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Valorization of wheat bread waste and cheese whey through cultivation of lactic acid bacteria for bio-preservation of bakery products

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ABSTRACT

In this work, three lactic acid bacteria (LAB) strains, specifically, *Lactiplantibacillus plantarum* UMCC 2996, *Furfurilactobacillus rossiae* UMCC 3002, and *Pediococcus pentosaceus* UMCC 3010, were tested in new bread-whey media composed by wheat bread and sweet cheese whey, designed as an alternative to the conventional MRS medium. The medium resulting from hydrolysis with amylase and neutrase (AN) was considered the best for the growth of all the strains. This medium was particularly optimal for the strain *F. rossiae* UMCC 3002, which showed an increase in growth of 114% compared to that in MRS medium. Additionally, the bio-preservative ability of the selected LAB was assessed in pectin-based coated sliced bread inoculated with *Aspergillus flavus* ITEM 7828, *Penicillium paneum* ITEM 1381, and *Aspergillus niger* ITEM 7090. Different LAB strain behavior was observed towards the specific molds. A good bio-preservation activity was shown from *F. rossiae* UMCC 3002 against *A. flavus* ITEM 7828 with results compared to the protection by ethanol treatment. The results obtained in this study suggest a novel strategy for the cultivation of selected starters with a bio-protection activity by valorizing bread waste and cheese whey by-products, in a circular economy perspective.

1. Introduction

Bread is one of the dominant food sources of the human diet (Hu et al., 2022), comprising several varieties with national and regional variants (Legan, 1993). It is an important calorie source, but also including protein, iron, calcium, and various vitamins (Zain, Shori, & Baba, 2021). The annual average consumption of bread in the world, is estimated to be around 70 kg per capita, and around 59 kg per capita just in Europe (De Boni, Pasqualone, Roma, & Acciani, 2019).

The bread loss of quality, caused by chemical, physical and microbiological agents, represents an important source of food waste both in the bakery sector and in the consumers' kitchen. Globally, around 10% of all manufactured bread is wasted (Narisetty et al., 2021). Mold and fungal deteriorations are the most important causes of bread loss. The high moisture content (about 40%) and water activity (about 0.94–0.97 a_w) of bread (Gobbetti et al., 2019) lead to mold attack susceptibility in 3–7 days.

Penicillium, Aspergillus, *Wallemia*, (Garcia et al., 2019; Quattrini et al., 2019), and other familiar molds such as *Rhizopus*, *Chrysonilia sitophila*, and *Mucor* are mainly involved in the spoiling of bakery products (Rahman et al., 2019). Chemical preservatives such as calcium propionate, ethanol, and sorbate are widely used to extend the microbiological lifetime of bread. However, in recent years, bio-preservation approaches have been evaluated to meet legislative issues, customer demand for clean-label products, and baking industry requirements (Akbar, Ali, & Anal, 2016; Fugaban, Holzapfel, & Todorov, 2022; Le Lay et al., 2016; Muhialdin, Hassan, & Saari, 2018; Nasrollahzadeh, Mokhtari, Khomeiri, & Saris, 2022; Russo et al., 2017; Sadiq et al., 2019). In particular, the sourdough technology, which involves yeasts and lactic acid bacteria (LAB), is reported to be a reasonable alternative to chemical preservatives while boosting flavor, texture, and nutritional characteristics (Axel, Zannini, & Arendt, 2017; De Vero, Iosca, Gullo, & Pulvirenti,

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2021; Hussein, 2022). The *in vitro* antifungal activity of various LAB strains has been widely assessed, however, only a few commercial products are available for bakery bio-preservation; an example is Hi Shield P from the company HI-FOOD S.p.A. (Leyva Salas et al., 2017). The limited number of solutions for bio-preservation on the market is mostly owing to several difficulties in developing effective and competitive solutions. The incorporation of live microorganisms in edible films and coatings has been recently reported as a promising strategy for bread bio-preservation (Guimarães, Abrunhosa, Pastrana, & Cerqueira, 2018). New developments in the usage of edible coatings include the conveyance of bioactive substances such as antioxidants, sensory enhancers, and antimicrobials (Ponce, Roura, del Valle, & Moreira, 2008) or the shelf-life extension, particularly for extremely perishable products. Coatings can also integrate live microorganisms to provide probiotic or antibacterial and -mold capabilities. Despite the numerous applications of antimicrobial edible coatings in meat, seafood, fruit, and vegetables, only some authors (Bartkiene et al., 2019; Ferreira Saraiva et al., 2016; Gregirchak, Stabnikova, & Stabnikov, 2020; Pruksarojanakul, Prakitchaiwattana, Settachaimongkon, & Borompichaichartkul, 2020) have utilized and highlight the potential of these new shelf-life extended techniques for bakery products.

Once the suitable strain with a desired bio-protective activity is selected, its maintenance at the industrial level, as a viable starter culture, requires a balance between cost and efficiency of cell production (Verni, Minisci, Convertino, Nionelli, & Rizzello, 2020).

Generally, conventional media, such as De Man, Rogosa and Sharpe (MRS) or M17, have been successfully used for LAB cultivation and ready-to-use commercial preparations are widely available. However, they are quite expensive, due to the high-priced components such as beef extract, yeast extract, and peptone, which are fundamental nitrogen sources for LAB. Moreover, to dissolve the powder it is necessary to use water, which is an important world resource.

The development of a zero-waste economy by 2025, including the increase of local profits and new technologies for waste valorization as tools of sustainable management, has been proposed by the European Union (EU) (Girotto, Alibardi, & Cossu, 2015). Like other countries, Italy is also making a great effort to support the bioeconomy by reconsidering the use of the main local wastes and by-products.

To reuse and valorize them, one of the strategies investigated recently, focused on the setup of culture media able to promote microbial growth (Benabda, Kasmi, Kachouri, & Hamdi, 2018; Boumaiza et al., 2018; Burns, Vinderola, Molinari, & Reinheimer, 2008; Johnson, Burgess, Shi, Li, & Blersch, 2022; Jung et al., 2022; Kusmiyati, Massora, & Wicaksono, 2022; Linares-Morales et al., 2022; Rahman et al., 2019; Verni et al., 2020; Yurliasni, Hanum, Firtiana, & Sy, 2022).

Regarding cheese whey, it is the most abundant dairy waste with a global production of around 10^8 tons/year. Approximately 10 L of cheese whey are produced to make 1 kg of cheese (Zotta, Solieri, Iacumin, Picozzi, & Gullo, 2020), with an estimated production of 40×10^6 tons/year in all Europe (with annual surplus calculated as on quarter of production) (Mollea, Marmo, & Bosco, 2013). Just in the Italian peninsula, cheese-whey production is estimated to be about 10^6 tons/-years with an annual surplus of more than 10^5 tons/years (Donzella et al., 2022). Despite being one of the most polluting products in the food industry, due to its high chemical and biological oxygen demands, as well as disposal issues (Sommella et al., 2016), cheese whey is considered a great source of vitamins and nitrogen that are essential for LAB development.

As a result of the previous statement, three LAB strains from the Unimore Microbial Culture Collection (https://umcc.bio-aware.com) were investigated in this study for the creation of an alternative growth medium capable of recycling wheat bread waste and Parmigiano Reggiano cheese whey by-product gathered from manufacturers in the province of Reggio Emilia (Italy). The strains' capacity to exert a bio-preservation activity on sliced bread, artificially contaminated with selected molds, was also evaluated and compared to the effect of ethanol, which is a typical exogenous chemical used in packaged sliced bread. In our earlier investigations, the employed strains, isolated from sourdough samples, were characterized for their ability to produce various aromas compounds (De Vero, Iosca, La China, et al., 2021). Furthermore, their potential *in vitro* anti-spoilage (bacteria and molds) activity, as well as their ability to produce exopolysaccharides was also assessed (Iosca et al., 2022). The final goal of the work is to develop a feasible strategy based on the use of specific waste for the cultivation of microbial starters with bioprotective activity. Both parts of the study aim to make new circular biological solutions for the bakery sector.

2. Materials and methods

2.1. Lactic acid bacteria strains

In this study *Lactiplantibacillus plantarum* UMCC 2996, *Furfurilactobacillus rossiae* UMCC 3002, and *Pediococcus pentosaceus* UMCC 3010, were used. The strains were revitalized from −80 °C conditions and grown on MRS broth (Biolife, Italy) at 30 °C for 48 h under microaerophilic conditions (using a jar).

2.2. Formulation of the alternative medium for LAB cultivation

Wheat bread waste, without signs of mold infestation, was provided by an artisan bakery in Emilia Romagna region (Panificio Fantuzzi, Montecchio Emilia, RE, Italy). Firstly, bread waste was finely ground to a particle size of about 2 mm and added to sweet cheese whey (pH 5.7 ± 0.01), resulted from Parmigiano Reggiano DOP production (Latteria Sociale di Migliara, Casina, RE, Italy), in a 1:4 ratio (w/w). The nutritional and chemical compositions of wheat bread and cheese whey are reported in Tables S1–S2. The pH of the cheese-whey was adjusted to 6.5 by using a pH-meter (XS Instruments mod. pH 6). Then, both proteolytic and amylolytic enzymes, singly or in combination were added. Specifically, neutrase, an endo-protease (1.5 AU/g) from *Bacillus amyloliquefaciens* (Novozymes, Denmark), was used at a final concentration of 1.5 mL/L, while amylase, a maltogenic enzyme (≥10000 MANU/g) from *Bacillus* sp. (Sigma-Aldrich®, Merck, Germany) was added at 0.5 g/L (final concentration). Some of the media were supplemented with yeast extract 1 g/100 mL (Oxoid, Thermo Scientific™, England) and 0.5 g/L of Tween 80 (Panreac Applichem, ITW Reagents, Germany). In detail, following a factorial experimental design 2^2, four different bread-whey media were prepared with addition of only amylase (A), addition of amylase and 1 g/100 mL yeast extract (AY1%), only amylase and neutrase (AN), addition of amylase, neutrase, and 1 g/100 mL yeast extract (ANY1%), respectively. To prepare the media, after adding all the components, they were incubated at 55 °C for 18 h, which are optimal conditions for the enzyme's activity according to the manufacturer's instructions. Then they were centrifuged at 6000 rpm for 20 min (centrifuge NEYA 16R, Remi Elektrotechnik LTD., India) to separate not hydrolyzed solid residues, and supernatants were collected. All the media were adjusted to pH 6.5 with NaOH (1 mol/L) before sterilization at 121 °C for 15 min. The precipitates were removed by filtering through a sterilized gauze followed by a centrifugation at 6000 rpm for 10 min. Aliquots of 5 mL of the different media were put in sterile tubes for the strains' inoculation. For each media, the chemical characterization and pH detection was done after autoclaving.

LAB strains were grown for 48 h on MRS agar plate at 30 °C, then cultures were suspended in saline solution (NaCl 0.9 g/L) and diluted up to 1 OD (optical density) detected at 600 nm by spectrophotometer UV/Vis UV-6300PC (VWR International Srl, Milan, Italy). The suspension was diluted at a ratio 1:10 in saline solution, and 100 μL were inoculated in tube test. The inoculum concentration (C_I) was assessed by CFU/mL count on MRS agar medium after incubation at 30 °C for 48 h, under microaerophilic conditions.

2.2.1. Bread-whey media characterization

Glucose content was determined using the Maltose/Sucrose/D-Glucose Assay Kit (Megazyme Int., Ireland) following the manufacturer's instructions, while the percentage of raw proteins was determined by Kjeldahl method. A nitrogen-to-protein conversion factor of 6.38 (Finete, Gouvêa, Marques, & Netto, 2013) was used to convert the total nitrogen in crude protein content following this formula:

$$\text{g} \Big/ 100\ \text{mL raw protein} = \frac{\text{mL HCl} \times \text{N HCl} \times 1.4007 \times 6.38}{\text{g of samples}}$$

The experiments were performed in triplicate.

2.2.2. LAB growth detection in the different media

The microbial growth and pH-value in the different bread-whey media was assessed after incubation at 30 °C for 48 h. Each strain culture was diluted in saline solution (NaCl 0.9 g/L) and serial dilutions from 10^{-1} to 10^{-7} were made. Appropriated dilutions were plated on MRS agar medium and incubated at 30 °C for 48 h under microaerophilic conditions. The results were converted in growth percentage (G%) with respect to those observed in the MRS medium, through the following equation:

$$\text{G}\ (\%) = \frac{(\log \text{C}_\text{I} - \log \text{C}_{\text{FBWx}})}{(\log \text{C}_\text{I} - \log \text{C}_{\text{FMRS}})} \times 100$$

where C_I was the inoculum concentration, C_{FBWx} was the final concentration in each type (x) of bread-whey medium and C_{FMRS} was the final concentration in MRS medium.

Similar to Verni et al. (2020), the effects of the independent variables, related to neutrase enzyme and yeast extract addition, on the microbial growth rate in each type of bread-whey medium, were evaluated by using a regression model with the equation:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2$$

where y was the dependent variable (growth rate) to be modeled; b_0, b_1, b_2, b_{12} were intercept, linear and interaction coefficients of the model, respectively; x_1 and x_2 were the independent variables (neutrase and yeast extract). For the assessment of the regression model, the application tool in Excel was used.

2.3. Formulation of the edible coating with LAB

Edible pectin-based coating was prepared by using for 100 mL of sterilized distilled water, 0.2 g yeast extract (Oxoid, Thermo Scientific™, England), 2.5 g low methoxy amidated pectin (Herbstreith & Fox GmbH & Co. KG, Germany), 0.75 g glycerol (Sigma-Aldrich®, Merck, Germany), 1 g sorbitol (Sigma-Aldrich®, Merck, Germany), 1 g oleic acid (Sigma-Aldrich®, Merck, Germany) and 0,1 g Tween 80 (Panreac Applichem, ITW Reagents, Germany). Distilled water and low methoxy amidated pectin were mixed on a magnetic stirrer at 45 °C and 700 rpm for 20 min. Glycerol, sorbitol, and yeast extract were added at intervals of 10 min at the same temperature and rpm. Oleic acid and Tween 80 were added in the final step of edible coating preparations and the emulsion was left for 1 h at 30 °C and 450 rpm.

2.3.1. Inoculation of the coating with LAB and bread sliced treatment

For the bread sliced treatment, edible coating was inoculated with LAB strains (10^8 CFU/mL). Each type of the inoculated coating was sprayed (1 mL) with a compressor system (ABAC G-541A, Air Powered, Turin, Italy) on each side of a sandwich-like bread slice (about 5 g, 0.9 cm of thickness) and dried for 10 min (25 °C). Wheat bread previously prepared at Fantuzzi bakery was cut in a sterile environment to obtain slices. The ingredients used for preparing the bread were: 400 g wheat flour, 200 g durum wheat semolina, 350 g water, 10 g extra virgin olive oil, 10 g bakery yeast, 4 g table salt, and 5 g bread improvers (amylase and ascorbic acid). The ingredients were mixed in an automatic bread maker for 10 min and dough was placed to rest for 10 min in a proofing cabinet at 25 °C. After the first rise, the dough was divided and settled down in a specific baking mold with a size of 40 × 12 × 12 cm (Fig. S1). The dough piece was placed back into the proofing cabinet for the second rise, gradually reached overnight at a temperature of 22 °C. Finally, the bread was baked in the oven at 200 °C for 35 min.

After the complete treatment with the LAB coatings (CLAB), the bread slices were put into sterile petri plates and incubated at 30 °C with 75% relative humidity (RH), for 9 d.

2.3.2. LAB viability in pectin-based edible coating

The LAB microbial count was performed in triplicate after 1 d (t_0), 3 d (t_3), 6 d (t_6), and 9 d (t_9). The slice of bread, about 5 g, and corresponding saline solution (NaCl 0.9 g/L) for a dilution 1:10 was homogenized by using a laboratory blender Stomacher. Serial dilutions from 10^{-1} to 10^{-5} were made, 100 μL of appropriated dilutions were plated in triplicate on MRS agar and incubated for 48 h at 30 °C under microaerophilic conditions. The results were expressed as a percentage of the cell viability (CV%):

$$\text{CV}\ (\%) = \frac{\log\left(\frac{\text{CFU}}{\text{mL}}\right)\text{t}_{3;6;9}}{\log\left(\frac{\text{CFU}}{\text{mL}}\right)\text{t}_0} \times 100$$

2.4. Anti-mold activity screening on bread sliced

Three molds provided by the Agro-Food Microbial Culture Collection (ITEM Collection, www.ispa.cnr.it/Collection) of the Institute of Sciences of Food Production, CNR (Bari, Italy), specifically, *Aspergillus flavus* ITEM 7828, *Aspergillus niger* ITEM 7090, and *Penicillium paneum* ITEM 13081, were used for the contamination of the sliced bread. The coating with LAB and the bread slides treatment were performed as described before. Then the slices were put into sterile Petri plates and incubated at 30 °C with 75% RH for 24 h. The fungi inoculum was prepared from spores of each mold collected after 7 d growing of the cultures on Potato Dextrose Agar medium (PDA) (Oxoid, United Kingdom). A concentration of 10^5 spores/mL was set with the Burker cell-counting chamber (BLAUBRAND, Germany) and 100 μl of spores' suspension was used; for each mold, two inocula were performed on each bread sliced. All plates were incubated in aerobic conditions at 25 °C and 75% RH in a conditioned chamber for 9 d. Contaminated slices were detected at 3, 6, and 9 d after the inoculum and compared with two controls: one without LAB coating bread control (BC) and the other one treated with vanilla-flavored ethanol (EBC) (Voltolini, Brescia, Italy) applied at the same conditions discussed above. Data were measured by AUTOCAD software (version 2022) following the shape of the mold and expressed as mold quantification area cm^2 (Fig. S2) (Ferreira Saraiva et al., 2016). The experiments were performed in duplicate.

2.5. Statistical analysis

Statistical analysis of data was determined by one-way analysis of variance (ANOVA) followed by Tuckey's test using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). *P-values $\leq$0.05 were considered statistically significant. The results were expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. Bread-whey medium chemical composition

In this study, four bread-whey media for LAB cultivation were formulated, based on several evidences reported in the literature (Benabda et al., 2018; Burns et al., 2008; Verni et al., 2020). Each type of formulated bread-whey medium was characterized by the content of glucose (g/L), total nitrogen converted to percentage of raw proteins, and pH. The data were compared to those observed in the MRS medium

Table 1
Chemical composition of bread-whey media and MRS medium.

Samples	Glucose g/L	g/100 mL Raw Proteins	pH
MRS	19.01 ± 0.16^{a}	2.24 ± 0.02^{c}	6.29 ± 0.12^{a}
A	8.07 ± 0.38^{b}	n.d.	5.75 ± 0.03^{b}
AY1%	8.47 ± 1.04^{b}	1.34 ± 0.06^{d}	5.77 ± 0.01^{b}
AN	8.22 ± 0.34^{b}	3.49 ± 0.08^{b}	5.76 ± 0.01^{b}
ANY1%	8.75 ± 0.22^{b}	4.19 ± 0.04^{a}	5.77 ± 0.04^{b}

Values are given as the means ± SD (n = 3). Mean values with different letters are significantly different (*P < 0.05). The letters represent the differences within columns). (A, AY1%, AN, ANY1%: experimental conditions produced by bread-whey medium).

(Table 1). The four bread-whey media contained from 8.07 to 8.75 g/L of glucose without statistically significant differences. However, a relevant difference was observed comparing these values with the glucose content in MRS. Regarding the value of raw proteins, it was statistically different for all media. In the bread-whey medium A, proteins could not be detected, but when 1 g/100 mL yeast extract was added (medium AY1%), the proteins content resulted to be 1.34 ± 0.06 g/100 mL. In the MRS medium, the value of raw proteins was 2.24 ± 0.02 g/100 mL, a lower quantity compared to the values observed in the two bread-whey media treated with neutrase (3.49 ± 0.08 g/100 mL and 4.19 ± 0.04 g/100 mL in AN and ANY1%, respectively). These results suggest the use of protease (neutrase) to supply the coagulation of serum proteins and their complete loss during filtration and centrifugation, as observed in the experimental conditions A and AY1%. Regarding the final pH of the media, it was observed a decrease of the values after sterilization. In particular, pH was significantly lower in the bread-whey media (5.76 ± 0.01) compared to the MRS medium (6.29 ± 0.12), maybe due to whey buffering capacity.

Considering the high biological value and essential components contained in bread waste and cheese-whey, their utilization after enzymes treatments can support the LAB growth by providing important nutrients. From the standpoint of cost/energy analysis, enzymatic hydrolyzation to produce glucose from bread waste might be a viable and cost-effective option (Zhang et al., 2020). To reduce water usage while maintaining suitable growth compounds for LAB, which require rich media with amino acids and peptides (Hayek & Ibrahim, 2013; Mokoena, 2017; Sauer, Russmayer, Grabherr, Peterbauer, & Marx, 2017), cheese-whey was used. This dairy by-product is composed by lactose (45–50 g/L), soluble proteins (6–8 g/L), lipids (4–5 g/L), mineral salts (including mainly NaCl and KCl), calcium salts (mainly phosphate), lactic (0.5 g/L) and citric acids, non-protein nitrogen compounds (urea and uric acid), and group B vitamins (Zotta et al., 2020). The enzymatic parameters were chosen to release the major quantity of raw proteins from the cheese whey, while the glucose content was assessed to 8–10 g/L, which is relatively low in comparison with the LAB optimal requirement in 1 L of fermentation media estimated at 20 g/L (e.g., MRS). This consideration has been made because the cheese whey provides a high quantity of lactose, and an excessive amount of carbon source may inhibit the cells growth. Lactose's utilization by LAB is mainly related to their fermentation processes. After internalization into the cell, lactose can be metabolized thanks to two pathways: Leloir and Tagatose-6-Phosphate pathways (Iskandar, Cailliez-Grimal, Borges, & Revol-Junelles, 2019).

3.2. Evaluation of the LAB growth in the bread-whey media

The load of *L. plantarum* UMCC 2996, *F. rossiae* UMCC 3002, and *P. pentosaceus* UMCC 3010 was measured after 48 h of incubation at 30 °C in the bread-whey media and compared to that obtained with MRS medium (Fig. 1, Table S3). The data were normalized and expressed as growth percentage (G%) with respect to MRS medium. For all the strains, the alternative media may be considered a good substitute of the

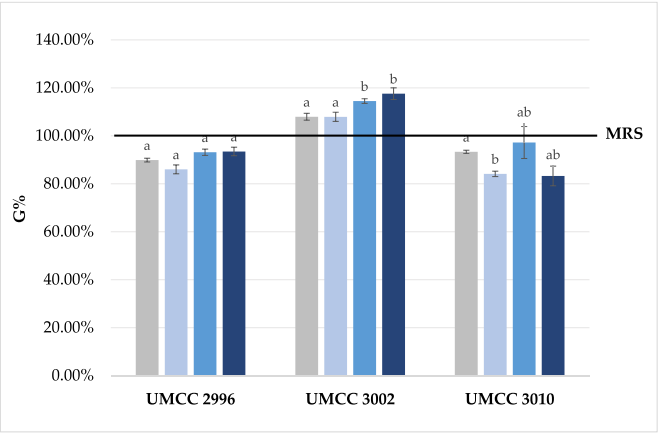

Fig. 1. Growth percentage (G%) of the strains *L. plantarum* UMCC 2996, *F. rossiae* UMCC 3002 and *P. pentosaceus* UMCC 3010, in the different bread-whey media. The different columns correspond to the medium with: Amylase (A) (), Amylase and 1 g/100 mL yeast extract (AY1%) (), Amylase and Neutrase (AN) (), Amylase, Neutrase and 1 g/100 mL yeast extract (ANY1%) (), respectively. The line corresponds to G% = 100% assessed for the growth in MRS. Values are given as the means ± SD (n = 2). The different letters over the columns indicate statistically significant differences (*P < 0.05).

MRS. Specifically in the cases of *L. plantarum* UMCC 2996, the G% was very high in the four bread-whey media (A: 89.88 ± 0.79%; AY1%: 86.03 ± 1.85%; AN: 93.15 ± 1.32%; AN1%: 93.48 ± 1.78%), almost comparable to those observed in MRS. Also, *P. pentosaceus* UMCC 3010 demonstrated a high G% with no significant difference among the experimental conditions (range from 83.27 ± 4.10% to 97.21 ± 6.66%), even if the regression model showed a negative coefficient related to the addition of yeast extract ($y = 8.94 - 0.18x_2$) (Table 3). For *F. rossiae* UMCC 3002, all the alternative media promoted cells viability with optimal results. The cellular growth in the bread-whey media containing neutrase was higher compared to the other media including MRS. Specifically, the values observed were 114.49 ± 0.98% and 117.57 ± 2.44% in the media AN and ANY1%, respectively. The regression model applied confirmed that only the presence of neutrase had a significant effect on the response function values (Table 2), while the addiction of yeast extract did not affect the growth, resulting unnecessary ($y = 9.22 + 0.12x_1$).

Successful application of alternative media for LAB cultivation have been previously reported in literature. Burns et al. (2008), showed that a medium composed by cheese-whey + 0.3 g/100 mL of yeast extract and cheese-whey + 0.3 g/100 mL of yeast extract + 1 g/100 mL of glucose could promote the growth and the acidification kinetics of *L. acidophilus* H5 and *L. paracasei* LS with data comparable to those observed in MRS medium. Verni et al. (2020) valuated several different alternative media composed by bread waste. The best one was composed of glucose 24.53 ± 0.29 g/L, peptides 37.08 ± 0.39 g/L, and 10.76 ± 0.12 g/L of amino acids, respectively and no statistical differences (*P < 0.05) with the MRS medium was observed in relation to the LAB growth. In our study, the bread-whey media formulated, thanks to the simultaneous use of a carbon and protein source, were able to promote the LAB growth without the addition of other ingredients. In particular, the medium

Table 2
Regression model used to describe LAB growth rate as a function of the independent variables (y: cellular growth log CFU/mL; x_1: Neutrase; x_2: yeast extract).

Strains	Regression model
***L. plantarum* UMCC 2996**	$y = 9.11 + 0.12\,x_1$
***F. rossiae* UMCC 3002**	$y = 9.22 + 0.12\,x_1$
***P. pentosaceus* UMCC 3010**	$y = 8.94 - 0.18\,x_2$

Table 3
Quantifications of LAB antimold activity after 3, 6, and 9 d of incubation.

MOLDS	BC	EBC	*L. plantarum* UMCC 2996	*F. rossiae* UMCC 3002	*P. pentosaceus* UMCC 3010
***A. flavus* ITEM 7828**					
t_3	2.43 ± 1.03^{c}	0 ± 0^{a}	1.26 ± 0.56^{c}	0.34 ± 0.16^{b}	2.37 ± 0.03^{c}
t_6	16.54 ± 1.82^{c}	2.18 ± 0.87^{a}	6.83 ± 1.03^{b}	7.65 ± 1.41^{b}	12.60 ± 2.83^{c}
t_9	30.86 ± 0.71^{b}	10.89 ± 0.49^{a}	33.11 ± 0.64^{b}	15.73 ± 2.60^{a}	33.57 ± 0.23^{b}
***A. niger* ITEM 7090**					
t_3	1.43 ± 0.11^{b}	0 ± 0^{a}	2.23 ± 0.40^{b}	2.55 ± 0.88^{b}	4.07 ± 0.28^{c}
t_6	15.40 ± 2.20^{c}	0 ± 0^{a}	10.22 ± 0.64^{b}	8.10 ± 0.21^{b}	11.71 ± 1.58^{bc}
t_9	33.94 ± 0.26^{d}	0 ± 0^{a}	32.93 ± 0.54^{d}	16.80 ± 2.16^{b}	22.68 ± 0.64^{c}
***P. paneum* ITEM 1381**					
t_3	3.54 ± 0.89^{b}	0 ± 0^{a}	1.66 ± 0.76^{b}	0 ± 0^{a}	3.13 ± 0.64^{b}
t_6	15.88 ± 1.41^{b}	1.59 ± 1.51^{a}	14.72 ± 1.40^{b}	7.40 ± 1.35^{a}	20.62 ± 0.52^{b}
t_9	28.16 ± 0.57^{b}	18.61 ± 1.48^{a}	27.48 ± 0.12^{b}	13.00 ± 2.21^{a}	28.72 ± 0.71^{b}

Values are expressed as mold propagation area $cm^2 \pm$ SD (n = 2). Mean values with different letters are significantly different (*P < 0.05). Small letters represent the differences within the rows.
BC: bread control; EBC: ethanol bread control; CLAB: coating with LAB.

resulting after hydrolysis with amylase and neutrase (AN) was considered optimal among the experimental conditions for all the strains, with likely results especially for *F. rossiae* UMCC 3002.

For each medium inoculated with a LAB strain, the pH value was detected after 48 h of incubation at 30 °C, and correlated to the strain's growth expressed as log CFU/mL (Fig. 2). The decrease of pH during fermentation is generally related to the production of organic acids, and values around 4 are archived for most of LAB (Wang et al., 2021). The strains showed a clustering on scatter plot, characterized by low pH values and high cellular concentration. The results highlighted the optimal fermentative process carried out by the LAB despite the substrate. The acidification detected for the strains was not statistically significant among the alternative media and MRS, despite the different initial pH values. For *L. plantarum* and *P. pentosaceus* the pH values detected were 4.08 ± 0.02 and 4.23 ± 0.04, respectively. Notwithstanding, only in the case of *F. rossiae* UMCC 3002, a linear behavior among pH values and cells concentration ($R^2 = 0.9239$) can be noted. In MRS the pH value was 4.65 ± 0.01, while in AN medium was 4.30 ± 0.02, with a correlation to a cell concentration of 8.86 ± 0.19 UFC/mL and 9.32 ± 0.04 UFC/mL, respectively.

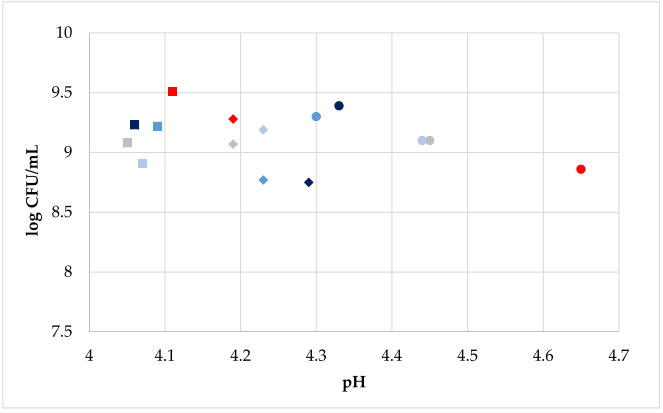

Fig. 2. Scatter plot of relations between cell concentration (log CFU/mL) and pH values detected after 48 h of incubation of *L. plantarum* UMCC 2996 (■), *F. rossiae* UMCC 3002 (●) and *P. pentosaceus* UMCC 3010 (◆) in the different media. The points with the same shape represent the growth on bread-whey (Amylase (A) (■), Amylase and 1 g/100 mL yeast extract (AY1%) (■), Amylase and Neutrase (AN) (■), Amylase, Neutrase and 1 g/100 mL yeast extract (ANY1%) (■)), and MRS (■) medium for the different strains. Values are given as the means ± SD (n = 3).

3.3. LAB cells viability in edible coatings

The viability of the three LAB strains, included into the edible coatings, was assessed during an incubation period of 9 d, in conditions which simulate a sliced bread storage at room temperature (25 °C; 75% RH) (Fig. 3). Cells viability was greater than 80% (corresponding to a concentration loss of ca. 1.4 log units) for all strains after 9 d despite the high moisture loss due to gas exchange. After three days each strain exhibited an unremarkable loss of viability. *L. plantarum* UMCC 2996 showed a decrease between the six and 9 d (from 100.00 ± 6.86% to 92.97 ± 6.55%), *F. rossiae* UMCC 3002 between three and six days (from 98.82 ± 7.43% to 79.76 ± 6.55%), instead *P. pentosaceus* UMCC 3010 did not show great variation and it exhibited the best viability at the end of the storage period (97.71 ± 3.18%). According to Rodrigues et al. (2011), high temperature and long storage periods are harmful to LAB survival. Pereira et al. (2016), reported a loss of viability of ca. 3 log units after 10 d of storage at 23 °C, while Gregirchak et al. (2020), stated a loss concentration of strains from ca. 1 to 3 after only 86 h compared four types of different coating. Nevertheless, in our study, the pectin-based edible coating contributed to have good viability for all the strains (greater than 80%) during the nine days of storage, therefore, it can be considered as a starting point for further optimization at an industrial scale.

Generally, pectin, as a polysaccharide, represents a suitable

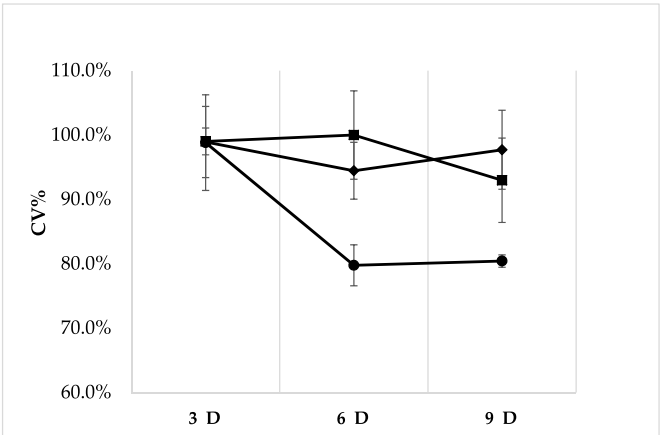

Fig. 3. Cells viability (CV%) of *L. plantarum* UMCC 2996 (■), *F. rossiae* UMCC 3002 (●) and *P. pentosaceus* UMCC 3010 (◆) incorporated in an edible coating of sliced bread during 9 d of storage (25 °C–75% RH). Values are given as the means ± SD (n = 3).

ingredient, due to its edibility and nutraceutical properties (Naqash, Masoodi, Rather, Wani, & Gani, 2017). Moreover, pectin can be easily extracted from fruit peels, and recent techniques have been developed to extract it with eco-sustainable processes and reagents (Cho et al., 2019). Pectics are polymers mainly composed of (1 → 4) α-D-galactopyranosyluronic acid units naturally esterified with methanol. According to their content of methyl esters or degree of esterification (DE), pectins are divided into high-methoxyl (HM, DE>50%) or low-methoxyl (LM, DE<50%). The DE has a decisive effect on pectin solubility and gelation properties (Campos, Gerschenson, & Flores, 2010). Pectin-based edible coatings have been already developed to improve the shelf-life of eggs (De Leo et al., 2018), different vegetables (Oms-Oliu, Soliva-Fortuny, & Martín-Belloso, 2008; Ranjitha et al., 2017) or application on peaches (Hussain, Suradkar, Wani, & Dar, 2016), grapes (Yinzhe & Shaoying, 2013) and orange (Strano et al., 2021) showing good maintenance of the nutritional quality of products. Sorbitol may have a protective effect on bacteria incorporated, the mechanism by which it exerts its protective effect could be related to the interaction between sugars and phospholipid membranes, helping to maintain its fluidity (Santivarangkna, Kulozik, Kienberger, & Foerst, 2009). In the studies of Gialamas, Zinoviadou, Biliaderis, and Koutsoumanis (2010), when sorbitol was added to film formulation, the viability of the bacterial cells during storage at 25 °C over 30 d was increased. Moreover, in the study carried out by López de Lacey, López-Caballero, Gómez-Estaca, Gómez-Guillén, and Montero (2012), from an initial concentration of 10^9 CFU/mL, both *L. acidophilus* and *B. bifidum* remained constant for 6 d in an edible coating containing sorbitol in its composition. Glycerol was added as a plasticizer to enhance the film flexibility, but it has also a role in cellular protection as sorbitol. Yeast extract was included as proteins and B vitamins source to enhance LAB viability, oleic acid was added as a surfactant to improve adhesion to the bread surface whereas Tween 80 as an emulsifying agent.

3.4. Assessment of the anti-mold activity on treated sliced bread

The preservation action of the edible coating containing *L. plantarum* UMCC 2996, *F. rossiae* UMCC 3002, and *P. pentosaceus* UMCC 3010 was tested on sliced bread intentionally contaminated with three filamentous fungi: *Aspergillus flavus* ITEM 7828, *Aspergillus niger* ITEM 7090, and *Penicillium paneum* ITEM 1381. The anti-mold activity of LAB was assessed after 3, 6 and 9 d of incubation at the optimal growth temperature (25 °C) for the molds and 75% RH (Table 3). A different LAB strain behaviour was observed in relation to a specific mold (Figs. S4–6). Regarding *A. flavus* ITEM 7828, the strain *F. rossiae* UMCC 3002, showed the best bio-preservation activity. After 9 d, also bread treated with ethanol (EBC) (10.89 ± 0.49 cm^2) was contaminated and the difference was not statistically different between its preservation and the action of *F. rossiae* UMCC 3002 (15.73 ± 2.60 cm^2), but truly significant respect to BC (30.86 ± 0.71 cm^2) and other coated treated with LAB. The same behavior of this strain, with respect to bread sprayed with ethanol, was observed for *P. paneum* ITEM 1381. Nevertheless, the previous study *in-vitro* did not encounter an antagonism among the LAB strains tested and this particular mold (Iosca et al., 2022). No contamination was visible on bread coated with *F. rossiae* UMCC 3002 after 3 d. After 9 d the contaminated area was only half respect to those observed in BC (13.00 ± 2.21 cm^2 and 28.16 ± 0.57 cm^2, respectively) and no statistical difference compared to EBC (18.61 ± 1.48 cm^2) (Fig. 4). Iosca et al. (2022), reported an antimold activity by *F. rossiae* UMCC 3002 and *P. pentosaceus* UMCC 3010 against *A. niger* ITEM 7090, which was confirmed in this screening. After 3 d of storage, on coated bread was observed a yellow halo in correspondence of the inoculum, while in BC was present the typical black fungal mycelium. That difference could be caused by the low pH characterized by the metabolic actions of LAB that produce organic acids with bio-preservation action. After 9 d was observed a statistical difference between coated bread with *F. rossiae* UMCC 3002 and *P. pentosaceus* UMCC 3010 and BC, whereas the presence of ethanol inhibited *A. niger* completely (Fig. 5).

A considerable number of studies have tested LAB strains for their antifungal activity, but, to date, only a few commercial products are available (Leyva Salas et al., 2017). Bio-preservations by LAB are highly documented for preventing molds contamination of the wheat bread, which causes economic and health problems (Rahman et al., 2019). Multiple studies have shown effects of bio-preservation with LAB. The obligatory and facultative heterofermentative LAB, such as *L. plantarum*, *F. sanfranciscensis*, *F. rossiae*, *L. citreum* and *L. parabuchneri*, seem to display a major antifungal activity than the homofermentative bacteria, such as *Lactobacillus delbrueckii* subsp. *lactis*, *Lacticaseibacillus paracasei* and *P. pentosaceus* (Bartkiene et al., 2019). In this study, the anti-mold activity results highlight that *F. rossiae* UMCC 3002 has demonstrated a strong action against *A. niger* ITEM 7090, *A. flavus* ITEM 7828 and *P. paneum* ITEM 1381 reducing the contaminated area on bread surface in respect to BC, and in some instance respect to EBC. This strain specific activity might be related to the production of different organic acids and antifungal compounds working in synergy (data not shown).

4. Conclusions

In a circular food system, the formulation of new bread-whey media able to promote the growth of LAB represents a reasonable strategy for the cultivation of selected starters with a bio protection activity by valorizing bread waste and cheese whey by-product, also avoiding the use of an important resource like water. Due to their compatibility with food, the bread-whey media allow the direct use of the cultivated LAB cultures in edible coatings designed for bio preservation purpose. In our study, the positive effects of the alternative media on bacterial growth

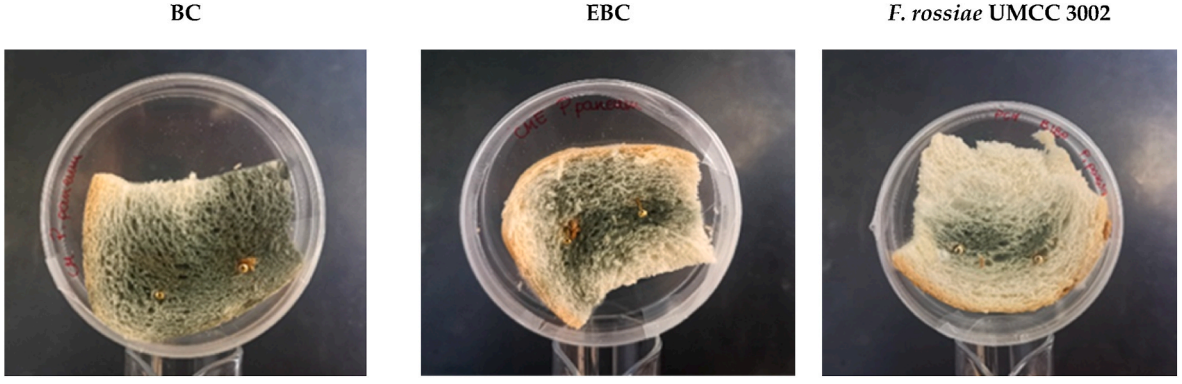

Fig. 4. Anti-mold activity against the simulation of microbial contamination of sliced bread with mycelium fungi of *Penicilium paneum* ITEM 1381 at the 9 d of storage (BC: bread control; EBC: ethanol bread control). The gold pin indicates the area where the mold was inoculated.

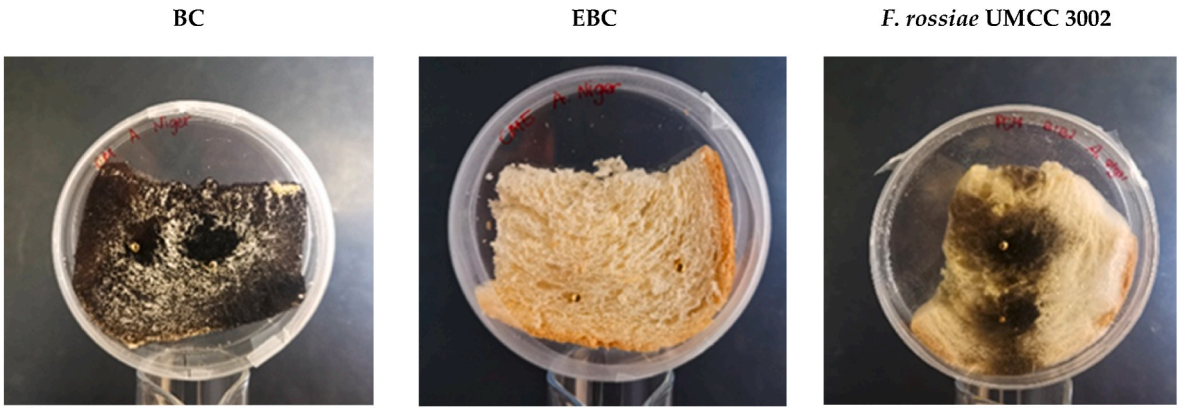

Fig. 5. Anti-mold activity against the simulation of microbial contamination of sliced bread with mycelium fungi of *Aspergillus niger* ITEM 7090 at the 9 d of storage (BC: bread control; EBC: ethanol bread control). The gold pin indicates the area where the mold was inoculated.

have been assessed. The medium AN, resulting from hydrolysis with amylase and neutrase, was considered the best for the growth of all the strains. In particular, the growth of *F. rossiae* UMCC 3002 was increased of 114% compared to that in the MRS medium. However, the microorganism metabolic activity in the alternative media could be affected by using different waste and by-product sources influencing the final growth ratio. Regarding the bio-preservation activity, some constraints also need to be assessed. Microorganisms' behavior might differ between the culture medium and the coated products. These differences could be linked to the complexity of the food or raw matrices, which are characterized by many abiotic and biotic variables that might affect LAB and mold development and metabolism, as well as the bioavailability of antifungal compounds. Furthermore, the maintenance of an available and stable microbial population during the whole storage period in the coating might be challenging due to variable environmental factors. Nevertheless, in the conditions described in this study, *F. rossiae* UMCC 3002 showed good bio-preservation activity against *A. flavus* ITEM 7828, suggesting an alternative to the use of chemical preservatives. Considering the evidence obtained from the reported results, the strain *F. rossiae* UMCC 3002 seems a promising candidate for the valorization and exploitation of food waste and by-products. Although, its bio-protection activity needs to be furtherly investigated, even against other spoilage agents, for the optimal employment of this strain in the manufacturing of clean-label bakery products.

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CRediT authorship contribution statement

Giovanna Iosca: Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Monica Turetta:** Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Luciana De Vero:** Conceptualization, Resources, Validation, Writing – review & editing, Supervision. **Claus Heiner Bang-Berthelsen:** Conceptualization, Resources, Validation, Writing – review & editing, Supervision. **Maria Gullo:** Writing – review & editing, Project administration, Funding acquisition. **Andrea Pulvirenti:** Writing – review & editing, Project administration, Funding acquisition.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.114524.

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