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Shelf-life extension of leavened bakery products by using bio-protective cultures and type-III sourdough

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ABSTRACT

Physicochemical and microbiological alteration of processed foods limits their shelf-life. To extend the shelf-life of two different types of bakery products, we evaluated the effectiveness of a type-III sourdough (tIII-SD) combined with a mixture of probiotics (*Lactobacillus acidophilus*, *L. casei*, *Bifidobacterium* spp, *Bacillus coagulans*) that were used as bioprotective-cultures (BCs). This innovative (I) dough was used to produce fresh *base-pizza* (BP) and *focaccia* (FO) that were inspected by a multi-omics approach aimful at monitoring features at different time-points of the shelf-life. Differences in physicochemical, protein, microbiological and volatile profiles were also investigated after 10-days of extended shelf-life. The addition of BCs and tIII-SD left unchanged the proximate composition. This, together with the absence of detected microbial contaminations, indicated the suitability of both I-BP and I-FO despite the shelf-life extension. Both I-samples accounted for a more stable and heterogeneous microbiota during the storage phase and showed lower scores of *Alternaria infectoria* and *A. alternata*. In I-samples, volatilomics showed an increased relative concentration of volatile carboxylic acids. Therefore, without resorting to chemical preservatives, the addition of BCs and tIII-SD led to specific microbiological and metabolite improvements in both BP and FO products, whose shelf-life was extended by 10-days under MAP.

1. Introduction

Wheat flour can be used to produce pasta, bread, and different types of sweet and savory baked goods (Pagani, Lucisano, & Mariotti, 2014). In Italy, pizza and focaccia are among the most popular bakery products. Being in close connection with the Italian history as the result of a centenary traditional socio-cultural background, the "art of Neapolitan *pizza*-makers" was recognised as "Intangible Cultural Heritage of Humanity" by the UNESCO agency (European Commission, 2010). On the other hand, *focaccia*, a circular flat bakery product accounting for various subtypes based on regional recipes, was recently included in the Italian list of typical agri-foods/local products with its Apulian variant (Ministerial Decree N. 60/2019, 7th Feb).

To meet the needs of both stores and consumers, the production of precooked *base-pizza* (BP) and *focaccia* (FO) registered an increased trade volume. Both the products are marketed as round or rectangular doughs already leavened, machine- or hand-flattened, packaged in protected or modified atmosphere, ready to be cooked and stuffed, and sold as fresh chilled (+4 °C) or frozen products (-20 °C). Otherwise, bakery products are a dynamic ecosystem suffering from physical (moisture redistribution, staling), chemical (nutraceutical value, rancidity), and microbiological alterations limiting their shelf-life (Melini & Melini, 2018). Physical and chemical variations affect the product freshness, texture, and taste, whereas microbiological spoilage can determine evident microbial growth, mycotoxin synthesis, and off-flavour (Melini & Melini, 2018). Whilst some species of *Bacillus* (e.g., *B. subtilis, B. licheniformis, B. megaterium, B. cereus*) led to bacterial-based rope spoilage (Thompson, Waites, & Dodd, 1998), among moulds, the main responsible of post-processing contamination are species

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Abbreviations

AFTs	total aflatoxins
ASVs	Amplicon Sequence Variants
a_w	water activity
BCs	bioprotective cultures
BP	base-pizza
Cd	cadmium
DON	deoxynivalenol
EVOO	extra virgin olive oil
FO	focaccia
I	innovative
LAB	lactic acid bacteria
MAP	modified atmosphere packaging
OTA	ochratoxin A
Pb	lead
PET/PE EVOH	Polyethylene Terephthalate/Poliethylene Ethylene Vinyl Alcohol
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
T	traditional
tIII-SD	type-III sourdough
UNESCO	United Nations Educational, Scientific and Cultural Organization
VOCs	volatile organic compounds
ZEA	zearalenone

belonging to *Aspergillus*, *Fusarium*, *Penicillium*, *Cladosporium*, and *Rhizopus* genera (Pitt & Hocking, 2009). Being involved in food waste and economic loss matters, spoiled goods are on the watch list of industries that are interested to identify innovative strategies allowing for shelf-life extension and avoiding changes of safety and organoleptic features (Axel, Zannini, & Arendt, 2017). To date, the adopted technology to extend the shelf-life is usually based on physical methods, chemical preservatives, or ecologically sustainable biological preservatives (Cheng, Sun, Zhu, & Zhang, 2017; Liu et al., 2022). However, due to the need of reducing the usage of chemical preservatives, the bio-protection *via* selected microorganisms and/or their metabolites represents a natural strategy to prevent food spoilage and harmful contamination (Pawlowska, Zannini, Coffey, & Arendt, 2012). As an example, within the group of lactic acid bacteria (LAB), various strains can produce antimicrobial metabolites that, when released in adequate amounts in foods, can be complementary to chemical preservatives or can even substitute their use (Melini & Melini, 2018). As showed for LAB, even specific strains of bifidobacteria, *Bacillus* spp, and yeasts can exert antimicrobial activities by producing organic acids, bacteriocins, antimicrobial peptides, or simple competitive exclusion (Saxelin, Tynkkynen, Mattila-Sandholm, & de Vos, 2005). Also, sourdough demonstrated the capability to extend the shelf-life of leavened goods thanks to the acid fermentation exerted by the metabolism of LAB and, for this reason, recent advances in food biotechnology supported its adoption as bio-agent and spoilage inhibitor (Rizzello, Cassone, Coda, & Gobbetti, 2011). Besides the antimicrobial activity, sourdough can also improve the functional/nutritional value of bakery products ensuring hygiene, rheology, sensory, and shelf-life improvements (Gobbetti et al., 2019). Although recently an addition type of sourdough was declared and labelled as "Type 0" (De Vuyst, Comasio, & Kerrebroeck, 2021), three different types of sourdough are known worldwide. Differences between these three types refer to the fermentation process. The "Type I" sourdough relies on spontaneously fermented flour-water mixture and daily back-slopping, the "Type II" sourdough is obtained from starter culture-initiated fermentation process, whereas the "Type III" sourdough labelling indicates a starter culture-initiated fermentation followed by back-slopping (De Vuyst et al., 2021). Whilst types I and II are generally used to provide metabolically active microorganisms, the type III sourdough (tIII-SD) is mostly used as flavour enhancer. In fact, as metabolically active microorganisms are no longer required, the tIII-SD is commercialized as spray- or freeze-dried allowing for the maintaining of a shelf-stable product (Reale et al., 2019; Caglar, Ermis, & Durak, 2021).

Because of the mandatory condition of the company involved in this study was the need of stable and ready-to-use ingredients, the tIII-SD and lyophilic BCs represented the optimal choice for maintaining multiple and timeless productions. Based on these considerations, the present study aimed at characterizing the combined effect of a multi-strain BCs and tIII-SD on the extension of the shelf-life in *base-pizza* (BP) and *focaccia* (FO) samples. A multi-omics approach was applied to assess the main chemical, microbiological, and metabolomic changes occurring during the phases of storage.

2. Materials and methods

2.1. Pizza base and focaccia making

Fresh samples of *base-pizza* (BP) and *focaccia* (FO) were produced in Apulia (Gravina di Puglia, Bari; Italy) at semi-industrial level and delivered in duplicate on three consecutive days.

The characteristics of the wheat flour used for BP and FO making were summarized (Appendix A).

Two different batches of both BP and FO samples were manufactured; the first, traditional (T), was obtained by conventional recipe used at company level and used as control for the present research, whereas the innovative (I) dough was obtained by the addition of BCs and tIII-SD.

BCs were freeze-dried multi-strain probiotics commercially available, containing *Lactobacillus acidophilus*, *Bifidobacterium animalis*, *Lacticaseibacillus paracasei* (basonym *Lactobacillus paracasei*), *Lacticaseibacillus casei* (basonym *L. casei*), and *Bacillus coagulans* strains (Montefarmaco OTC SpA, Bollate, Milan, Italy). Immediately before the use, BCs were prepared dissolving the lyophilic mixture in isotonic sterile solution following the manufacturer's instructions. Spray dried tIII-SD (Puratos, Groot – Bijgaarden, Belgium) was a wheat flour fermented by lactic and propionic acid bacteria in powder form (moisture, 6.0 g/100 g; total protein, 10.1 g/100 g; total fat, 1.0 g/100 g; ash, 7.3 g/100 g, total fibres, 5.9 g/100 g, and total carbohydrates, 49.8 g/100 g) accounting for total mesophilic bacteria <5 log cfu/g, yeasts and moulds <3 log cfu/g, *Escherichia coli*, total coliforms, and *Staphylococcus aureus* <1 log cfu/g, while *Salmonella* was absent (in 25g).

The two dough-variants were: *i)* dough made with durum wheat semolina, water, salt, EVOO, baker yeast, and sunflower oil for both traditional BP and FO samples (T-BP and T-FO, respectively); *ii)* dough made with the addition of 1.0% (wt/wt) of BCs to wheat flour (final cell densities of lactobacilli and bifidobacterial ~7 log CFU/g of dough and *B. coagulans* ~5 log spores/g of dough) and 1% (wt/wt of wheat flour) of tIII-SD for both innovative BP and FO samples (I-BP and I-FO, respectively). After mixing all the ingredients, the doughs were divided and rounded. Fermentation was carried out at 30 °C for 5.5 h and 6.5 h for BP and FO samples, respectively. Doughs were handmade stretched. In FO, the topping was represented by tomato sauce. BP-samples were baked at 320–350 °C on stone, in a tunnel oven, whereas FO-samples were baked at 220–250 °C in a static oven. The size was 250g × 33 cm (diameter) and 600g × 33 cm for BP and FO, respectively. After baking, each product was immediately cooled at +4 °C and placed under MAP (gas concentration 50%CO_2:50%N_2) in PET/PE EVOH sealed plastic bags.

BP-samples were profiled at 0, 60 (current shelf-life) and 70 (extended shelf-life) days of refrigerated storage, instead FO-samples

were profiled at 0, 30 (current shelf-life) and 40 (extended shelf-life) days of refrigerated storage. All samples were shipped to the laboratory under refrigerated conditions and chemical and microbiological analyses were immediately performed. An aliquot of each sample was frozen (−80 °C) until metagenomic and metabolomic analysis were carried out.

2.2. Chemical characterization

Lipids, proteins (total nitrogen × 6.25), ash, and total dietary fibers were determined according to AOAC methods 945.38F, 979.09, 923.03, 991.43. Moisture content was determined by an automatic moisture analyzer at 105 °C (Mod. MAC 110/NP, Rodwang Wagi Elektroniczne, Poland). In 100g of sample, the carbohydrate content was determined as residual amount after the estimation of the afore-mentioned macronutrients and water. The energy value was determined multiplying the protein and carbohydrate values of the bakery products by their calorific value (4 kcal/100g), and the fat value by its calorific value (9 kcal/100g). Water activity (a_w) in BP and FO was determined according to UNI EN ISO 18787:2017.

The gas concentration (O_2 and CO_2) in headspace of packaged samples were measured according to L-MI056 rev.0 Ed.2018 method. Mycotoxins (AFTs, DON, OTA, and ZEA) and heavy metals (Cd and Pb) were determined according to the L-MI067 rev.0 2020 method.

2.3. Protein characterization

Protein fractions (albumins, gliadins, and glutenins) were extracted from 1g of product according to Weiss, Vogelmeier, and Görg (1993). The protein concentration was determined following the Bradford's method (Bradford, 1976). Using ca. 15 μg, the SDS-PAGE protein profiling was carried out as previously described (Laemmli, 1970). Two-dimensional electrophoresis (2-DE) of ca. 30 μg of proteins was carried out with the Immobiline-polyacrylamide system as described by De Angelis et al. (2008). The second-dimension electrophoresis was carried out in a Laemmli system (1970). Before spot-intensity normalization, as described by Bini et al. (1997), gels were silver-stained.

2.4. Cultivable microbiota of bakery products

Microbiological analyses were performed. The count of total viable microorganisms at 30 °C was carried out according with the method UNI EN ISO 4833–1:2013; yeasts and moulds according to standard method ISO 21527–2:2008; coliforms with ISO 4832:2006 method; *Enterobacteriaceae* according to ISO 21528–2:2017; *Salmonella* by AFNOR BIO 12/32-10/11 method; beta-glucuronidase-positive *E. coli* and coagulase-positive staphylococci according to ISO 16649–2:2001 and UNI EN ISO 6888–2:2004, respectively; mesophilic lactic acid bacteria according to ISO 15214:1998 and *Listeria monocytogenes* according by AFNOR BIO 12/27-02/10 method. *Bifidobacterium* were enumerated according to Tharmaraj and Shah (2003). For the enumeration of presumptive *B. coagulans*, dilutions were heat-treated (75 °C for 30 min in a water bath), spread-plated on glucose yeast extract agar, and incubated at 40 °C for 48–72 h (Konuray & Erginkaya, 2018).

2.5. Microbiome of bakery products

Forty-five ml of NaCl (0.9%, vol/vol) were added to 5g of each sample, twice homogenized for 3 min, centrifuged (1000g, 5 min, 4 °C) recovering supernatants, in turn, again centrifuged (5000g, 15 min, 4 °C). The pellet was suspended in 500 μl of saline solution. DNA was extracted using the FastDNA SPIN kit for soil (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's procedure. DNAs were eluted in 100 μl of nuclease-free water. Negative controls (sterile distilled water) were also processed with samples. Qualitative and quantitative analyses of extracted DNAs were performed using agarose-gel (1%) electrophoresis and the Quant-iTTM PicoGreen® dsDNA Assay Kit (Invitrogen, Carlsbad, California), respectively. Before library preparation, DNAs were stored at 4 °C. The 16S rDNA amplicon sequencing of V5–V6 hypervariable regions and the ITS1 (Internal Transcribed Spacer) region, within the ITS region of the gene locus for ribosomal RNA, were respectively chosen as targets for prokaryotic and fungal characterization (Bokulich & Mills, 2013; Manzari et al., 2015).

In the first PCR-round, the overhang primer pairs, BV5 (Next For) and AV6 (Next Rev) (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[ATTAGATACCCYGGTAGTCC]-3'/5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[ACGAGCTGACGACARCCATG]-3′) were used to analyse V5–V6 regions, while BITS (Next For) and B58S3 (Next Rev) (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG[ACCTGCGGARGGATCA]-3'/5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG[GAGATCCRTTGYTRAAAGTT]-3′) for the ITS1 region. Negative reaction control samples (sterile distilled water) were also processed. The detailed methods used to carry out the bioinformatic analysis were included in Appendix B. The 16S rRNA and ITS1 data were analysed by using two different pipelines, respectively, QIIME2 (Bolyen et al., 2019) and BioMaS (Fosso et al., 2015). Before 16S rDNA ASV-table normalization (depth 50,000 sequences) to perform diversity analyses, mitochondrial and chloroplast ASVs were filtered out. Similarly, to obtain the ITS1-seq table, sequences were filtered to exclusively retain those belonging to *fungi*, then these were normalized to an equal depth of 80,000 sequences for diversity analysis.

2.6. Profile of volatile organic compounds

To evaluate the volatile organic compounds (VOCs) by gas chromatography–mass spectrometry (GC-MS) analysis, 0.75g of crashed FO and BP were placed into 20 mL vials with 10 μL 4-methyl-2-pentanol (internal standard, IS; final concentration of 33 mg/g) (Molfetta, Celano, & Minervini, 2021). Detailed settings used to profile BP and FO samples were included in Appendix C. Each chromatogram was analysed for peak identification by using the National Institute of Standard and Technology (NIST) 2016 library. A peak area threshold of >1 000 000 and a match criterion of >85% was used for VOCs identification followed, when necessary, by manual visual inspection of the fragment patterns. Quantitative analyses of selected VOCs were carried out by using the internal standard procedure and expressed as μg/g of IS equivalents.

2.7. Statistical analyses

Data were subjected to one-way ANOVA analysis. Pair-comparison for mean values was achieved by Tukey's procedure at $P < 0.05$, using the statistical software, Statistica 12.5 (TIBCO Software Inc., Palo Alto, USA) for Windows. The heatmap with hierarchical clustering (i.e., Ward's method) based on Euclidean distance and the principal component analysis were carried out by running the PCA.anal and the PlotHeatMap R-functions (MetaboAnalyst v.5.0).

3. Results and discussion

Being ready-to-use, precooked bakery products are suitable to the actual intense rhythms of consumers. However, the bakery environment represents a source of contamination. Even usual practices like cooling, packaging, and storage can affect the products' shelf-life (Pateras, 1998); therefore, the adoption of innovative strategies to reduce potential microbial spoilage is required (Oliveira, Zannini, & Arendt, 2014). In this field, BCs and sourdough biotechnology have been previously proposed as promising approaches leading to bio-preservation (Axel et al., 2016; Coda et al., 2011; Rizzello et al., 2011). In the present research, experimental samples were produced at semi-industrial plant level according to previous evidence reporting exhaustive differences between laboratory and bakery outcomes (Minervini, Lattanzi, De

Table 1
Proximate composition of pizza base and focaccia samples obtained by traditional (T-BP and T-FO, respectively) and innovative making (I-BP and I-FO, respectively). The innovative making was based on the addition of probiotic cultures and spray dried sourdough (type III).

Parameter	T-BP	I-BP	T-FO	I-FO
Energy value (kcal/100 g)	370.0 ± 0.35	370.0 ± 0.38	407.0 ± 0.44	407.0 ± 0.49
Proteins (g/100 g)	12.0 ± 0.12	12.0 ± 0.14	14.0 ± 0.10	14.0 ± 0.13
Carbohydrates (g/100 g)	40.8 ± 0.16	41.5 ± 0.13	27.5 ± 0.22	29.6 ± 0.20
of which sugars (g/100 g)	0.6 ± 0.02	0.6 ± 0.02	1.3 ± 0.02	1.3 ± 0.03
Fats (g/100 g)	2.9 ± 0.08	2.9 ± 0.08	3.8 ± 0.11	3.8 ± 0.15
of which saturated fatty acids (g/100 g)	0.1 ± 0.01	0.1 ± 0.01	0.8 ± 0.01	0.8 ± 0.01
Fibers (g/100 g)	4.0 ± 0.14	4.0 ± 0.16	4.6 ± 0.18	4.6 ± 0.20
Ashes (g/100 g)	2.6 ± 0.08	2.6 ± 0.04	2.9 ± 0.07	2.9 ± 0.12

Data are showed as means of three independent experiments (n = 3) ± standard deviations (SD).

Table 2
Chemical characterization of pizza base (BP) obtained following the traditional (T-) or innovative (I-) protocol. The innovative making was based on the addition of probiotic cultures and spray dried sourdough (type III). Samples were analysed at the beginning (T0) and at the end of the actual and extended (T60 and T70 days, respectively) shelf life.

Determinations	T-BP-T0	T-BP-T60	T-BP-T70	I-BP-T0	I-BP-T60	I-BP-T70
A_w	0.85 ± 0.01^{b}	0.94 ± 0.01^{a,*}	0.95 ± 0.01^{a,*}	0.84 ± 0.01^{b}	0.86 ± 0.01^{b}	0.92 ± 0.01^{a}
Moisture (g/100 g)	37.72 ± 0.02*	37.73 ± 0.04*	38.08 ± 0.09*	36.49 ± 0.02	36.72 ± 0.03	36.21 ± 0.01
pH	5.67 ± 0.03^{a,*}	5.70 ± 0.03^{a,*}	5.51 ± 0.03^{b,*}	5.52 ± 0.03	5.50 ± 0.03	5.56 ± 0.03
O_2 (%)	2.12 ± 0.02^{c,*}	2.22 ± 0.02^{b,*}	3.89 ± 0.01^{a,*}	1.72 ± 0.03^{c}	2.42 ± 0.02^{a}	2.02 ± 0.02^{b}
CO_2 (%)	34.92 ± 0.02^{a}	33.91 ± 0.01^{a}	27.53 ± 0.04^{b}	38.34 ± 0.06^{a,*}	32.03 ± 0.04^{c,*}	33.32 ± 0.02^{b,*}
AFs (μg/Kg t. q.)	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.10 ± 0.01	0.08 ± 0.01
DON (μg/Kg t. q.)	49.00 ± 1.41	47.00 ± 1.01	55.00 ± 2.41	45.00 ± 1.11	49.5 ± 0.71	50.5 ± 0.71
OTA (μg/Kg t. q.)	0.45 ± 0.01	0.50 ± 0.01	0.52 ± 0.02	0.51 ± 0.02	0.53 ± 0.01	0.44 ± 0.01
ZEN (μg/Kg t. q.)	9.51 ± 0.71*	9.50 ± 0.45*	9.30 ± 0.71*	3.95 ± 0.07	3.85 ± 0.02	3.99 ± 0.01
Cd (mg/Kg)	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01
Pb (mg/Kg)	0.02 ± 0.02	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.01 ± 0.02	0.02 ± 0.01

Abbreviations: **AFs**, total aflatoxins; **A_w**, activity water; **DON**, deoxynivalenol, **OTA**, ochratoxin A; **ZEN**, zearalenone; **Cd**, cadmium; **Pb**, lead.
Data are showed as means of three independent experiments (n = 3) ± standard deviations (SD).
Within the same protocol of manufacturing (traditional (T) or innovative (I)), different superscripts letters in the same row indicate a significant difference ($P < 0.05$, Tukey's test) between the different times of shelf-life.
Comparing the same time shelf-life, "*" indicates a significant difference ($P < 0.05$; Tukey's test) between traditional (T) and innovative (I) bakery product.

Angelis, Di Cagno, & Gobbetti, 2012). The used BCs accounted for strains with proven beneficial activity. The ability to inhibit many food-pathogens exerted by *Bifidobacterium* species (Igbafe, Kilonzo-Nthenge, Nahashon, Mafiz, & Nzomo, 2020) and LAB (Pawlowska et al., 2012) led to their application as protective cultures in food and feed industry. Similarly, strains of spore-forming/lactic acid--producer B. coagulans (Konuray & Erginkaya, 2018) can metabolize specific compounds with antimicrobial effect (Le Marrec, Hyronimus, Bressollier, Verneuil, & Urdaci, 2000).

3.1. Chemical characterization

When the chemical composition was inspected, no difference in macronutrient was found between traditional (T) and innovative (I) products relatively to both BP and FO samples. In fact, as also supported by the absence of differences in the energy values, the protein, lipid, carbohydrate, and ash contents resulted to be similar ($P > 0.05$; Table 1).

With the aim of elucidating the packaging film properties, the water-vapor transmission rate (WVTR) and the oxygen transmission rate (OTR) were studied in BP (Table 2) and FO (Table 3) samples. As expected, the a_w increased during all product storage. However, the evaluation of the last sampling time allowed for assessing a slight but significantly lower value of a_w in I- than T-products as a positive outcome (Erkmen & Bozoglu, 2016). The moisture did not change during storage. Besides, all I-BP and I-FO samples showed lower values of moisture than their T-relatives. This indicated a further positive result because of high moisture percentages negatively impacted the stability of different foods during storage, whereas low values reduced the incidence of microbial overgrowths (Bavaro et al., 2021).

Useful in the monitoring of microbial spoilage, the OTR is a keystone parameter whose assessment during storage phase can be inspected through headspace-gas analysis (Zardetto, Fregonese, & Pasini, 2022). The O_2 concentration linearly increased during T-BP and T-FO storage. On the contrary, although maintaining values higher than T0, the I-BP products exhibited an O_2 percentage decrease from T60 to T70. No significant differences were, instead, detected between O_2 values of I-FO samples collected at T0 and T70.

Concerning CO_2 concentrations, T-products showed the absence of differences between the baseline (T0) and the relative following sampling times, more precisely T0 *vs* T60 and T0 *vs* T30 for BF and FO, respectively. Differently, significant lower values were found when longer time periods were compared against the T0 (T70 for BP, T40 for FO).

No differences existed in pH values between T-BP and I-BP at T0. However, while the latter group did not exhibit differences during storage, the former displayed a significant acidification at T70 if compared to the baseline. Stable pH values were, instead, detected during the storage of all FO samples. Noteworthy, comparing the same storage time, it should be noted how I-products had lower pH values than their T-relatives.

Until the end of the storage, all samples exhibited mycotoxins and heavy metal values lower than the safety limits (CE Reg. N. 1881/2006 of 19 December 2006), even though we found zearalenone (ZEN) values to be significantly lower in all the I-products than their T-relatives.

3.2. Protein characterization

Albumin, globulin, and gliadin fraction concentration was comparable and stable during storage both in FO and BP (data not showed; *d.n.s.*). Protein fractions were separated and visualized in SDS-PAGE analysis (Supp. Fig. S1). All samples had a similar banding pattern, and no changes occurred until T70 (for BP-samples) and T40 (for FO-samples). Protein fractions were also visualized by 2DE. Based on SDS-PAGE, no differences were found for total spot numbers and expression of proteins between samples having different time of storage. Therefore, due to the absence of substantial differences on wheat protein fractions during storage of T-samples, no further samples were profiled. Therefore, considering that protein modifications are subjected to the impact of metabolically active microorganisms (De Angelis et al., 2021; Gobbetti,

Table 3
Chemical characterization of focaccia (FO) obtained following the traditional (T-) or innovative (I-) protocol. The innovative making was based on the addition of probiotic cultures and spray dried sourdough (type III). Samples were analysed at the beginning (T0) and at the end of the actual and extended (T30 and T40 days, respectively) shelf life.

Determinations	T–FO–T0	T–FO–T30	T–FO–T40	I-FO-T0	I-FO-T30	I-FO-T40
A_w	0.82 ± 0.01^{b}	0.91 ± 0.01^{b}	0.93 ± 0.02^{a}	0.87 ± 0.01^{b}	0.90 ± 0.01^{a}	0.90 ± 0.01^{a}
Moisture (g/100 g)	47.22 ± 0.02*	46.99 ± 0.01*	47.12 ± 0.02*	45.12 ± 0.03	44.21 ± 0.01	45.42 ± 0.03
pH	5.20 ± 0.03*	5.21 ± 0.03*	5.18 ± 0.03*	5.02 ± 0.03	5.00 ± 0.03	5.06 ± 0.03
O_2 (%)	1.01 ± 0.01^{c}	$1.43 \pm 0.04^{b,*}$	$3.33 \pm 0.04^{a,*}$	1.03 ± 0.04^{a}	0.73 ± 0.04^{c}	0.83 ± 0.04^{b}
CO_2 (%)	31.73 ± 0.04^{a}	31.73 ± 0.04^{a}	24.82 ± 0.02^{b}	$35.52 \pm 0.02^{c,*}$	$37.09 \pm 0.01^{a,*}$	$35.81 \pm 0.01^{b,*}$
AFs (μg/Kg t.q.)	0.10 ± 0.01^{a}	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.09 ± 0.02
DON (μg/Kg t.q.)	49.00 ± 1.41^{c}	$160.5 \pm 2.12^{a,*}$	$102.5 \pm 0.71^{b,*}$	$61.00 \pm 1.41^{c,*}$	129.00 ± 6.41^{a}	76.00 ± 2.31^{b}
OTA (μg/Kg t.q.)	0.50 ± 0.01	0.50 ± 0.01	0.50 ± 0.01	0.50 ± 0.01	0.50 ± 0.01	0.50 ± 0.02
ZEN (μg/Kg t.q.)	9.50 ± 0.71*	9.50 ± 0.71*	9.50 ± 0.71*	3.95 ± 0.07	3.95 ± 0.07	3.95 ± 0.01
Cd (mg/Kg)	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.02 ± 0.01	0.01 ± 0.00	0.02 ± 0.01
Pb (mg/Kg)	0.02 ± 0.01	0.02 ± 0.0	0.02 ± 0.02	0.02 ± 0.03	0.02 ± 0.01	0.04 ± 0.01

Abbreviations: **AFs**, total aflatoxins; **A_w**, activity water; **DON**, deoxynivalenol, **OTA**, ochratoxin A; **ZEN**, zearalenone; **Cd**, cadmium; **Pb**, lead.
Data are showed as means of three independent experiments (n = 3) ± standard deviations (SD).
Within the same protocol of manufacturing (traditional (T) or innovative (I)), different superscripts letters in the same row indicate a significant difference ($P < 0.05$, Tukey's test) between the different times of shelf-life.
Comparing the same time shelf-life, "*" indicates a significant difference ($P < 0.05$, Tukey's test) between traditional (T) and innovative (I) bakery product.

Table 4
Viable cell counts (log CFU/g) of different microbial groups found in pizza base (BP) and focaccia (FO) samples. Doughs were made following the traditional or innovative (T and I, respectively) protocols. The innovative making was based on the addition of probiotic cultures and spray dried sourdough (type III). BP-samples were analysed at the beginning (T0) and at the end of the actual and extended (T60 and T70 days, respectively) shelf life, whereas FO-samples were analysed at the beginning (T0) and at the end of the actual and extended (T30 and T40 days, respectively) shelf life.

	T-BP-T0	T-BP-T60	T-BP-T70	I-BP-T0	I-BP-T60	I-BP-T70
TAMC	$2.49 \pm 0.03^{b,*}$	$3.03 \pm 0.04^{ab,*}$	$4.08 \pm 0.03^{a,*}$	1.59 ± 0.01^{c}	1.55 ± 0.21^{a}	2.01 ± 0.01^{b}
LAB	$2.10 \pm 0.02^{b,*}$	$2.08 \pm 0.11^{b,*}$	$3.05 \pm 0.04^{a,*}$	1.02 ± 0.02^{b}	1.44 ± 0.06^{a}	0.99 ± 0.01^{c}
Bacillus	<1	<1	<1	3.91 ± 0.13*	4.01 ± 0.23*	4.01 ± 0.11*
Yeasts	1.54 ± 0.03*	1.57 ± 0.04*	1.47 ± 0.04*	0.97 ± 0.04	0.95 ± 0.01	1.05 ± 0.01
Moulds	1.00 ± 0.02^{b}	$1.84 \pm 0.02^{a,*}$	$2.01 \pm 0.03^{a,*}$	0.91 ± 0.13	0.95 ± 0.1	0.93 ± 0.11
	T-FO-T0	**T-FO-T30**	**T-FO-T40**	**I-FO-T0**	**I-FO-T30**	**I-FO-T40**
TAMC	$1.56 \pm 0.06^{c,*}$	$2.63 \pm 0.04^{b,*}$	$3.20 \pm 0.03^{a,*}$	0.99 ± 0.01^{b}	1.46 ± 0.03^{a}	1.48 ± 0.04^{a}
LAB	$1.02 \pm 0.02^{b,*}$	$2.02 \pm 0.02^{a,*}$	$2.01 \pm 0.01^{a,*}$	0.92 ± 0.11	1.28 ± 0.03	0.95 ± 0.08
Bacillus	<1	<1	<1	4.02 ± 0.03*	3.92 ± 0.01*	3.88 ± 0.05*
Yeasts	$1.00 \pm 0.01^{b,*}$	$1.15 \pm 0.02^{ab,*}$	$1.55 \pm 0.11^{a,*}$	0.86 ± 0.20	0.87 ± 0.18	0.98 ± 0.15
moulds	$1.05 \pm 0.07^{b,*}$	$1.61 \pm 0.01^{a,*}$	$1.81 \pm 0.01^{a,*}$	0.92 ± 0.11	0.82 ± 0.12	0.95 ± 0.01

Abbreviations: **TAMC**, Total aerobic mesophilic microorganisms at 30 °C; LAB, mesophilic lactic acid bacteria.
Data are showed as means of three independent experiments (n = 3) ± standard deviations (SD).
Within the same protocol of manufacturing (traditional (T) or innovative (I)), different superscripts letters in the same row indicate a significant difference ($p < 0.05$, Tukey's test) between the different times of shelf-life.
Comparing the same time shelf-life, "*" indicates a significant difference ($p < 0.05$, Tukey's test) between traditional (T) and innovative (I) bakery product.

De Angelis, Corsetti, & Di Cagno, 2005) and considering the proteomic profile overlapping in terms of presence and intensity of gel-bands and albumin, gliadin and glutenin spots, we supposed that the microbial contamination in T-products exclusively involved the product's surface.

3.3. Cultivable microbiota

Pathogens (*Salmonella* and *L. monocytogenes*) were absent in all samples, whereas densities of beta-glucuronidase-positive *E. coli*, coagulase-positive Staphylococci, coliforms, and *Enterobacteriaceae* did not exceed the detection threshold (1 *log* CFU/g) till the end of the storage (*d.n.s.*), thus emphasizing the adoption of good manufacturing practices during sample making.

In T-BP, total aerobic mesophilic microorganism (TAMC) grew about 1.5-fold more (from 2.49 to 4.08 *log*) during 70 days of storage, whereas in I-BP, TAMC cell density was almost unchanged (max value found: ~2.01 *log*) till the end of the storage (Table 4). In T-BP, the LAB cell density was 1.5-fold higher (~3.05 *log*) at T70 than T0, whereas in I-BP, LAB showed densities of ~1.02 *log* during all the 70 days of storage. Viable bifidobacterial cells were not detected (*d.n.s.*). Due to their use in spore-forms, the greatest difference between T- and I-products was revealed for *Bacillus* cell densities, which were <1 *log* during T-BP storage, whereas values close to 4 *log* were found in all I-BP products. Both yeasts and moulds showed densities significantly higher in T-BP than I-BP. Although different times of storage were considered for FO samples, and although different counts were assessed, the ratios (T-to I-FO) of culturable densities were equal to those found in BP-samples. In details, both TAMC and LAB were about 1.5-fold higher in T-FO than I-FO, densities of *Bacillus* were lower than 1 *log* in T-FO, whereas they approximately were 4 *logs* in I-FO. Moreover, both yeasts and moulds showed higher densities in T-FO than I-FO.

Based on the collected results, although the high cell densities of BCs added in I-doughs, viable cells in samples were measured at very low values. Exception made for *Bacillus* counts, which increased significantly in both I-BP and I-FO, neither TAMC nor LAB increased significantly. Because of significantly lower cell counts were here assessed in comparison to other heat-treated products supplemented with BCs (Minervini, Siragusa, et al., 2012; Tabanelli et al., 2020) we can speculate on the impact of the baking process on the cell viability, that here exceeded 70 °C. Oppositely, we found that presumptive *Bacillus* cell density significantly increased in I-samples. In fact, as also assessed by previous studies, if used as spore, *B. coagulans* can preserve its viability during the heat process and storage (Konuray & Erginkaya, 2018), making it suitable for the production of bakery products (Jao, Huang, Wu, & Kuo-Chiang, 2011). Thanks to culturomics, we also observed significantly lower viable mould densities in I- than T-products allowing us to speculate about the presence of antifungal activity in the former. The persistence of an antifungal activity in I-products was monitored by extending the storage of 10 days, and the absence of significant differences emphasized the relevant contribution of BCs and tIII-SD during the

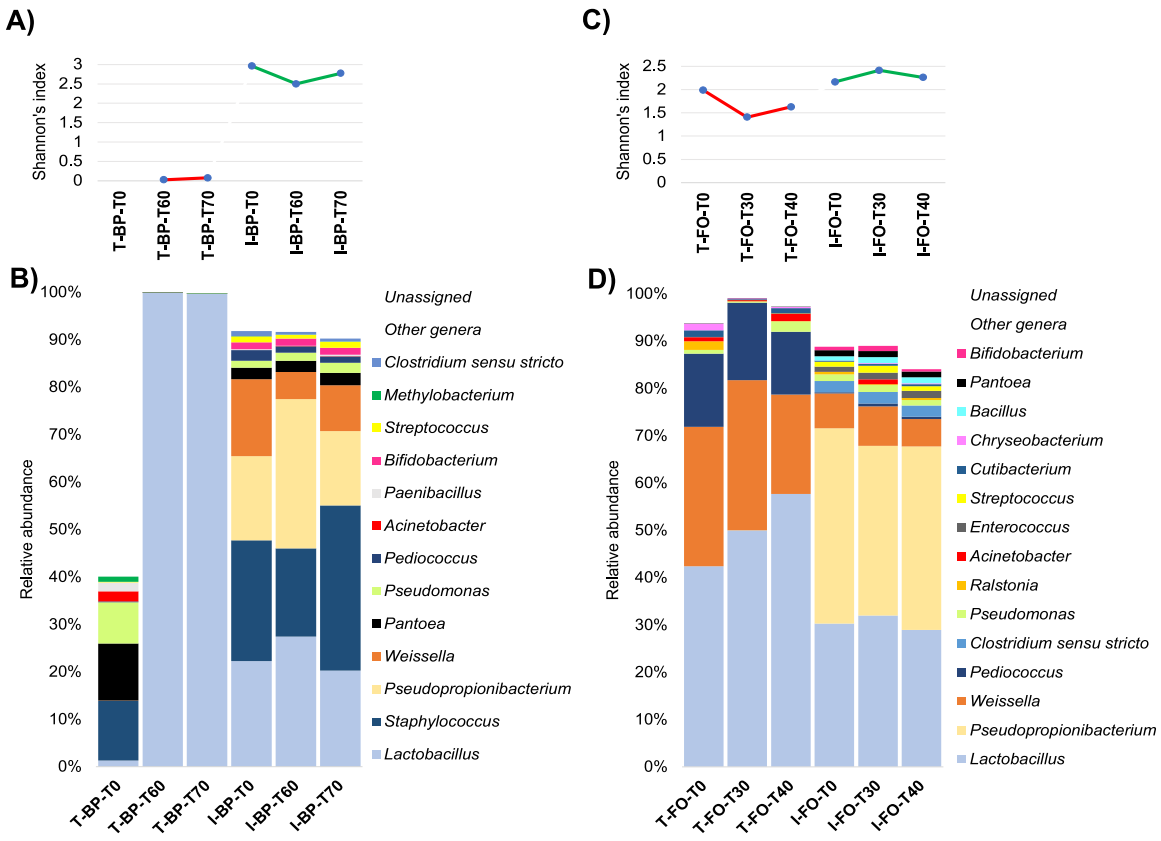

Fig. 1. Metataxonomic results. Based on 16S rDNA gene-seq, panels A and B respectively show values of Shannon's index found in pizza base (BP) and focaccia (FO) samples made following the traditional (T-) or innovative (I-) protocols. Based on 16S rDNA gene-seq, panels C and D respectively show the relative abundance of genera found in BP and FO made following the T- or I-protocol. The innovative making was based on the addition of probiotic cultures and spray dried sourdough (type III). BP-samples were analysed at the beginning (T0) and at the end of the actual and extended (T60 and T70 days, respectively) shelf life, whereas FO-samples were analysed at the beginning (T0) and at the end of the actual and extended (T30 and T40 days, respectively) shelf life.

pre-heating fermentation phase, leading to a reduced viability of microbial contaminants (Ribes, Fuentes, Talens, & Barat, 2018). The anti-microbial and anti-mycotoxigenic (considering the lower ZEN levels) activity of BCs and tIII-SD can be related to the acidification and, directly, to the presence of bioactive molecules (Bourdichon et al., 2021). Evidently, microbial secondary metabolites produced during fermentation of tIII-SD (Pandey, Larroche, & Soccol, 2017) or following the LAB stationary phase (Reale et al., 2019) can continue to be present even after spray-dried or freeze-dried sourdough stabilization and despite the microbial metabolism deactivation.

3.4. 16S rRNA gene-target microbiota analysis

After the filter quality check, metataxonomic reads were 200,000 ± 57,000 (mean ± SD) *per* sample. Because of the removal of mitochondrial and chloroplast ASVs affected the number of reads to lower value than the rarefaction threshold, it was not possible to compare the alpha diversity of T-BP against I-BP samples at T0. Besides the missing comparison at T0, a higher alpha diversity was on average observed between I- and T-samples (Fig. 1A). The taxonomy at the genus level mirrored these tendencies. On the one hand, T-BP-T0 was characterized by the presence of *Lactobacillus* (1.3%), *Staphylococcus* (12.6%), *Pantoea* (12%), *Pseudomonas* (8.7%), *Acinetobacter* (2.2%), *Paenibacillus* (1.9%), and *Methylobacterium* (1.1%). Then (at T60 and T70), the number of different taxa reduced showing that samples were mainly featured by lactobacilli (>99%) (Fig. 1B). On the other hand, in I-BP samples, the microbiota maintained a more stable and heterogenous profile from T0 till T70, while genera characterizing BCs and tIIISD were found as main colonizers in all I-BP.

Shannon's indices of T- and I-samples were almost similar for FO at T0 (Fig. 1C). However, while in the former group of samples the alpha-diversity decreased at T30 then increasing at T40, the latter group exhibited stable alpha-diversity values from T0 till T40. Based on a higher abundance of LAB (i.e., lactobacilli: 42.4%, *Weissella*: 29.5%, and pediococci: 15.5%) (Fig. 1D) microbiota profiles at T0, mainly distinguished T-FO from I-FO samples. Additionally, *Acinetobacter* (0.9%), *Chryseobacterium* (1.4%), *Cutibacterium* (1.4%), *Pseudomonas* (0.8%), *Ralstonia* (1.9%), and "other genera" (3.9%) were detectable in T-FO at T0. Differently, an unchanged taxon profile accounting for the presence of lactobacilli (~30.4%, on average), *Bifidobacterium* (~0.8%, on average), *Bacillus* (~1.2%, on average), *Weissella* (~7.2%, on average), and *Pseudopropionibacterium* (~38.7%, on average) featured all I-FO samples mirroring the addition of BCs and tIII-SD in doughs. Further genera, i.e., *Clostridium sensu stricto* (~2.4%, on average), *Enterococcus* (~1.3%, on average), *Streptococcus* (~1.2%, on average), and *Pantoea* (~1.2%, on average), characterized I-FO while not T-FO samples.

Therefore, in I-FO and I-BP, the BCs and tIII-SD addition led to the establishment of a more heterogenous and stable microbial community featured by the presence of different taxa belonging to LAB, i.e., *Weissella*, *Staphylococcus*, *Lactobacillus*, *Pedicoccus,* and *Bacillus*, which are all genera that usually can belong to the core of a sourdough microbiota (Calabrese et al., 2022). Instead, although not featuring the sourdough core microbiota, *Pseudopropionibacterium* genus specifically featured the used tIII-SD and, for this reason, it was found in all I-samples.

3.5. ITS1 gene-target mycobiota analysis

After the filter quality check, ITS1 gene-based reads were 192,000 ±

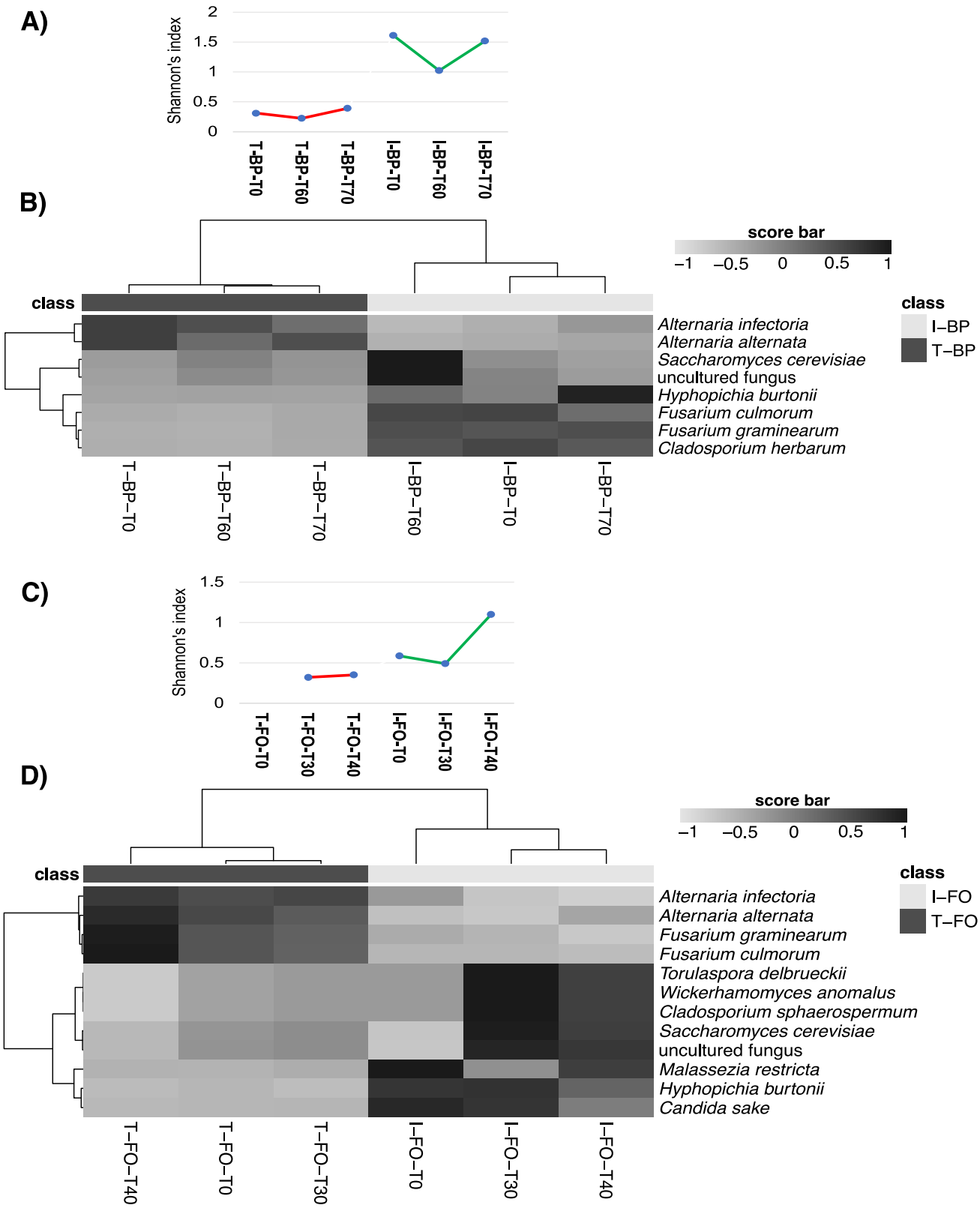

Fig. 2. Mycobiota results. Based on ITS1 gene-seq, panels A and C respectively show values of Shannon's index found in pizza base (BP) and focaccia (FO) samples made following the traditional (T-) or innovative (I-) protocols. Panels B and D respectively show the heatmap and clustering of BP and FO samples made following the T- or I-protocol. The innovative making was based on the addition of probiotic cultures and spray dried sourdough (type III). BP-samples were analysed at the beginning (T0) and at the end of the actual and extended (T60 and T70 days, respectively) shelf life, whereas FO-samples were analysed at the beginning (T0) and at the end of the actual and extended (T30 and T40 days, respectively) shelf life. The colour scale-bar ranged between low (white) to high (black) relative abundances of fungal species (filtered for those showing values > 0.1% at least in one sample) that were used to perform the clustering (Ward's method) based on Euclidean distance. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

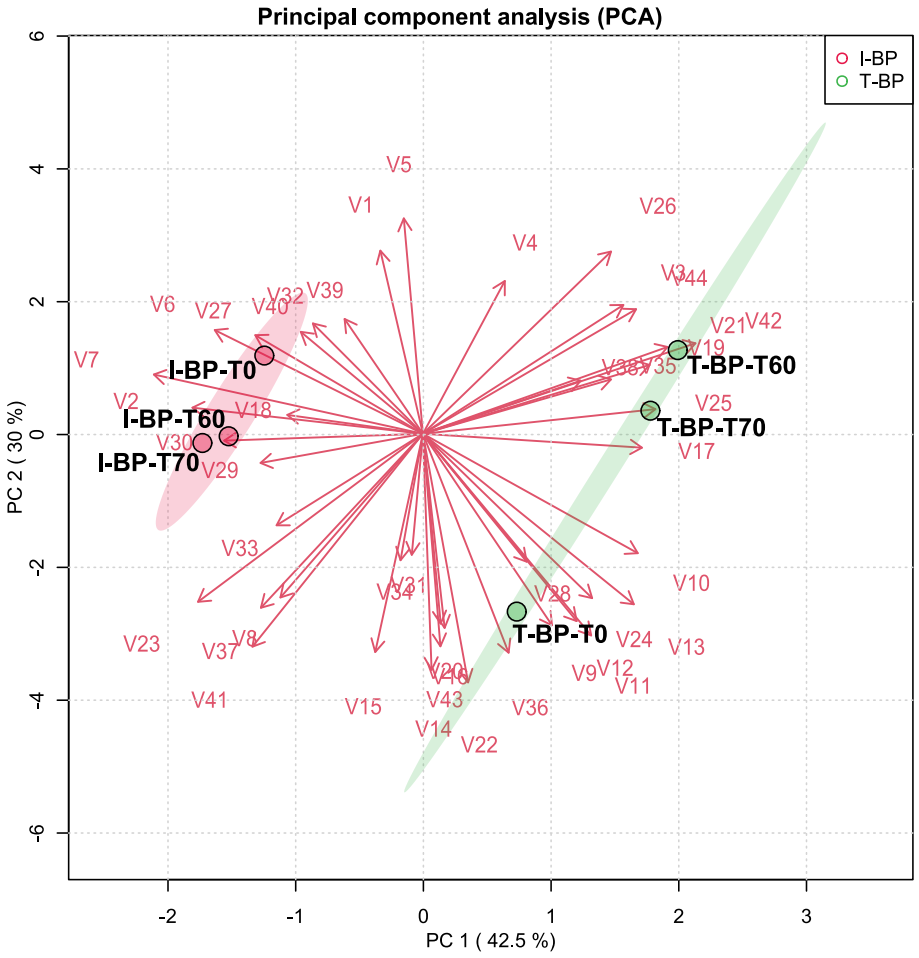

Fig. 3. Principal component analysis (PCA, both score plot and loading plot) based on volatile components found in pizza base (BP) samples made following the traditional (T-) or innovative (I-) protocols. The innovative making was based on the addition of probiotic cultures and spray dried sourdough (type III). BP-samples were analysed at the beginning (T0) and at the end of the actual and extended (T60 and T70 days, respectively) shelf life. V1, Acetic acid; V2, Propanoic acid; V3, Hexanoic acid; V4, Heptanoic acid; V5, Octanoic acid; V6, Nonanoic acid; V7, 1-Propanol, 2-methyl-; V8, 1-Butanol, 3-methyl-; V9, 1-Pentanol; V10, 1-Hexanol; V11, 1-Octen-3-ol; V12, 1-Octanol; V13, 3-Nonen-1-ol, (E)-; V14, Hexanal; V15, 2-Butenal, 3-methyl-; V16, Heptanal; V17, 2-Hexenal; V18, Octanal; V19, 2-Heptenal, (Z)-; V20, Nonanal; V21, 2-Octenal; V22, Furfural; V23, Benzaldehyde; V24, 2-Nonenal; V25, 2-Decenal; V26, 2,4-Decadienal; V27, Ethyl Acetate; V28, Hexanoic acid, ethyl ester; V29, Hex-4-enoic acid, ethyl ester; V30, Octanoic acid, ethyl ester; V31, 2,4-Hexadienoic acid, ethyl ester; V32, Glyceryl diacetate; V33, Heptane, 2,2,4,6,6-pentamethyl; V34, 2,2,4,4-Tetramethyloctane; V35, 2,4-Heptadienal, (E,E)-; V36, Acetoin; V37, 3,5-Octadien-2-one; V38, 6-Methyl-3,5-heptadiene-2-one; V39, Benzene, 1,3-bis(1,1-dimethylethyl)-; V40, Benzene, (1-ethylheptyl)-; V41, Formamide, N,N-dibutyl-; V42, 4-Cyanocyclohexene; V43, Linalool; V44, Safranal.

101,000 (mean ± SD) *per* sample. Due to the used rarefaction threshold, the Shannon's index of T-FO at T0 cannot be retained. Although this missing value, values of alpha-diversity were higher in I- than T-samples (Fig. 2A and C). Fungal species with a relative abundance greater than 0.1% at least in one sample were used as the most representative of the sample's mycobiota. Although no substantial differences were detected between T- and I-samples, *Saccharomyces cerevisiae* was found in all samples as the main fungal colonizer, exception made for I-BP-T70, in which *Saccharomyces cerevisiae* (42.1%) and *Hyphopichia burtonii* (42.0%) had a similar relative abundance. Nonetheless, T-BP and I-BP samples were featured by a different mycobiota profile, as highlighted by the clustering (Fig. 2B). While T-BP samples showed high scores of *Alternaria infectoria* and *A. alternata*, not the same was found in I-BP. The afore-mentioned species, supported by high scores of *Fusarium graminearum* and *F. culmorum*, also distinguished T-FO and I-FO samples (Fig. 2D). Therefore, in line with culturomics data, the ITS1 gene-seq showed how BCs and tIII-SD reduced the abundance of specific mould-contaminants. Further, the sequencing revealed product type species-specific differences. In fact, mostly accounting for changes in *Alternaria* species, which is a mould frequently isolated in bakery products (Pitt & Hocking, 2009), BCs and tIII-SD were also able to negatively affect the abundance of *Fusarium* species in FO-samples.

3.6. Profiles of volatile organic compounds

T- and I-products were characterized by different qualitative and quantitative VOC composition. More than 60 volatile components belonging to various chemical classes were identified (Supp. Tables S1 and S2). In I-BP samples, the greatest relative concentration of carboxylic acids was detected. Among carboxylic acids, acetic, propanoic, octanoic and nonanoic acids had the highest relative concentrations. The total relative concentration of aldehydes in T-BP was higher than that found in I-BP samples, while hexanal ($P < 0.05$) was highest in T-BP-T0. The highest relative concentration of alcohols was observed in T-BP samples; nonetheless, ethanol and phenylethyl alcohol were higher ($P < 0.05$) in I-BP than T-BP. 1-Hexanol was higher in T-BP and increased during storage. VOC relative concentrations were used to carry out a principal component analysis (PCA). Concerning BP samples, the PCA-biplot accounted for the 72.5% of the total variance (PC1: 42.5% and PC2: 30%; Fig. 3). The sample distance based on PC1 axis revealed how the VOC-profiles relative to T-BP and I-BP slightly differed, while the PC2 mainly discriminated samples based on the time of storage.

A conspicuous incidence of carboxylic acids in all I-FO samples was found. Propanoic, hexanoic, heptanoic, and nonanoic acids had a higher concentration in I-FO than T-FO, while alcohols mainly characterized all FO samples. Ethanol exhibited the highest concentration, while other alcohols characterizing the FO samples were 1-hexanol, 3-heptanol, 1-octanol, 2-phenylethanol, 1-hexanol and (E)-3-nonen-1-ol. The highest concentration of total aldehydes was detected in I-FO-T70. The highest concentration of hexanal ($P < 0.05$) was found in T-BP-T0. The PCA-biplot relative to FO samples explained 77.1% of the total variance distribution (PC1: 60.4% and PC2: 17.9%; Fig. 4). The PC1 described the VOC profiles distinguishing T-FO from I-FO samples, while PC2 discriminates samples based on the time of storage. Although used as flavour enhancer, the tIII-SD directly provide organic acids (mainly lactic and propionic) during the dough-making process allowing to restrict the optimum of growth for undesired microbes (bacteria and

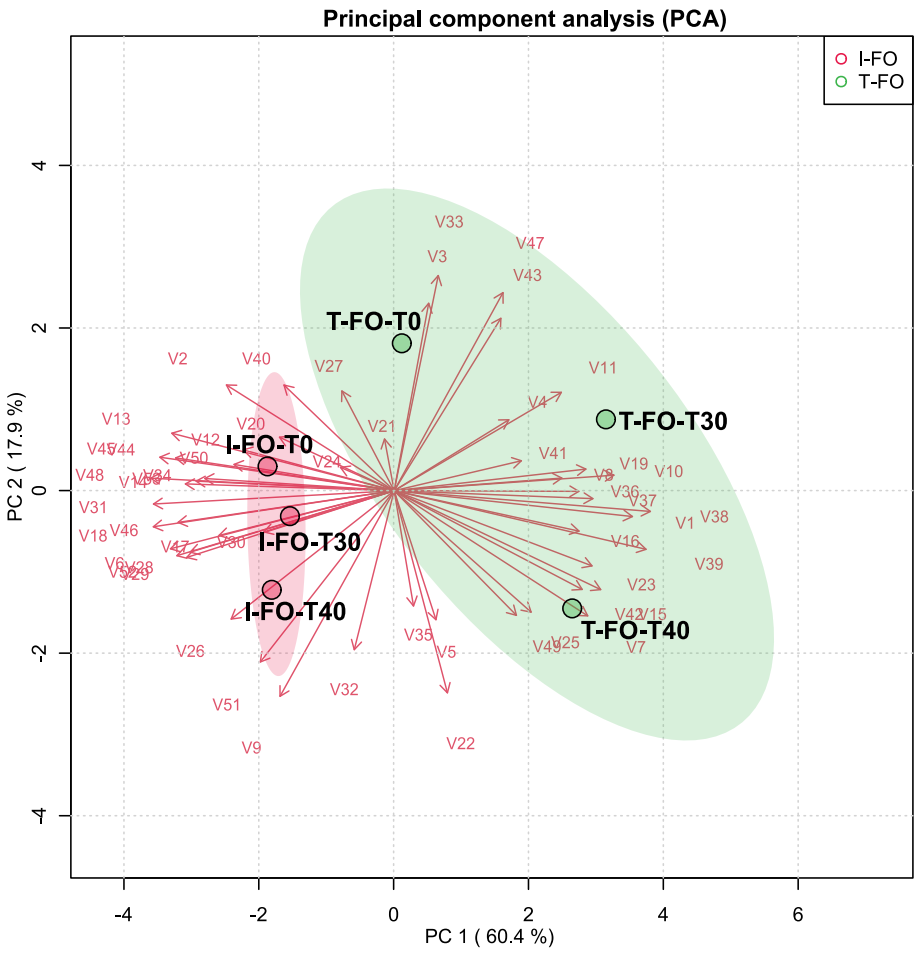

Fig. 4. Principal component analysis (PCA, both score plot and loading plot) based on volatile components found in focaccia (FO) samples made following the traditional (T-) or innovative (I-) protocols. The innovative making was based on the addition of probiotic cultures and spray dried sourdough (type III). FO-samples were analysed at the beginning (T0) and at the end of the actual and extended (T30 and T40 days, respectively) shelf life. V1, Acetic acid; V2, Propanoic acid; V3, Hexanoic acid, anhydride; V4, Hexanoic acid; V5, Heptanoic acid; V6, Octanoic acid; V7, Nonanoic acid; V8, Ethanol; V9, Eucalyptol; V10, 1-Butanol, 3-methyl-; V11, 1-Hexanol; V12, 1-Octen-3-ol; V13, 1-Heptanol; V14, 1-Octanol; V15, Terpinen-4-ol; V16, 3-Nonen-1-ol, (E)-; V17, L-alfa-Terpineol; V18, 1-Decanol; V19, Hexanal; V20, Octanal; V21, 2-Heptenal, (Z)-; V22, Nonanal; V23, 2-Octenal; V24, Furfural; V25, Benzaldehyde; V26, 2-Nonenal; V27, Benzeneacetaldehyde; V28, 2-Decenal; V29, 2-Undecenal; V30, 2,4-Decadienal; V31, Hexanoic acid, ethyl ester; V32, Octanoic acid, ethyl ester; V33, Glyceryl diacetate; V34, Furan, 2-pentyl-; V35, 2-n-Octylfuran; V36, Heptane, 2,2,4,6,6-pentamethyl; V37, 2,2,4,4-Tetramethyloctane; V38, Undecane; V39, Dodecane; V40, Acetoin; V41, 5-Hepten-2-one, 6-methyl-; V42, 6-Methyl-3,5-heptadiene-2-one; V43, gamma-Caprolactone; V44, gamma-Heptanolactone; V45, γ-Butyl-γ-butyrolactone; V46, Gamma-n-Amylbutyrolactone; V47, Benzene, 1,3-bis(1,1-dimethylethyl)-; V48, 2(3H)-Benzofuranone, hexahydro-3-methylene; V49, D-Limonene; V50, Linalool; V51, Beta-Citral; V52, Citral; V53, Safranal.

fungi) in preliminary phases of fermentation (Ross, Morgan, & Hill, 2002). This condition favours the LAB growth. Thanks to this strategy, many pathogenic and spoilage microbes altering bakery goods during storage can be kept under control before the complete activation of the LAB metabolism. Therefore, both LAB and organic acids can be used as bio-preservatives (Crowley, Mahony, & Van Sinderen, 2012), as also demonstrated by previous studies on low molecular mass metabolites (e.g., cyclic dipeptides, hydroxyl-fatty acids, phenyl, and substituted phenyl derivatives) that exert anti-fungal activities (Melini & Melini, 2018; Oliveira et al., 2014). In I-BP and I-FO samples, we detected the greatest concentration of carboxylic propanoic, hexanoic, heptanoic, and nonanoic acids. Reasonably, these results indicated the contribution of organic acids that, increasing the plasma membrane permeability, neutralized the electrochemical proton gradient (Caglar et al., 2021) and killed undesired microorganisms, leading to a longer shelf-life. To note, the pre-baking did not affect the potential bio-preservative effect exerted by these metabolites. In fact, many antifungal compound(s) from LAB are heat-stable at very high temperatures and, for this reason, they can be thus used to obtain clean-label heat-treated products (Crowley et al., 2012; Valerio et al., 2009).

4. Conclusion

The present study successfully combined commercial probiotic BCs with tIII-SD to extend the shelf-life of pre-cooked *base-pizza* and *focaccia* bakery products stored in MAP under refrigerated conditions. The used approach allowed for increasing by additional 10 days the shelf-life of two different bakery products. The here used approach, relying on the combination of BCs and tIII-SD, led to the production of a microbiologically stabilized pre-cooked bakery product thanks to a clean-label enrichment in antimicrobial compounds since from the firsts phases of dough making and throughout the fermentation time. Therefore, the testing for the applicability of this approach on a large-scale will help to extend the product shelf-life without including further adjuvants and/or preservatives.

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CRediT authorship contribution statement

Maria Calasso: Data curation, Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Marinella Marzano:** Methodology, Investigation, Formal analysis, Writing – original draft. **Giusy Rita Caponio:** Methodology, Investigation, Formal analysis, Writing – original draft. **Giuseppe Celano:** Methodology, Investigation, Formal analysis. **Bruno Fosso:** Methodology, Investigation, Validation. **Francesco Maria Calabrese:** Writing – review & editing. **Domenico De Palma:** Methodology, Resources, Validation. **Mirco Vacca:** Data curation, Writing – original draft, Writing – review & editing. **Elisabetta Notario:** Methodology, Investigation, Formal analysis. **Graziano Pesole:** Methodology, Resources,

Validation. **Maria De Angelis:** Funding acquisition, Conceptualization, Methodology, Supervision, Project administration, Writing – review & editing. **Francesca De Leo:** Funding acquisition, Conceptualization, Methodology, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declared that there is no conflict of interest.

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.2023.114587.

References

Axel, C., Brosnan, B., Zannini, E., Furey, A., Coffey, A., & Arendt, E. K. (2016). Antifungal sourdough lactic acid bacteria as biopreservation tool in quinoa and rice bread. *International Journal of Food Microbiology, 239*, 86–94.

Axel, C., Zannini, E., & Arendt, E. K. (2017). Mold spoilage of bread and its biopreservation: A review of current strategies for bread shelf-life extension. *Critical Reviews in Food Science and Nutrition, 57*(16), 3528–3542.

Bavaro, A. R., Di Biase, M., Conte, A., Lonigro, S. L., Caputo, L., Cedola, A., et al. (2021). Weissella cibaria short-fermented liquid sourdoughs based on quinoa or amaranth flours as fat replacer in focaccia bread formulation. *International Journal of Food Science and Technology, 56*(7), 3197–3208.

Bini, L., Magi, B., Marzocchi, B., Arcuri, F., Tripodi, S., Cintorino, M., et al. (1997). Protein expression profiles in human breast ductal carcinoma and histologically normal tissue. *Electrophoresis, 18*(15), 2832–2841.

Bokulich, N. A., & Mills, D. A. (2013). Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. *Applied and Environmental Microbiology, 79*(8), 2519–2526.

Bolyen, E., Rideout, J. R., Dillon, M. R., Bokulich, N. A., Abnet, C., Al-Ghalith, G. A., … Caporaso, J. G. (2019). QIIME 2: Reproducible, interactive, scalable, and extensible microbiome data science. *Nature Biotechnology, 37*(8), 852–857.

Bourdichon, F., Arias, E., Babuchowski, A., Bückle, A., Bello, F. D., Dubois, A., et al. (2021). The forgotten role of food cultures. *FEMS Microbiology Letters, 368*(14), fnab085.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*(1–2), 248–254.

Caglar, N., Ermis, E., & Durak, M. Z. (2021). Spray-dried and freeze-dried sourdough powders: Properties and evaluation of their use in breadmaking. *Journal of Food Engineering, 292*, Article 110355.

Calabrese, F. M., Ameur, H., Nikoloudaki, O., Celano, G., Vacca, M., Junior, W. J., et al. (2022). Metabolic framework of spontaneous and synthetic sourdough metacommunities to reveal microbial players responsible for resilience and performance. *Microbiome, 10*(1), 1–37.

Cheng, L., Sun, D. W., Zhu, Z., & Zhang, Z. (2017). Emerging techniques for assisting and accelerating food freezing processes: A review of recent research progresses. *Critical Reviews in Food Science and Nutrition, 57*(4), 769–781.

Coda, R., Cassone, A., Rizzello, C. G., Nionelli, L., Cardinali, G., & Gobbetti, M. (2011). Antifungal activity of wickerhamomyces anomalus and Lactobacillus plantarum during sourdough fermentation: Identification of novel compounds and long-term effect during storage of wheat bread. *Applied and Environmental Microbiology, 77*(10), 3484–3492.

Crowley, S., Mahony, J., & Van Sinderen, D. (2012). Comparative analysis of two antifungal Lactobacillus plantarum isolates and their application as bioprotectants in refrigerated foods. *Journal of Applied Microbiology, 113*(6), 1417–1427.

De Angelis, M., Minervini, F., Caputo, L., Cassone, A., Coda, R., Calasso, M. P., et al. (2008). Proteomic analysis by two-dimensional gel electrophoresis and starch characterization of Triticum turgidum L. var. durum cultivars for pasta making. *Journal of Agricultural and Food Chemistry, 56*(18), 8619–8628.

De Angelis, M., Siragusa, S., Vacca, M., Di Cagno, R., Cristofori, F., Schwarm, M., … Gobbetti, M. (2021). Selection of gut-resistant bacteria and construction of microbial consortia for improving gluten digestion under simulated gastrointestinal conditions. *Nutrients, 13*(3), 992.

De Vuyst, L., Comasio, A., & Kerrebroeck, S. V. (2021). Sourdough production: Fermentation strategies, microbial ecology, and use of non-flour ingredients. *Critical Reviews in Food Science and Nutrition*, 1–33.

Decreto Ministeriale 7 Febbraio. (2019). *Aggiornamento dell'elenco nazionale dei prodotti agroalimentari tradizionali ai sensi dell'articolo 12, comma 1, della legge 12 dicembre 2016, N. 238. Gazzetta ufficiale N. 60 del 12 marzo 2019–supplemento ordinario N. 9* Accessed online (2022, Sept).

Erkmen, O., & Bozoglu, T. F. (2016). Food preservation by reducing water activity. In O. Erkmen, & T. F. Bozoglu (Eds.), *Food microbiology: Principles into practice* (chapter 4).

European Commission. (2010). Commission Regulation (EU) No 97/2010 of 4 February 2010 entering a name in the register of traditional specialties guaranteed [Pizza Napoletana (TSG)]. *Official Journal of the European Union, L34*, 7–16.

Fosso, B., Santamaria, M., Marzano, M., Alonso-Alemany, D., Valiente, G., Donvito, G., et al. (2015). BioMaS: A modular pipeline for bioinformatic analysis of metagenomic AmpliconS. *BMC Bioinformatics, 16*(1), 1–11.

Gobbetti, M., De Angelis, M., Corsetti, A., & Di Cagno, R. (2005). Biochemistry and physiology of sourdough lactic acid bacteria. *Trends in Food Science & Technology, 16*(1–3), 57–69.

Gobbetti, M., De Angelis, M., Di Cagno, R., Calasso, M., Archetti, G., & Rizzello, C. G. (2019). Novel insights on the functional/nutritional features of the sourdough fermentation. *International Journal of Food Microbiology, 302*, 103–113.

Igbafe, J., Kilonzo-Nthenge, A., Nahashon, S. N., Mafiz, A. I., & Nzomo, M. (2020). Probiotics and antimicrobial effect of Lactiplantibacillus plantarum, Saccharomyces cerevisiae, and Bifidobacterium longum against common foodborne pathogens in poultry. *Agriculture, 10*(9), 368.

Jao, C. L., Huang, S. L., Wu, S. C., & Kuo-Chiang, H. (2011). The study on SFLAB GanedenBC30 viability on baking products during storage. *Procedia Food Science, 1*, 1601–1609.

Konuray, G., & Erginkaya, Z. (2018). Potential use of Bacillus coagulans in the food industry. *Foods, 7*(6), 92.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685.

Le Marrec, C., Hyronimus, B., Bressollier, P., Verneuil, B., & Urdaci, M. C. (2000). Biochemical and genetic characterization of coagulin, a new antilisterial bacteriocin in the pediocin family of bacteriocins, produced by Bacillus coagulans I4. *Applied and Environmental Microbiology, 66*(12), 5213–5220.

Liu, A., Xu, R., Zhang, S., Wang, Y., Hu, B., Ao, X., … Liu, S. (2022). Antifungal mechanisms and application of lactic acid bacteria in bakery products: A review. *Frontiers in Microbiology, 13*.

Manzari, C., Fosso, B., Marzano, M., Annese, A., Caprioli, R., D'Erchia, A. M., et al. (2015). The influence of invasive jellyfish blooms on the aquatic microbiome in a coastal lagoon (Varano, SE Italy) detected by an Illumina-based deep sequencing strategy. *Biological Invasions, 17*(3), 923–940.

Melini, V., & Melini, F. (2018). Strategies to extend bread and GF bread shelf-life: From Sourdough to antimicrobial active packaging and nanotechnology. *Fermentation, 4*(1), 9.

Minervini, F., Lattanzi, A., De Angelis, M., Di Cagno, R., & Gobbetti, M. (2012). Influence of artisan bakery-or laboratory-propagated sourdoughs on the diversity of lactic acid bacterium and yeast microbiotas. *Applied and Environmental Microbiology, 78*(15), 5328–5340.

Minervini, F., Siragusa, S., Faccia, M., Dal Bello, F., Gobbetti, M., & De Angelis, M. (2012). Manufacture of Fior di Latte cheese by incorporation of probiotic lactobacilli. *Journal of Dairy Science, 95*(2), 508–520.

Molfetta, M., Celano, G., & Minervini, F. (2021). Functional, nutritional, and sensory quality of mixed flours-based breads as compared to durum wheat semolina-based breads. *Foods, 10*(7), 1613.

Oliveira, P. M., Zannini, E., & Arendt, E. K. (2014). Cereal fungal infection, mycotoxins, and lactic acid bacteria mediated bioprotection: From crop farming to cereal products. *Food Microbiology, 37*, 78–95.

Pagani, M. A., Lucisano, M., & Mariotti, M. (2014). Italian bakery products. *Bakery products science and technology*, 685–721.

Pandey, A., Larroche, C., & Soccol, C. R. (Eds.). (2017). *Current developments in biotechnology and bioengineering: Current advances in solid-state fermentation*.

Pateras, I. M. C. (1998). Bread spoilage and staling. In *Technology of breadmaking* (pp. 240–261). New York, NY, USA: Springer, ISBN 978-1-4613-5922-7.

Pawlowska, A. M., Zannini, E., Coffey, A., & Arendt, E. K. (2012). Green preservatives": Combating fungi in the food and feed industry by applying antifungal lactic acid bacteria. *Advances in Food & Nutrition Research, 66*, 217–238.

Pitt, J. I., & Hocking, A. D. (2009). Spoilage of stored, processed and preserved foods. In *Fungi and food spoilage* (pp. 401–421). Boston, MA: Springer.

Reale, A., Di Renzo, T., Preziuso, M., Panfili, G., Cipriano, L., & Messia, M. C. (2019). Stabilization of sourdough starter by spray drying technique: New breadmaking perspective. *Lebensmittel-Wissenschaft und -Technologie, 99*, 468–475.

Ribes, S., Fuentes, A., Talens, P., & Barat, J. M. (2018). Prevention of fungal spoilage in food products using natural compounds: A review. *Critical Reviews in Food Science and Nutrition, 58*(12), 2002–2016.

Rizzello, C. G., Cassone, A., Coda, R., & Gobbetti, M. (2011). Antifungal activity of sourdough fermented wheat germ used as an ingredient for bread making. *Food Chemistry, 127*(3), 952–959.

Ross, R. P., Morgan, S., & Hill, C. (2002). Preservation and fermentation: Past, present and future. *International Journal of Food Microbiology, 79*(1–2), 3–16.

Saxelin, M., Tynkkynen, S., Mattila-Sandholm, T., & de Vos, W. M. (2005). Probiotic and other functional microbes: From markets to mechanisms. *Current Opinion in Biotechnology, 16*(2), 204–211.

Tabanelli, G., Barbieri, F., Campedelli, I., Venturini, M. C., Gardini, F., & Montanari, C. (2020). Effects of bioprotective cultures on the microbial community during storage of Italian fresh filled pasta. *Food Control, 115*, Article 107304.

Tharmaraj, N., & Shah, N. P. (2003). Selective enumeration of Lactobacillus delbrueckii ssp. bulgaricus, Streptococcus thermophilus, Lactobacillus acidophilus, bifidobacteria, Lactobacillus casei, Lactobacillus rhamnosus, and propionibacteria. *Journal of Dairy Science, 86*(7), 2288–2296.

Thompson, J. M., Waites, W. M., & Dodd, C. E. R. (1998). Detection of rope spoilage in bread caused by Bacillus species. *Journal of Applied Microbiology, 85*(3), 481–486.

Valerio, F., Favilla, M., De Bellis, P., Sisto, A., de Candia, S., & Lavermicocca, P. (2009). Antifungal activity of strains of lactic acid bacteria isolated from a semolina ecosystem against Penicillium roqueforti, Aspergillus Niger and Endomyces fibuliger contaminating bakery products. *Systematic & Applied Microbiology, 32*(6), 438–448.

Weiss, W., Vogelmeier, C., & Görg, A. (1993). Electrophoretic characterization of wheat grain allergens from different cultivars involved in bakers' asthma. *Electrophoresis, 14*(1), 805–816.

Zardetto, S., Fregonese, M., & Pasini, G. (2022). Effects of modified atmospheric packaging configuration on spoilage mould growth in damaged packages of fresh pasta. *Journal of Food Engineering, 314*, Article 110760.