm$ 17 <sup>ef</sup>	787 $\pm$ 11 <sup>e</sup>	646 $\pm$ 22 <sup>c</sup>	368 $\pm$ 18 <sup>a</sup>	638 $\pm$ 17 <sup>c</sup>	488 $\pm$ 11 <sup>b</sup>
1-Butanol, 3-methyl	318 $\pm$ 17 <sup>d</sup>	209 $\pm$ 12 <sup>bc</sup>	452 $\pm$ 23 <sup>e</sup>	300 $\pm$ 12 <sup>d</sup>	258 $\pm$ 18 <sup>cd</sup>	143 $\pm$ 13 <sup>ab</sup>	295 $\pm$ 34 <sup>d</sup>	139 $\pm$ 15 <sup>a</sup>
1-hexanol	84 $\pm$ 12 <sup>a</sup>	233 $\pm$ 22 <sup>c</sup>	145 $\pm$ 22 <sup>b</sup>	114 $\pm$ 17 <sup>ab</sup>	109 $\pm$ 11 <sup>ab</sup>	112 $\pm$ 10 <sup>ab</sup>	116 $\pm$ 16 <sup>ab</sup>	109 $\pm$ 11 <sup>ab</sup>

Table 3 (Continued)

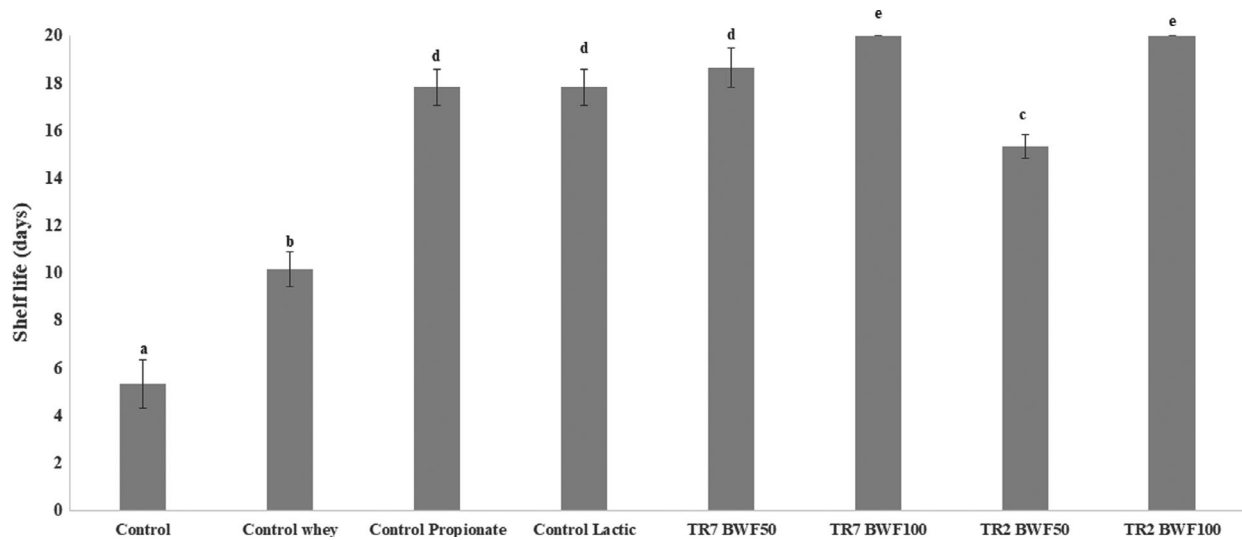
(b) VOCs	Bread							
	Control	Control propionate	Control Lactic	Control whey	TR7 BWF50	TR7 BWF100	TR2 BWF50	TR2 BWF100
1-Hexenol, 2-ethyl	245 ± 11 <sup>b</sup>	245 ± 33 <sup>ab</sup>	250 ± 15 <sup>b</sup>	249 ± 17 <sup>b</sup>	127 ± 17 <sup>a</sup>	84 ± 7 <sup>a</sup>	133 ± 10 <sup>a</sup>	270 ± 16 <sup>b</sup>
1-Octanol	206 ± 6 <sup>c</sup>	202 ± 12 <sup>b</sup>	210 ± 11 <sup>c</sup>	213 ± 18 <sup>c</sup>	81 ± 16 <sup>ab</sup>	113 ± 16 <sup>b</sup>	89 ± 9 <sup>ab</sup>	65 ± 6 <sup>a</sup>
Phenylethylalcohol	473 ± 17 <sup>d</sup>	204 ± 25 <sup>c</sup>	280 ± 17 <sup>bc</sup>	499 ± 11 <sup>d</sup>	314 ± 17 <sup>bc</sup>	330 ± 23 <sup>c</sup>	331 ± 22 <sup>c</sup>	259 ± 16 <sup>ab</sup>
1-propanol, 2-methyl	53 ± 13 <sup>a</sup>	213 ± 18 <sup>b</sup>	47 ± 7 <sup>a</sup>	47 ± 8 <sup>a</sup>	nd	nd	nd	nd
1-octen-3-ol	87 ± 10 <sup>a</sup>	113 ± 8 <sup>a</sup>	113 ± 18 <sup>a</sup>	94 ± 6 <sup>a</sup>	nd	nd	nd	nd
Acids								
Octanoic acid, ethylester	119 ± 12 <sup>d</sup>	117 ± 16 cd	117 ± 8 cd	120 ± 22 <sup>d</sup>	36 ± 7 <sup>ab</sup>	61 ± 8 <sup>ab</sup>	77 ± 11 <sup>bc</sup>	24 ± 5 <sup>a</sup>
Acetic acid, 2-phenylethylester	92 ± 6 <sup>d</sup>	93 ± 7 <sup>d</sup>	51 ± 3 <sup>bc</sup>	54 ± 10 <sup>c</sup>	31 ± 4 <sup>ab</sup>	24 ± 8 <sup>a</sup>	44 ± 6 <sup>abc</sup>	24 ± 4 <sup>a</sup>
Aldehydes								
Heptanal	57 ± 9 <sup>bc</sup>	65 ± 11 <sup>c</sup>	65 ± 11 <sup>c</sup>	35 ± 5 <sup>ab</sup>	19 ± 5 <sup>a</sup>	31 ± 5 <sup>a</sup>	27 ± 5 <sup>a</sup>	28 ± 4 <sup>a</sup>
Nonanal	519 ± 22 <sup>d</sup>	448 ± 28 <sup>c</sup>	847 ± 17 <sup>e</sup>	696 ± 19 <sup>e</sup>	297 ± 12 <sup>a</sup>	527 ± 17 <sup>d</sup>	401 ± 22 <sup>bc</sup>	348 ± 17 <sup>ab</sup>
2-Nonenal	267 ± 12 <sup>e</sup>	216 ± 16 <sup>a</sup>	236 ± 8 <sup>de</sup>	185 ± 12 <sup>bc</sup>	117 ± 14 <sup>a</sup>	155 ± 12 <sup>ab</sup>	117 ± 14 <sup>a</sup>	154 ± 11 <sup>ab</sup>
2-Decenal	815 ± 16 <sup>d</sup>	812 ± 11 <sup>d</sup>	823 ± 29 <sup>d</sup>	822 ± 29 <sup>d</sup>	98 ± 8 <sup>a</sup>	260 ± 17 <sup>c</sup>	147 ± 9 <sup>ab</sup>	174 ± 12 <sup>b</sup>
2, 4-Decadienal	154 ± 14 <sup>c</sup>	78 ± 7 <sup>ab</sup>	110 ± 10 <sup>b</sup>	263 ± 14 <sup>d</sup>	77 ± 9 <sup>ab</sup>	66 ± 12 <sup>a</sup>	108 ± 12 <sup>b</sup>	94 ± 7 <sup>ab</sup>
Hexanal	nd	nd	nd	nd	56 ± 8 <sup>ab</sup>	57 ± 7 <sup>ab</sup>	65 ± 7 <sup>b</sup>	38 ± 3 <sup>a</sup>
Benzeneacetaldehyde	nd	nd	nd	nd	65 ± 5 <sup>a</sup>	124 ± 16 <sup>b</sup>	65 ± 6 <sup>a</sup>	88 ± 9 <sup>a</sup>
Octanal	47 ± 8 <sup>a</sup>	67 ± 9 <sup>a</sup>	115 ± 17 <sup>b</sup>	225 ± 22 <sup>c</sup>	nd	nd	nd	nd
3-Hexen-1-al, acetate	109 ± 11 <sup>a</sup>	109 ± 12 <sup>a</sup>	110 ± 11 <sup>a</sup>	113 ± 17 <sup>a</sup>	nd	nd	nd	nd
Benzaldehyde	nd	nd	nd	nd	34 ± 4 <sup>a</sup>	49 ± 2 <sup>b</sup>	39 ± 6 <sup>ab</sup>	41 ± 3 <sup>ab</sup>
Ketones								
5-hepten-2-one, 6-methyl	34 ± 7 <sup>ab</sup>	34 ± 6 <sup>c</sup>	35 ± 6 <sup>ab</sup>	34 ± 4 <sup>ab</sup>	27 ± 9 <sup>a</sup>	39 ± 4 <sup>ab</sup>	48 ± 4 <sup>b</sup>	38 ± 5 <sup>ab</sup>
3-heptanone	14 ± 4 <sup>a</sup>	14 ± 3 <sup>a</sup>	14 ± 2 <sup>a</sup>	14 ± 3 <sup>a</sup>	nd	nd	nd	nd
3-Hexanone, 2, 5-dimethyl	9 ± 2 <sup>a</sup>	9 ± 2 <sup>a</sup>	9 ± 3 <sup>a</sup>	9 ± 2 <sup>a</sup>	nd	nd	nd	nd
Others								
Pyrazine tetramethyl	nd	nd	nd	nd	204 ± 21 <sup>b</sup>	398 ± 32 <sup>c</sup>	98 ± 19 <sup>a</sup>	189 ± 11 <sup>b</sup>
Hexane	68 ± 9 <sup>ab</sup>	109 ± 12 <sup>c</sup>	87 ± 11 <sup>bc</sup>	47 ± 7 <sup>a</sup>	57 ± 8 <sup>ab</sup>	74 ± 6 <sup>ab</sup>	66 ± 9 <sup>ab</sup>	114 ± 10 <sup>c</sup>
Heptane, 2, 4-dimethyl	77 ± 11 <sup>b</sup>	175 ± 12 <sup>c</sup>	24 ± 8 <sup>a</sup>	36 ± 5 <sup>a</sup>	77 ± 11 <sup>b</sup>	151 ± 13 <sup>c</sup>	88 ± 12 <sup>b</sup>	154 ± 12 <sup>c</sup>
Dodecane	93 ± 7 <sup>a</sup>	550 ± 24 <sup>d</sup>	359 ± 16 <sup>c</sup>	94 ± 7 <sup>a</sup>	96 ± 6 <sup>a</sup>	383 ± 17 <sup>c</sup>	147 ± 8 <sup>b</sup>	338 ± 14 <sup>c</sup>
Tetradecane	367 ± 22 <sup>b</sup>	446 ± 17 <sup>c</sup>	381 ± 17 <sup>d</sup>	152 ± 8 <sup>a</sup>	128 ± 13 <sup>a</sup>	353 ± 22 <sup>b</sup>	179 ± 10 <sup>a</sup>	332 ± 13 <sup>b</sup>
Octane	nd	nd	nd	nd	144 ± 9 <sup>a</sup>	220 ± 12 <sup>b</sup>	138 ± 6 <sup>a</sup>	194 ± 7 <sup>b</sup>
Nonane, 2, 6-dimethyl	nd	nd	nd	nd	33 ± 5 <sup>a</sup>	59 ± 7 <sup>b</sup>	33 ± 5 <sup>a</sup>	45 ± 4 <sup>ab</sup>

The organic acid content and pH of BWF are shown in Table 2a. The pH value of the non-fermented buffalo whey was 4.73, whereas the BWFs had a pH in the range of 3.38–4.63. The lowest pH value was obtained for the whey fermented by *L. plantarum* TR7. Non-fermented buffalo whey showed an average concentration of lactic acid of 6.51 g L<sup>-1</sup> produced during the production process of MBC DOP. Lactic acid production by isolated bacteria in BWF ranged from 7.18 to 15.00 g L<sup>-1</sup>. The LAB with the highest lactic acid production were *Lactobacillus plantarum* TR7 (15.00 g L<sup>-1</sup>), *Lactobacillus ghanensis* TR2 (12.49 g L<sup>-1</sup>) and *Lactobacillus plantarum* TR14 (11.78 g L<sup>-1</sup>).

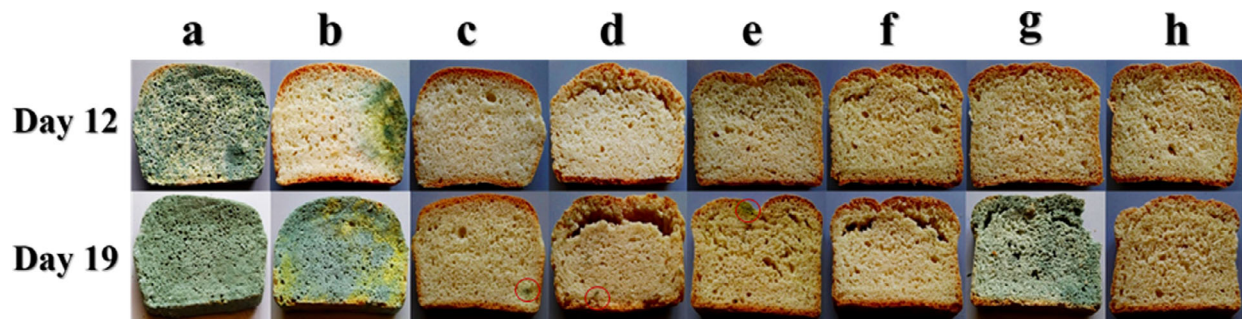
The lactic acid concentrations and pH of the eight types of bread studied are shown in Table 2b. Control lactic bread evidenced the lowest pH value (4.5) and a lactic acid concentration of 3.42 g kg<sup>-1</sup>. The control

and control propionate breads showed non-significant differences in pH value ( $p > 0.01$ ) and non-detectable lactic acid. Bread elaborated using fermented and non-fermented buffalo milk whey presented significant differences in pH and lactic acid values. As expected, the BWF100 breads exhibited lower pH values (4.62–4.79) and higher concentrations of lactic acid (2.85–3.97 g kg<sup>-1</sup>) compared with control whey and BWF50 breads.

A total of 39 VOCs were identified in the BWF and non-fermented buffalo whey. However, the control media showed a lower number of compounds. The profile of VOCs quantified in BWF and non-fermented buffalo milk whey is reported in Table 3a. The compounds were classified into five groups according to their chemical class: alcohols, acids, aldehydes, ketones and others. The fermentation of buffalo milk whey with TR7 and TR2 evidenced a significant increase in



**Figure 1** Shelf life expressed in days of each typologies of breads contaminated naturally. Different letters represent a significant difference among the treatments ( $p \leq 0.01$ ). The experiment was carried out three times with analyses in triplicate ( $n = 9$ ). Results are expressed as mean  $\pm$  standard deviation



**Figure 2** Appearance of bread slices contaminated naturally a) Control, b) Control whey, c) Control propionate, d) Control lactic, e) TR7 BWF50, f) TR7 BWF100, g) TR2 BWF50 and h) TR2 BWF100 at 12 and 19 days of incubation

the content of alcohols, acids, aldehydes, ketones and pyrazines. The average value of ethanol in the BWF by TR7 was 2.3-fold higher than in control whey. The fermentation process by LAB showed a significant increment of acetic acid, hexanoic acid and octanoic acid in the BWFs. The acetic acid concentration ranged between 95 and 325 mg kg<sup>-1</sup>, being 3.4-fold higher in the BWF by TR2. A similar result was found for aldehydes such as benzaldehyde, benzeneacetaldehyde, benzaldehyde, 2, 4-dimethyl and nonanal. Nevertheless, there was a significant reduction in benzaldehyde, 4-methyl by LAB fermentation. In general, the compounds belonging to the ketone group were increased by fermentation, except 2-undecanone and 2-tridecanone. Also, the amount of pyrazine

tetramethyl before fermentation showed a concentration ranging between 689 and 1215 mg kg<sup>-1</sup>. Many studies have reported inhibition of fungal growth by VOCs produced by bacteria (Morita *et al.*, 2019). Other authors have reported the antimicrobial activity of VOCs (1-octen-3-ol and 2,5-dimethyl pyrazine) against the fungal pathogen *Phaeoemoniella chlamydospora* involved in grapevine trunk diseases (Haidar *et al.*, 2016). Rybakova *et al.* (2016) reported the production of VOCs by *Paenibacillus*, in particular, pyrazine derivatives as a potential biocontrol agent in agriculture.

In the case of bread, a total of 30 VOCs were identified (Table 3b). The quantified compounds were classified in the same way as those detected in the BWF.

The use of calcium propionate and BWF at different concentrations evidenced a significant reduction in the final ethanol content in the breads. Specifically, the ethanol content of the breads TR7 BWF100 and TR2 BWF100 was 1.8-fold and 2.4-fold smaller than the control bread. Ethanol is a compound produced primarily by yeast during the dough fermentation process. Capozzi *et al.* (2016) reported that the ingredients, the inoculum of starter culture and technological process directly can influence on bread VOC profile. In particular, the composition of the dough, among other factors, can modify ethanol production during fermentation and produce doughs with different ethanol contents (Pizarro & Franco, 2017). Also, a reduction in the content of some aldehydes, such as 2-decenal, octanal and 3-hexen-1-al, acetate, was observed using BWF as an ingredient. However, hexanal, benzeneacetaldehyde and benzaldehyde showed a significant increase in this condition. Furthermore, the presence of pyrazines in BWF discussed previously was reflected in the composition of VOCs of the breads. Luz *et al.* (2020a) also showed an increment of pyrazine derivatives in MRS broth after fermentation by the same selection of lactic acid bacteria. Specifically, the strain *L. plantarum* TR2 evidenced the highest concentration of pyrazines in the MRS fermented.

#### BWF as a bread biopreservative

A study of the shelf life of bread naturally contaminated and treated with BWF as a biopreservative showed visible fungal growth at day 5 of storage in the control bread. For the control propionate, the shelf life was increased by 13 days in comparison with the control untreated bread. Similar results were observed for control lactic and TR7 BWF50 breads. Nevertheless, a reduction in the shelf life was observed on days 10 and 15 in the control whey and TR2 BWF50 breads, respectively. In general, the breads in which 100% of the water was replaced with BWF by *L. plantarum* TR7 and *L. ghanensis* TR2 showed fungal growth at 20 days of storage, evidencing an improvement in the shelf life of 15 days and a greater effect biopreservative compared with the other treatments (Fig. 1). Figure 2 shows the appearance and visible fungal growth on bread on days 12 and 19 in a constant climate chamber. Luz *et al.* (2020b) showed an increase in the shelf life of two days of bread inoculated with *Penicillium expansum* using cow's milk whey fermented with *L. plantarum* CECT 221.

#### Conclusions

Mozzarella di Bufala whey fermentation by *L. plantarum* TR7 and *L. ghanensis* TR2 produced several

compounds such as organic acids and VOCs. There was also evidence of an increment in the antioxidant activity and shelf life of bread elaborated with BWF as a biopreservative ingredient. This study demonstrates the importance of the reevaluation of whey by-product through the use of biotechnology, and the application of this fermented product as a biopreservative of bread contributes to meeting the demand of consumers to reduce the use of food additives. Further research is warranted to expand our knowledge on the application of BWF as a biopreservative of bread on a larger industrial scale and influence of this about the breads flavours.

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#### Author Contributions

**Carlos Luz:** Conceptualization (lead); Data curation (lead); Formal analysis (lead); Funding acquisition (supporting); Investigation (lead). **Juan M. Quiles:** Writing-original draft (supporting); Writing-review & editing (supporting). **Raffaele Romano:** Methodology (lead); Resources (supporting); Supervision (supporting); Writing-review & editing (supporting). **Giuseppe Blaiotta:** Resources (supporting); Supervision (supporting). **Lorena Rodriguez:** Writing-original draft (supporting); Writing-review & editing (supporting). **Giuseppe Meca:** Conceptualization (supporting); Funding acquisition (lead); Project administration (lead); Supervision (lead).

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