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Dynamics of microbial ecology and their bio-preservative compounds formed during the panettones elaboration using sourdough-isolated strains as starter cultures

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ABSTRACT

The present study evaluated the application of *Limosilactobacillus fermentum* IAL 4541 and *Wickerhamomyces anomalus* IAL 4533 isolated from sourdoughs and their combinations, for bio-conservative action in panettones, as well as to identify the antifungal volatile compounds. Samples from different fermentation steps were submitted to physicochemical and microbiological evaluation, as to know: sourdough after 48 h (A), 96 h (B), and 144 h (C); dough of the first fermentation (D); final dough after second fermentation (E), and baked panettone (F). In all treatments, lactic bacteria and yeast counts demonstrated a mechanism of proto-cooperation. Panettones containing L. *fermentum* or *W. anomalus* in their composition had longer shelf life, which indicates their effect as potential biopreservatives. The genetic sequencing data demonstrated the dominance of the Lactobacilli throughout the process, corroborating the data obtained by cultivable methods. A total of 59 volatile organic compounds were found in this study, and a hierarchical cluster analysis presented the separation between the production stages (C, E) and the most recurrent compounds in the final product (F). Of these compounds, the following stood out (n = 26): acids (7.7%), alcohols (23.1%), aldehydes and ketones (34.6%), and esters (34.6%). The treatments containing *L. fermentum* presented more diversity of volatile organic compounds with potential antifungal effect. There was a higher production of acetic (1.17–8.85 mmol/kg), phenyllactic (5–10.4 mmol/kg), and propionic (3.5–4.3 mmol/kg) acids in the final product. This study demonstrates the feasibility of applying endogenous starter cultures from sourdoughs, with great biopreservative activity, enabling the production of healthier and more natural panettones.

1. Introduction

Panettone is a leavened cake of Italian origin, traditionally eaten at Christmas parties in Italy and in some Latin American countries, such as Peru and Brazil. It can be prepared with sourdough and added candied fruit, nuts, drops or chocolate filling (Benejam et al., 2009; Stefanello et al., 2019). In Brazil, due to its wide acceptance, this product is available on the market almost all year round.

This product is considered an intermediate moisture food, with moisture content usually ranging from 23% to 26%, water activity (Aw) between 0.83 and 0.85, and pH from 4.0 to 5.0 (Stefanello et al., 2019; Valcárcel-Yamani & Lannes, 2013). These parameters make them susceptible to filamentous fungi spoilage, becoming among the five main products in terms of consumer complaints due to mold spoilage (Lemos et al., 2018). The fungal deterioration leads to economic losses for the baking industry (Smith et al., 2004), causing adverse effects (Gray &

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Bemiller, 2003) and, sometimes, the production of hazardous mycotoxins to consumers' health (Dalié et al., 2010).

Consumers generally require high-quality, preservative-free, microbiologically safe, and extended shelf-life products. A possible preservative-free solution can be offered by using biopreservation methods based on microorganisms or their metabolites naturally produced during some fermentative microorganism growth in foods (Sadiq et al., 2019). The most studied microbial interaction during bread's fermentation is sourdough (Stefanello et al., 2019; Garofalo, Silvestri, Aquilanti, & Clementi, 2008), which produces inhibitory compounds that act against spoilage microorganisms, increasing product stability.

The sourdough is the result obtained by the spontaneous fermentation of a mixture of wheat flour, water, and salt (Torrieri et al., 2014), and their use has been extensively studied in recent years due to the growing demand for foods with fewer chemical-added preservatives (Vera et al., 2009). Besides the old process of spontaneous fermentation (Venturini Copetti, 2019), nowadays essential cultures are being selected and applied in sourdough fermentation to increase the shelf life of baked goods, aiming its stability against fungal spoilage, in addition to improving their sensory characteristics (Coda et al., 2011; Gobbetti & Gänzle, 2013; Stefanello et al., 2019). Also, functional/nutritional features of sourdough, such as the potential to lower glycemic index, increase mineral bioavailability, and decrease the gluten content, have been recently highlighted (Gobbetti & Gänzle, 2013).

Lactic acid bacteria (LAB) are interesting because they can produce molecules with antifungal effects (Dalié et al., 2010). Some studies have demonstrated the antifungal activity of some LAB species under laboratory conditions (Hassan & Bullerman, 2016; Lavermicocca et al., 2000), while other investigations have highlighted the ability of LAB antifungal strains to inhibit fungal growth in baked fermented products (Coda et al., 2008; Ryan et al., 2011; Stefanello et al., 2019). The species most present in bakery products belong to the LAB with emphasis on *Lactobacillus sanfranciscensis*, *Levilactobacillus brevis*, and *Lactiplantibacillus plantarum* (De Vero et al., 2021), main responsible for the acidification of the dough. Yeasts, in turn, are present in smaller fractions but are essential for the technological characteristics of the dough, such as gas production (leavening process), moisture retention, and flavor formation, mostly *Saccharomyces cerevisiae*, *Kazachstania exigua*, and *Kazachstania humilis* (De Vero et al., 2021; Garofalo, Silvestri, Aquilanti, & Clementi, 2008).

Few studies correlate the microbial ecology present in the sourdough by molecular methods with the characteristics of the final product, with a lack of data outside Europe. Thus, the present study aimed to apply three microbial strains isolated from lyophilized natural yeast in Brazil to obtain different sourdoughs for panettone production. During the preparation stages of sourdoughs and panettones, ecology and microbial diversity were studied to elucidate the modification in the fermentation process over time. Further, the shelf life and physicochemical aspects of these panettones were assessed.

2. Material and methods

2.1. Microorganisms

LAB and yeasts strains isolated from sourdough and belonging to the Microorganism Collection Nucleus – NCMO (Institute's Adolfo Lutz), Sao Paulo, Brazil, were used (Table 1). The strains used in this study were *Wickerhamomyces anomalus* IAL 4533, and *Limosilactobacillus fermentum* IAL 4541 (former *Lactobacillus fermentum* IAL 4541) isolated from a sourdough spontaneously produced from the blend of refined wheat flour and whole wheat flour, according to Stefanello et al. (2019).

Actively growing single strains of *W. anomalus* IAL 4533, and *L. fermentum* IAL 4541 were inoculated (inoculum level 1.0%, v/v) into Erlenmeyer flasks containing 100 mL of Sabouraud Dextrose (Difco, Detroit, Mich) and De Man, Rogosa and Sharpe (MRS) Broth (Oxoid, Basingstoke, Hampshire, United Kingdom), and incubated for 24 h at 25 and 30 °C for yeast and LAB, respectively (Paramithiotis et al., 2005). Twelve-hour cells were harvested (5000×*g*, 15 min, 4 °C), washed twice with distilled sterile water, and suspended in distilled water to an optical density at 620 nm (OD_{620nm}) corresponding to about 10^8 colony forming units per milliliter (CFU/mL) of *W. anomalus* IAL 4533 and commercial yeast; and 10^{10} CFU/mL of *L. fermentum* IAL 4541.

Five sourdoughs were tested, differing in the composition of the inoculum, as follows treatments: I: commercial yeast, II: *L. fermentum* IAL 4531; III: *L. fermentum* IAL 4531 + commercial yeast; IV: *L. fermentum* IAL 4531 + *W. anomalus* IAL 4533 and V: *L. fermentum* IAL 4531 + commercial yeast + *W. anomalus* IAL 4533 (see Table 1), according to the methodology of Stefanello et al. (2019).

Sourdough samples were collected for microbiological, sequencing, volatile compounds, organic acids, and physicochemical analysis after 48 h, 96 h, and 144 h of sourdough preparation in triplicates. Samples for the microbiological and physicochemical analyses were immediately prepared. The other samples were rapidly frozen using an ultra-freezer (Prática Klimaquip, Pouso Alegre, MG, Brazil) for later use.

2.2. Panettone elaboration

The ingredients used in preparing panettones and their formulation (flour basis) are listed in Table 2. Panettone preparation was divided into two steps: the first one was the preparation of the sponge, and the second step was the dough elaboration, according to Stefanello et al., 2019, and the flowchart presented in Fig. 1. The sourdough microorganisms used in the sponge step varied according to Table 1, considering that for the traditional sponge 3.0% of commercial yeast was used, 40% of refined wheat flour, and 60% of water. The panettones dough was portioned, rounded, and shaped proofed during the 2nd fermentation (32 °C/80%/2–4 h) and baked using a turbo-electric oven (G-Paniz FTE-120) at 180 °C for 40 min, packed, and stored for up to 180 days.

2.3. Microbiological analysis

2.3.1. Microbiological analysis of sourdough and fermented doughs

Microbiological analyses were performed after 48 h (A), 96 h (B), and 144 h (C) for sourdough sponge and fermented dough (D, E) preparation in triplicates. Analysis was performed after a 25 g sample homogenization with 225 mL of 1 mg/L peptone water in a lab blender stomacher (Seward Medical, London, UK) for 2 min. Afterward, decimal serial dilutions were carried out in triplicate. LAB was counted using MRS agar (Oxoid, Basingstoke, Hampshire, United Kingdom) through the pour plate method (Rizzello et al., 2016), incubated at 30 °C for 48 h,

Table 1
The number of microorganisms used in each treatment (I to V) for the preparation of sourdough of the panettones.

Treatments	*Limosilactobacillus fermentum* IAL 4531 (10^{10} CFU/mL)	*Saccharomyces cerevisae* commercial (10^8 CFU/mL)	*Wickerhamomyces anomalus* IAL 4533 (10^8 CFU/mL)	Inoculum (mL)	Wheat flour (g)	Tap water (mL)
I	–	X	–	5	100	95
II	X	–	–	5	100	95
III	X	X	–	10	100	90
IV	X	–	X	10	100	90
V	X	X	X	15	100	85

Table 2
Panettone formulations using traditional and sourdough sponge with five microorganisms combinations in step 1 (Sponge) and step 2 (Dough) for panettone making.

Ingredients	*Step 1 - Sponge % (b.f.)*[a]	*Step 2 - Dough % (b.f.)*[a]
Wheat flour	76.0	24.0
Fungal α-amylase	0.045	–
Ascorbic acid	0.005	0.008
DATEM**	0.5	–
Distilled monoglycerides	2.0	–
Wheat gluten	3.5	–
Pasteurized egg yolk	9.5	–
Dry malt	1.0	–
Sourdough***	0–30.0	–
Commercial yeast****	0–3%	–
Water	38.0	–
Sugar	20.5	17.5
Vegetable fat	–	15.0
Salt	–	1.0
Sorbitol	–	0.15
Panettone essence	–	0.35

[a] b.f. = base flour, i.e., the weight of the ingredients was calculated based on the total weight of the wheat flour. ** Emulsifier DATEM = Diacetyl Tartaric Acid Ester of Mono-and Diglycerides. ***Sourdough microorganisms combinations: II: L. fermentum IAL 4531; III: *L. fermentum* IAL 4531 + *S. cerevisiae* commercial, IV: L. fermentum IAL 4531 + *W. anomalus* IAL 4533 and V: *L. fermentum* IAL 4531 + *S. cerevisiae* commercial + *W. anomalus* IAL 4533. ****For the traditional sponge preparation was used 3.0% of commercial instant yeast, 40% of refined wheat flour, and 60% of water were used.

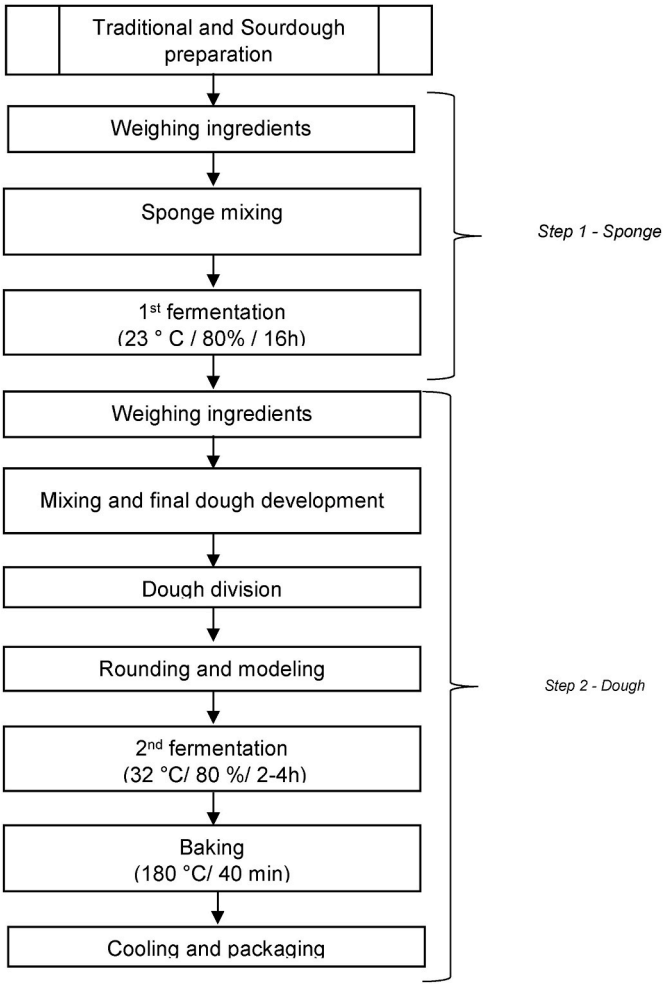

Fig. 1. Traditional and Sourdough panettone process steps.

under anaerobiosis. Yeasts were counted on acidified (pH 3.5; 10% tartaric acid) Potato Dextrose agar (PDA) (Difco, Detroit, Mich.) using a spread plate technique (Coda et al., 2011), incubated at 25 °C for 24–72 h.

2.3.2. Shelf life and mycological analysis of panettone

The panettones (F) were stored at 28 °C/75% relative humidity (RH) and monitored for up to 180 days after manufacture were monitored daily with a visual check done by the naked eye for the presence or absence of fungal colonies. The shelf life of the panettones was determined by the time elapsed from their manufacture to the visual observation of visible and apparent fungal growth (colonies with a size equal to or greater than 3 mm). The spreading plate method was chosen for analyses of moldy panettones. Briefly, 25 g of each sample was weighed and aseptically added to 225 mL of peptone water (0.1%). After homogenization, serial dilutions were carried out with subsequent surface inoculation in plates of Dichloran Glycerol Agar at 18% with chloramphenicol (DG18) and incubated at 25 °C for seven days. Results were expressed in colony-forming units per gram of product (CFU/g) (Pitt & Hocking, 2009). The colonies were isolated in Czapek Yeast Autolysate Extract Agar (CYA) and subsequently identified, according to each genus, following the incubation regimen (time, temperatures, and media) recommended for each fungal genus or section according to Klich and Pitt (1988); Frisvad and Samson (2004) and; Chen et al. (2017).

2.4. Sequencing

For the analysis and study of ecology and microbial diversity, sourdough samples were used as samples of the panettone dough during manufacture. Therefore, aliquots were obtained in five stages of the process, thus being described: (A) sourdough after 48 h; (B) sourdough after 96 h; (C) sourdough after 144 h; (D) dough of the first fermentation; (E): final dough after the second fermentation. In each stage, all treatments described in item 2.11 were evaluated (I, II, III, IV, or V). A resume of all samples is described in the supplementary material (Table S1).

2.4.1. DNA extraction

Sourdough samples were thawed in the refrigerator for 12 h. Samples (10 g) were homogenized with 90 mL of peptone saline solution (0.1% peptone + 0.85% NaCl) for 1 min. Each sample was divided into two falcon tubes (50 mL) and centrifuged at 1000×*g* for 5 min and then at 5000×*g* for 15 min for pellet formation (Bessmelteseva et al., 2014). The resulting pellet was resuspended in 200 μL of enzymatic lysis solution (Tris-HCl 20 mM pH 8.0. sodium EDTA 2 mM, Triton X-100 12 g/L, lysozyme 20 g/L) and incubated at 37 °C for 1 h. 0.3 g of glass beads (150–200 μm diameter) were added, and cells were broken (2 min shaking, 2 min pausing on ice, 2 min shaking) in a bead beater (TissueLyserII/QIAGEN). DNA was purified using the DNeasy® Blood & Tissue kit (Qiagen Cat nº 69504), according to the manufacturer's instructions. DNA quantity was assessed using Quant-iT™ PicoGreen® double-stranded DNA (dsDNA) reagent (Invitrogen, USA), following manufacturer's guidelines and in conjunction with a NanoDrop 2000c (Thermo-Fischer Scientific, Wilmington, MA, USA). The quality of DNA was checked by agarose gel electrophoresis. PCR analysis was performed according to the manufacturer (GoTaq Flexi Polymerase - Promega), and the gel order was: Sample Marker from 01 to 19 - Marker/Marker - Sample from 20 to 25 - Positive Control - Negative Control.

2.4.2. Amplicon library preparation and sequencing

The bacteria were identified via high-throughput sequencing of 16S rRNA V3/V4 region performed using a methodology with a proprietary protocol (Neoprospecta Microbiome Technologies, Brazil). The rRNA 16S V3/V4 region was amplified using the 341F (CCTACGGGRSGCAGCAG) (Wang & Qian, 2009) and 806R (GGACTACHVGGGTWTCTAAT)

(Caporaso et al., 2011) primers, with Illumina adapters, necessary for sequencing. For the amplification of the ITS1 was used ITS1 (GGAACCWGCGGARGGATCA) (Schmidt et al., 2013) and ITS2 (GCTGCGTTCTTCATCGATGC) primers (White et al., 1990). The amplification was performed in 35 cycles at 50 °C of annealing temperature, where each sample was amplified in triplicate. The 16S rRNA and ITS libraries were sequenced using the MiSeq Sequencing System (Illumina Inc., USA) with the V2 kit, 300 Cycles, and single-end sequencing with 100bp.

2.4.3. Bioinformatics analysis

The Quantitative Insights Into Microbial Ecology (QIIME) software version 1.9.0 (Caporaso et al., 2011) was used to perform bioinformatic analysis. Raw data were demultiplexed, and the initial sequences were checked for quality by FASTQC and subsequently filtered with Trimmomatic (0.36). The sequences were then processed using Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.0 software (Caporaso et al., 2010a). Operational Taxonomic Units (OTUs) were clustered at 97% identity using an open reference approach by the UCLUST algorithm (Edgar, 2010). The bacterial taxonomic annotation was performed against the Greengenes bacterial 16S rRNA database (13_8 release) and SILVA database. Also, the fungal taxonomic annotation of the rDNA ITS gene was performed using the RDP classifier. The detection and removal of chimeras were processed using UCHIME (Edgar et al., 2011). Representative sequences were aligned using PyNAST (Caporaso et al., 2010b). The alpha and beta diversity metrics were used to analyze the taxonomic composition during panettone production. The alpha diversity evaluation was done through QIIME to generate rarefaction curves, Good's coverage, Chao1 richness (Chao & Bunge, 2002), and Shannon & Simpson diversity indexes (Shannon, 1948). Raw sequence reads were submitted in the Sequence Read Archive of the National Center for Biotechnology Information (NCBI) under the Bio-Project ID PRJNA1013384 and PRJNA1013494.

After extensive studies using polyphasic approaches (Zheng et al., 2020), the former genus *Lactobacillus* was reclassified into 25 genera. However, according to the same authors, the generic term 'lactobacilli' remains useful to designate organisms in all 25 genera that used to be classified as *Lactobacillus* species and will be adopted in this manuscript.

2.5. Physicochemical analysis

2.5.1. Volatile compounds

The volatile compounds from sourdough after 144 h (C), final dough after second fermentation (E), and panettone (F) were extracted using the headspace solid-phase microextraction (HS-SPME) technique, as described in another study (Aponte et al., 2014), with modifications. Panettone, sourdough, and final dough samples (2.0 g) using different natural ferments were evaluated (according to Table S1). A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) 50/30 mm SPME fiber (Supelco Co., Bellefonte, PA, U.S.A.) was used to extract volatile constituents from headspace. The samples were placed in a 15 mL hermetically sealed flask and heated for 15 min at 40 °C to reach the sample headspace equilibrium. The SPME fiber exposition extracted the volatile compounds into the headspace for 30 min at 40 °C.

The compounds were analyzed using a Shimadzu GC model QP2010 equipped with mass spectrometry (MS) and a capillary column of silica Carbo-Wax 20M (30 m × 0.25 mm; 0.25 μm). The temperature program began with 3 min at 40 °C, followed by a gradient of 40 °C–240 °C at 5 °C/min; the temperature was then maintained at 240 °C for 10 min. The injector and detector temperatures were maintained at 230 °C. The carrier gas (He) was used at a 1.2 mL/min flow rate. Injections were performed by fiber exposition for 2 min. Volatile compounds were identified by comparing the mass spectra.

2.5.2. Organic acids

Organic acid determination was adapted from Zhao et al. (2006) and da Silva et al. (2014). Samples of sourdough after 144 h (C), final dough after the second fermentation (E), and panettone (F) (about 0.5 g) were homogenized with 2 mL of ultrapure water, and the pH was adjusted to 2–3 using an aqueous solution of 2 M HCl. Then, samples were centrifuged at 1700×*g* for 20 min. The supernatant was collected, and an internal standard, 2-ethyl-butyric acid (54.75 mM in 12% formic acid), was added to reach a final concentration of 1 mM. Samples were injected in an Agilent Technologies gas chromatograph (HP 6890 N) equipped with a capillary column Nukol TM (30 m × 0.25 mm; 0.25 μm) and flame ionization detector (FID). The injection port temperature was maintained at 100 °C, and the carrier gas was nitrogen (1 mL/min). After injection (0.5 μL, split ratio 10:1), the oven temperature was heated from 50 °C to 100 °C at 6 °C/min. It was held for 3 min, increased to 180 °C at 8 °C/min, and maintained at this temperature for 2 min, and then the temperature was increased to 200 °C at 50 °C/min and maintained at 200 °C for 1 min. A standard mix of volatile-free fatty acids (46975-U), sorbic acid (47845), and DL-3-phenyl lactic acid (P7251) was obtained from Sigma-Aldrich (St. Louis, MO, USA) diluted in a solution of formic acid (12%) and used as external standard.

2.6. Statistical analysis

Data were submitted to analysis of variance (ANOVA) using Statistica software version 7.0 (2004, Statsoft Inc., Tulsa, OK, USA). Means were compared using Tukey's test, considering a significance level of 5% ($p < 0.05$). All data were performed in triplicate. Results were expressed as mean ± standard deviation. Multivariate statistics (hierarchical cluster and principal component analysis) were performed in PAST software (vs 3.26).

3. Results and discussion

3.1. Microbiological analysis and shelf life of panettone

The values of the microbiological analyses carried out in the

Table 3
Microbiological counts for lactic acid bacteria (LAB) and yeasts (Log CFU/g) performed after 48h (A), 96h (B) and 144h (C) for sourdough sponge and fermented dough (D, E) of panettone production steps.

Lactobacilli (Log CFU/g)							
	I	II	III	IV	V	SEM	Sig
A	8.13^{bB}	9.25^{aA}	9.55^{aA}	9.39^{aA}	9.16^{aA}	0.261	***
B	7.00^{cCD}	9.26^{aA}	8.84^{abA}	6.52^{cD}	7.53^{bcB}	0.459	***
C	6.45^{bD}	7.22^{bB}	7.00^{bB}	6.96^{bC}	8.25^{aAB}	0.341	***
D	7.53^{cBC}	8.95^{aA}	5.90^{dC}	7.79^{cB}	8.35^{bAB}	0.184	***
E	9.18^{aA}	7.95^{cB}	7.67^{cB}	9.53^{aA}	8.55^{bAB}		
SEM	0.225	0.307	0.278	0.127	0.458		
Sig	***	***	***	***	**		
Yeasts (Log CFU/g)							
	I	II	III	IV	V	SEM	Sig
A	8.86^{aA}	8.80^{aA}	8.43^{abA}	8.20^{bAB}	8.20^{bB}	0.167	**
B	8.58^{abA}	8.97^{aA}	8.47^{cA}	8.10^{cBC}	8.36^{cB}	0.148	***
C	8.68^{abA}	8.98^{aA}	8.42^{bAB}	8.26^{bA}	8.63^{abA}	0.132	***
D	8.70^{abA}	8.84^{aA}	8.29^{bcBC}	8.05^{cC}	8.38^{bcB}	0.138	***
E	8.62^{abA}	8.92^{aA}	8.22^{bcC}	8.09^{cBC}	8.35^{bcB}	0.141	***
SEM	0.283	0.158	0.046	0.050	0.067		
Sig	n.s.	n.s.	***	***	***		

a-d line
A-D column.
*$P < 0.05$.
*** $P < 0.001$.
** $P < 0.01$.
[a] Traditional and sourdough sponge using microorganisms' combinations: I: *S. cerevisiae* commercial; II: *L. fermentum* IAL 4531; III: *L. fermentum* IAL 4531 + *S. cerevisiae* commercial, IV: *L. fermentum* IAL 4531 + *W. anomalus* IAL 4533 and V: *L. fermentum* IAL 4531 + *S. cerevisiae* commercial + *W. anomalus* IAL 4533.

sourdough during its manufacture and in the later stages are shown in Table 3. Regarding the microbiological count for LAB, it was observed a significant difference among the treatments of the panettone production steps (lines) and also between treatments (inoculum starters) in the same stage of the processes (columns) ($p < 0.001$; $p < 0.01$) (Table 3). The counts are expected to be very similar to the concentration of the inoculum added at the beginning of the process. At the time of panettone production, these values have been increasing and decreasing, depending on the inherent metabolism of each type of microorganism in each treatment. It was possible to observe a reduction in treatments I (commercial yeast), IV (*L. fermentum* IAL 4531 + *W. anomalus* IAL 4533) and V: (*L. fermentum* IAL 4531 + commercial yeast + *W. anomalus*) IAL 4533 in the first hours of the fermentation process (Step B - Sourdough after 96 h) for the microbiological count of LAB (Table 3). No significant variation in treatments I (commercial *S. cerevisiae*) and II (*L. fermentum* IAL 4531) between treatments and along the steps for panettone production (n.s) (column x line) was observed for the microbiological count of yeasts (Table 3). Also, in step C (Sourdough after 144 h), significant variation was not observed compared to other treatments (n.s). On the other hand, a significant reduction was observed in the first hours of the panettone production in treatment IV ($p < 0.001$). Also, in this treatment, between steps A to B (Sourdough after 96 h), there was a reduction of the microbiological count of yeast (from 8.20 to 8.10 CFU/g) (Table 3). In addition, an increase in the count for yeast in step C was observed (8.26 CFU/g), followed by a significant reduction in step D (8.05 CFU/g) ($p < 0.001$). There was a significant correlation between both counts in treatments II, III (*L. fermentum* IAL 4531 and commercial *S. cerevisiae* yeast), and V (−0.56, 0.55, and −0.44, respectively) for LAB and yeast counts.

The relative abundance of yeasts during all phases of panettone production may be related to the fact that this microorganism establishes a non-competitive and stable association with the LAB present (Venturi et al., 2021). Microbial populations can establish a non-competitive and stable association based on competition for substrate if population growth rates are similar and if fermentable carbohydrates remain available at all stages of production. It is still necessary to consider the incubation interval and temperature in the propagation steps to obtain reasonable rates between LAB and yeasts (Galli et al., 2019). The LAB dominates in the sourdough environment due to its high metabolic versatility and adaptation abilities in diverse stress conditions. Their metabolism interacts with autochthonous and added yeasts following a proto-cooperation mechanism during sourdough fermentation (Gänzle & Gobbetti, 2013).

All prepared panettones presented quality parameters within the acceptable range, such as specific volume (2.88–3.25 cm/g^3) analyzed 2 h after the panettone production, and follow-up during 140 days to pH (3.90–5.13), total titratable acidity (TTA) (1.9–4.1 mL NaOH), water activity (0.77–0.85), instrumental firmness (3.27–12.73 N), and L color parameter (74.90–77.79). These complementary data were previously published in the Stefanello et al. (2019).

Regarding the shelf life of panettone, the first perception of fungal spoilage occurred after at least 55 days, and some batches remained stable even after 126 days. The level of fungal counts (average, expressed in CFU/g) in moldy panettone samples ranged from 10^4 (II) to 10^7 (V) CFU/g (Table 4). The minimal fungal count was recovered from spoiled panettone when *Saccharomycopsis fibuligera* or *Cladosporium* sp. was the sole spoilage microorganism isolated. Regarding diversity, at least 11 species were present, and xerophilic Aspergilli were predominant. No toxigenic species of relevance were present in the spoiled panettones. Most of the species deteriorating panettones have also been found in a diversity of spoiled bread in Brazil (Garcia et al., 2019). However, in that study, the predominant spoiler was the sorbic and propionic acid tolerant species *Penicillium roqueforti* (Moro et al., 2022) and *Hyphopicchia burtonii*, both absent from panettones.

The panettones produced using a starter with the sole addition of the lactic acid bacterium *L. fermentum* (II) and the ones made with a starter containing a combination of three different microorganisms (*L. fermentum; W. anomalus* and *S. cerevisiae* commercial) (V) were the most resistant to fungal deterioration (Table 4). In comparison, the panettones produced with a starter solely composed of the yeast *S. cerevisiae* commercial (I) and the ones added of the starter containing *S. cerevisiae* commercial and *L. fermentum* (III) had the shortest shelf life.

Table 4
Fungi isolated and their counts when colonies became visible on the surface of spoiled panettones using sourdough with five microorganisms' combinations[a].

Treatment	Fungal counts (CFU/g)	Shelf life (days)	Isolated fungi (in order of appearance)
I	3.05×10^6	65	*Talaromyces* sp., *Cladosporium* sp., *Aspergillus montevidensis, Aspergillus chevalieri, Wallemia sebi, Saccharomycopsis fibuligera*
II	6.00×10^4	184	*Cladosporium* sp., *Aspergillus chevalieri, Talaromyces* sp., *Saccharomycopsis fibuligera, Aspergillus montevidensis.*
III	1.05×10^6	64	*Aspergillus chevalieri, Cladosporium* sp., *Saccharomycopsis fibuligera, Wallemia sebi, Aspergillus versicolor, Talaromyces* sp., *Aspergillus sydowii*
IV	2.30×10^5	124	*Aspergillus nidulans, Penicillium fellutanum, Aspergillus chevalieri, Cladosporium* sp., *Wallemia sebi.*
V	1.10×10^7	185	*Aspergillus chevalieri, Aspergillus versicolor, Cladosporium* sp., *Aspergillus restrictus, Aspergillus montevidensis*

[a] I: *S. cerevisiae* comercial, II: *L. fermentum* IAL 4531; III: *L. fermentum* IAL 4531 + *S. cerevisiae* commercial; IV: *L. fermentum* IAL 4531 + *W. anomalus* IAL 4533 and V: *L. fermentum* IAL 4531 + *S. cerevisiae* commercial + *W. anomalus* IAL 4533.

The stability conferred by sourdough fermentation against fungi, the most relevant microbial deteriorative group of bread, can be attributed to a variety of compounds, including biologically active low-molecular-mass compounds, bacteriocins, and organic acids (such as lactic, acetic, caproic, formic, propionic, and butyric) produced during fermentation, as well as the pH decrease induced by them (Hassan et al., 2016). These later compounds will be discussed later.

3.2. 16S rRNA and ITS amplicon sequencing

The average number of OTUs was 57.0–192 for the 16S rRNA gene. Also, the average was 1.0–3650 for the ITS gene for all the samples (Tables S2 and S3). The high Shannon index values were found in I(B), V (D), and V(E) samples for the 16S rRNA gene, also found in V(D), V(E), and IV(C) samples for ITS gene amplicon sequencing (Tables S2 and S3). Regarding taxonomic affiliation of the 16S rRNA gene amplicon sequence, it was observed that the main bacteria found in samples of all experimental periods were lactobacilli, presented in high relative abundance (from 61.5% to 99.8%) (Fig. 2A). Sample I(B) has shown a lower abundance of lactobacilli (61.5%); on the other hand, the prevalence of *Pediococcus* genera (35%) was observed. In sample I(A), there is more diversity of genera, such as *Clostridium* (8.1%), *Enterobacter* (2.7%), *Pediococcus* (1.7%), *Bacillus* (1.6%), *Citrobacter* (1.4%). Also, sample III(A) showed the considered presence of *Enterobacter* (3.9%) and *Citrobacter* (1.8%) (Fig. 2A).

When the taxonomic affiliation of the ITS gene amplicon sequence was examined, it was observed that the main genera found in high prevalence in samples of all experimental periods were *Aspergillus* sp. (from 7.1% to 99.8%) (Fig. 2B). *Wickerhamomyces* sp. was found in high abundance in samples V(A), II(E), V(B), II(B), IV(A) and II(C) (58%, 25%, 24.6%, 15.1%, 9.9% and 9.7%, respectively). On the other hand, *Saccharomyces* was found in high prevalence in samples I(E), III(D), V (C), II(C), I(C) and III (A) (23.8%, 11.8%, 10%, 9.3%, 8.9% and 4.2% respectively). The presence of Saccharomycetales order was also

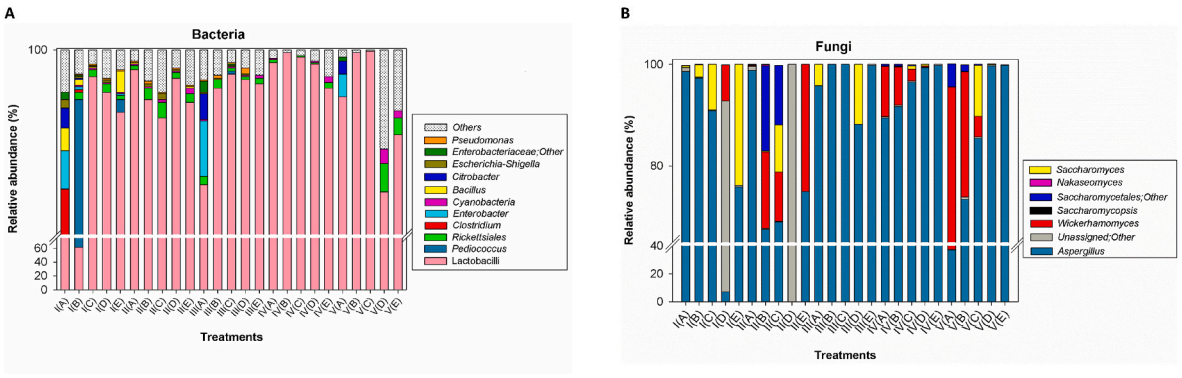

Fig. 2. Relative abundance of bacterial genera inferred from 16S rRNA and fungal genera inferred from ITS gene amplicon sequencing in the group with different combinations of start cultures (I to V) at different stages of the panettone processing line.

observed in samples II (B) and II (C) (16.7% and 11.7%, respectively) (Fig. 2B). Similarly, the *Saccharomyces* and *Wickerhamomyces* could be related to establishing a symbiotic association with LAB (Venturi et al., 2012). The population growth rates are similar if fermentable carbohydrates are available in all production steps. Also, the incubation period and temperature should be considered to obtain good growth rates between LAB and yeast (Venturi et al., 2012).

3.3. Volatile compounds

A total of 59 volatile compounds were identified in panettone, sourdough, and final dough samples from the 5 treatments with the different microorganisms by comparing their mass spectrometry with those standards of mass spectrometry stored in the GC-MS database. These compounds were classified as acids, alcohols, aldehydes, ketones, esters, and others, as shown in Table S4. Of both sample treatments, 17 and 14 volatile compounds were identified in sourdough (X^C) and panettone (X^F). Most of the compounds observed in all samples were alcohols and acids.

Both treatments showed close amounts of volatile compounds; however, treatment II presented a higher formation of volatile organic compounds in panettone, while treatment I presented the lowest formation according to Table S4. 11 compounds were found only in panettone, whereas 6 in sourdough. The alcohol 2-furan-methanol and aldehyde 2-furan carboxaldehyde, 5-methyl- were found in all panettone samples. These furan derivatives are commonly formed during thermal food production processes (baking, frying, roasting, and cooking) (Ozolina et al., 2011). According to these authors, the furfural is described as a pungent, but sweet, bread-like, caramelic, cinnamon-almond-like odor of poor tenacity. T.

A hierarchical cluster analysis was performed with the principal compounds found (n = 26), according to Fig. S1. Of these compounds, the following stood out: acids (7.7%), alcohols (23.1%), aldehydes and ketones (34.6%), and esters (34.6%). According to Fig. 3, there was a well-defined separation between the groups according to the steps along the process: before (E, C) and after cooking the panettone (F). The treatment I (containing only *S. cerevisiae* as initial inoculum) had the lowest diversity of these compounds (n = 13) in the final product (F). In contrast, the other treatments, II, III, IV, and V, containing *L. fermentum* in their composition, had the highest number of VOCs: 18, 15, 15, and 16, respectively.

A principal component analysis was performed to visualize better the correlation between the compounds present in the different treatments at each stage of the process. Fig. 4 showed that the compounds present in the sponge fermented for 144 h (treatment C), which stood out the most, were alcohols (1-butanol, 3-methyl-, 1-hexanol, phenyl ethyl alcohol, and ethanol) and some esters and acids (hexadecanoic acid, ethyl ester, dodecanoic acid).

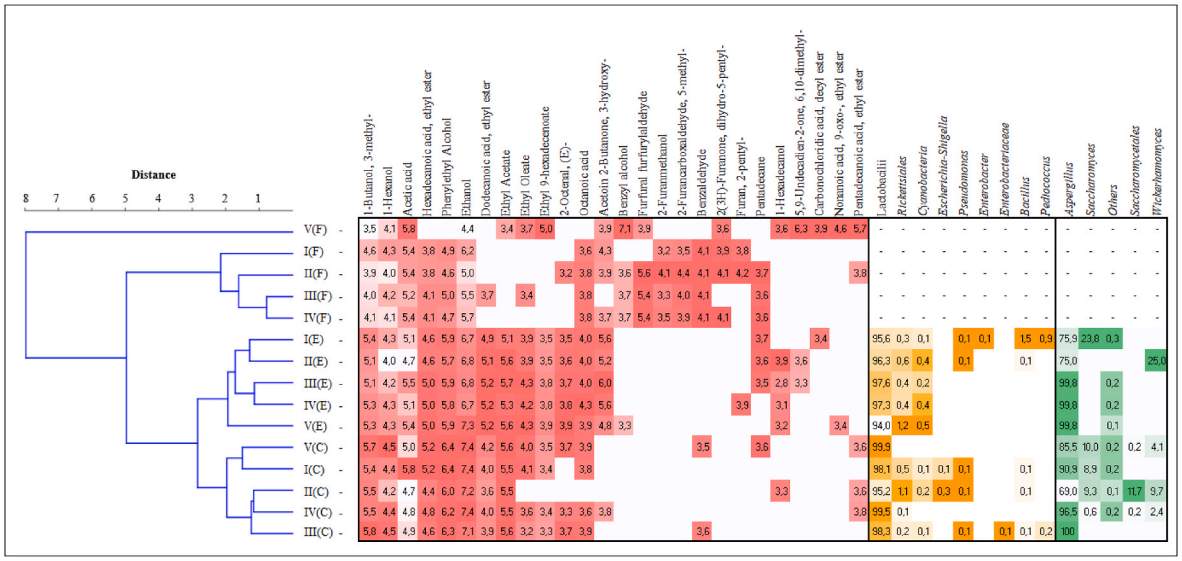

Fig. 3. Dendrogram resulting from the hierarchical cluster analysis (HCA) to group different combinations of starter cultures (I to V) at different stages of the fermentation process (C, E) and the final product (F), based on their similarity regarded to volatile organic compounds found. All sample cod s can be visualized in the supplementary material (Table S1).

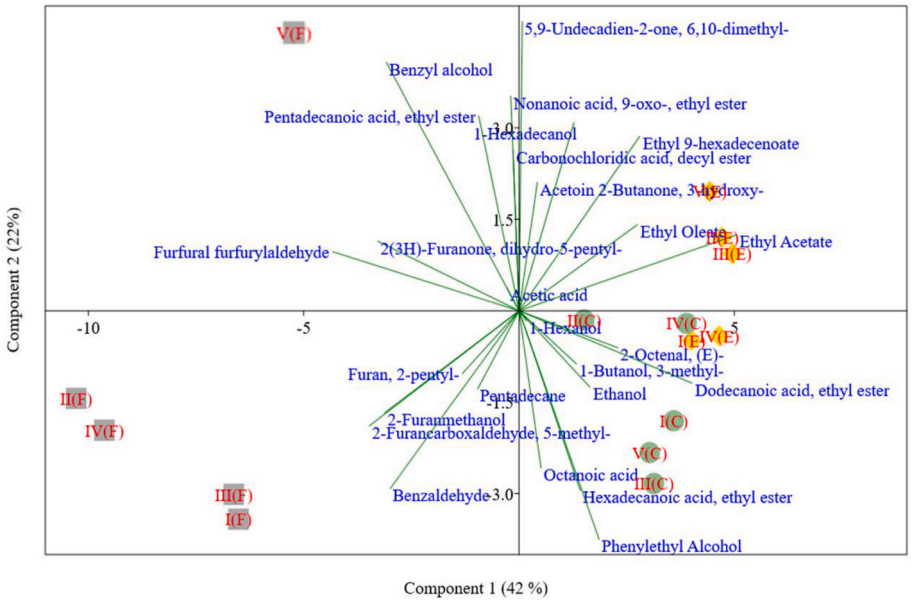

Fig. 4. Principal component analysis (PCA) score graph of volatile organic compounds obtained from five sourdough fermentation starter combinations, at different stages of the panettone processing line. C: Sourdough after 144 h; E: Final dough after second fermentation; F: Panettone. I: *Saccharomyces cerevisiae*; II: *Limosilactobacillus fermentum*; III: *Limosilactobacillus fermentum* + *Saccharomyces cerevisiae*; IV: *Limosilactobacillus fermentum* + *Wickerhamomyces anomalus*; V: *Limosilactobacillus fermentum* + *Wickerhamomyces anomalus* + *Saccharomyces cerevisiae*.

In the fermented dough after 4 h (treatment E), there is a more significant correlation with the following esters, aldehydes, and ketones: hexadecanoic acid, ethyl ester, dodecanoic acid, ethyl ester, ethyl acetate, ethyl oleate, ethyl 9-hexadecenoate, 2 -octenal, (E)-, octanoic acid, acetoin 2-butanone, 3-hydroxy-. In the final product (treatment F), there was a more significant correlation with the following furfural compounds, alcohols, aldehydes, and ketones: 2-furan-methanol, 2-furancarboxaldehyde, 5-methyl-, benzaldehyde, furan, 2-pentyl-, and pentadecane, for treatments I-IV.

The panettone elaborated with the combination of *L. fermentum*, *S. cerevisiae* commercial, and *W. anomalus* (treatment V), showed a more significant correlation with the following compounds: Acetic acid, Ethyl 9-hexadecenoate, Benzyl alcohol, 2(3H)-Furanone, dihydro-5-pentyl -, 1-Hexadecanol, 5,9-Undecadien-2-one, 6,10-dimethyl-, Carbonochloridic acid, decyl ester, Nonanoic acid, 9-oxo-, ethyl ester, Pentadecanoic acid, ethyl ester. The traditional sponge (I) and different sourdough cultures tested (treatments II to V) considerably affect the relative abundance of the VOCs present in the samples after baking, in agreement with Lutter et al. (2023).

Several factors are associated with the diversity of flavor in bread and baked goods, such as raw material, the extent of dough fermentation, cooking time, microbiota diversity of LAB and yeasts, etc. Among the main aromatic compounds present in flour and flour blends, we can highlight terpenes, carboxylic acids, aliphatic aldehydes, alcohols (e.g., pentanal and hexanal, formed as fatty acid oxidation products), alkanes, and esters (Warburton et al., 2022), as found in this paper. These compounds originate primarily from fermentation, lipid oxidation, and Maillard reactions by sourdough microorganisms and enzymatic processes during fermentation, as described by Lutter et al. (2023).

3.4. Organic acids

The most abundant organic acids found in samples were acetic, propionic, and phenyllactic acids (Table 5), which are compounds with antifungal properties. The content of acetic acid decreased during the production of panettone (Table 5). For all treatments, except number I, the final dough (E) and panettone (F) had lower acetic acid content than the sourdough after 144 h (C) ($p < 0.05$). This difference can be both due to changes in the microbial ecology and the addition and mixing of ingredients, which can dilute the acetic acid content and allow their volatilization. Besides that, acetic acid volatilization during panettone baking could reduce levels of this acid in the final panettone (F).

In addition, acetic acid content in the panettones was significantly ($p < 0.05$) affected by the type of microorganism used to prepare sourdough. Treatment III yielded the highest levels of acetic acid in panettone samples, whereas treatment IV yielded the lowest level, and the other treatments yielded intermediate levels ($p < 0.05$). Despite differences observed among panettone samples, the treatment did not affect the acetic acid content in the sourdough after 144 h or in the final dough ($p < 0.05$).

The propionic acid content was higher in the final dough than the sourdough after 144 h for treatments IV and V ($p < 0.05$). In addition, treatment significantly affected propionic acid content in some sourdoughs after 144 h: treatment I > IV and V ($p < 0.05$). No difference in propionic acid concentration was observed among the final doughs (E) and respective panettones (F).

Although phenyllactic acid was the most abundant organic acid in panettone samples and had an essential contribution to the organic acid content of sourdough and dough, its content did not significantly change during the steps of panettone preparation or among the different treatments. This phenomenon probably occurred due to the more significant variability in the content of phenyllactic acid among samples.

The content detected of isobutyric, butyric, isovaleric, valeric, isocaproic, caproic, and heptanoic acids was low (<0.1 mM/kg). Sorbic acid was not detected in all the samples evaluated. It is essential to mention that the antifungal properties of these organic acids depend on the substrate pH since it is related to their undissociated form (Alcano et al., 2016). So, the final pH of panettones also significantly influences the product stability.

The sensitivity of some bread spoilage species (*P. roqueforti, Penicillium paneum, H. burtonii, Aspergillus montevidensis*, and *Aspergillus pseudoglaucus*) to some organic acids with antifungal properties (acetic and propionic) detected in this study was evaluated by Moro et al. (2022). The minimal inhibitory concentration (MIC) of the undissociated form (showing antifungal properties) of acetic acid was between 40 and 90 mM/L for *P. roqueforti, P. panicum,* 40 to 60 for *H. burtonii* and 20 to 30

Table 5
Content of organic acids (mmol/kg of sample) monitoring during the panettone processing steps: using traditional and sourdough sponge with five micro-organims combinations.

Treatments							
	I	II	III	IV	V	SEM	Sig
Acetic acid							
C	24.92^{aA}	33.76^{aA}	34.40^{aA}	32.14^{aA}	26.70^{aA}	7.31	n.s.
E	8.50^{aA}	10.92^{aB}	6.14^{aB}	11.62^{aB}	6.76^{aB}	2.04	n.s.
F	3.47^{abA}	6.16^{abB}	8.85^{aB}	1.17^{bC}	2.14^{abB}	2.22	**
SEM	7.89	2.98	3.84	2.80	3.22		
Sig	n.s.	***	***	***	***		
Propionic acid							
C	7.12^{aA}	3.69^{abA}	2.16^{abA}	2.02^{bB}	2.02^{bB}	1.51	*
E	2.73^{aA}	3.28^{aA}	3.11^{aA}	5.60^{aA}	5.20^{aA}	1.12	n.s.
F	4.25^{aA}	4.20^{aA}	3.90^{aA}	3.45^{aAB}	3.45^{aAB}	0.92	n.s.
SEM	1.87	0.96	1.16	0.74	1.00		
Sig	n.s.	n.s.	n.s.	*	*		
Isobutyric acid							
C	n.d.^{aA}	0.016^{aA}	0.033^{aA}	0.010^{aA}	0.026^{aA}	0.012	n.s.
E	0.030^{aA}	0.016^{aA}	0.043^{aA}	0.046^{aA}	n.d.^{aA}	0.021	n.s.
F	0.056^{aA}	0.023^{aA}	0.016^{aA}	0.013^{aA}	0.010^{aA}	0.029	n.s.
SEM	0.037	0.014	0.010	0.024	0.014		
Sig	n.s.	n.s.	n.s.	n.s.	n.s.		
Butyric acid							
C	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	0.017	n.s.
E	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	0.03^{aA}	0.04^{aA}	0.019	n.s.
F	0.03^{aA}	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	0.018	n.s.
SEM	0.024	0.022	0.023	0.014	0.021		
Sig	n.s.	n.s.	n.s.	n.s.	n.s.		
Isovaleric acid							
C	0.09^{abA}	0.08^{abA}	0.12^{aA}	0.03^{cB}	0.05^{bcA}	0.013	**
E	0.05^{bA}	0.09^{abA}	0.06^{abA}	0.09^{abAB}	0.10^{aA}	0.011	*
F	0.08^{aA}	0.08^{aA}	0.07^{aA}	0.10^{aA}	0.05^{aA}	0.022	n.s.
SEM	0.017	0.013	0.016	0.022	0.009		
Sig	n.s.	n.s.	n.s.	*	*		
Valeric acid							
C	0.02^{abA}	0.04^{aA}	n.d.^{bA}	n.d.^{bA}	0.02^{abAB}	0.008	*
E	0.01^{bA}	n.d.^{bB}	n.d.^{bA}	n.d.^{bA}	0.04^{aA}	0.006	***
F	0.02^{aA}	0.01^{aB}	n.d.^{aA}	n.d.^{bA}	n.d.^{aB}	0.016	n.s.
SEM	0.021	0.009	0.009	0.009	0.008		
Sig	n.s.	*	n.s.	n.s.	**		
Isocaproic acid							
C	n.d.^{aA}	n.d.^{aA}	0.01^{aA}	n.d.^{aA}	0.01^{aA}	0.006	n.s.
E	0.01^{aA}	n.d.^{aA}	n.d.^{aA}	0.03^{aA}	0.02^{aA}	0.001	n.s.
F	0.11^{aA}	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	0.01^{aA}	0.044	n.s.
SEM	0.057	0.002	0.006	0.016	0.013		
Sig	n.s.	n.s.	n.s.	n.s.	n.s.		
Caproic acid							
C	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	0.01^{aA}	0.005	n.s.
E	0.02^{aA}	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	0.01^{aA}	0.005	n.s
F	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	n.d.^{aA}	0.011	n.s
SEM	0.012	0.011	0.010	0.009	0.012		
Sig	n.s.	n.s.	n.s.	n.s.			
Heptanoic acid							
C	0.06^{aA}	0.05^{aB}	0.06^{aA}	0.07^{aA}	0.07^{aA}	0.008	n.s.
E	0.06^{bA}	0.10^{abA}	0.06^{bA}	0.13^{aA}	0.11^{abA}	0.015	*
F	0.08^{aA}	0.05^{aB}	0.07^{aA}	0.06^{aA}	0.08^{aA}	0.029	n.s.
SEM	0.029	0.008	0.017	0.024	0.016		
Sig	n.s.	*	n.s.	n.s.	n.s.		
Phenyl lactic acid							
C	3.41^{aA}	12.72^{aA}	6.43^{aA}	19.01^{aA}	10.48^{aA}	5.76	n.s.
E	1.98^{aA}	2.35^{aA}	1.09^{aA}	19.06^{aA}	7.51^{aA}	7.08	n.s.
F	4,98^{aA}	10.41^{aA}	6.10^{aA}	6.29^{aA}	7.71^{aA}	7.86	n.s.
SEM	6.97	7.81	2.66	9.17	6.41		
Sig	n.s.	n.s.	n.s.	n.s.	n.s.		

$^{a-b}$ Superscript indicates differences within the same line.
$^{A-C}$ Superscript indicates differences within the same column. n.d.: not detected. n.s.: not significant. ***P < 0.001. **P < 0.01. *P < 0.05.
I: *S. cerevisiae* commercial; II: *L. fermentum* IAL 4531; III: *L. fermentum* IAL 4531 + *S. cerevisiae* commercial; IV: *L. fermentum* IAL 4531 + *W. anomalus* IAL 4533 and V: *L. fermentum* IAL 4531 + *S. cerevisiae* commercial + *W. anomalus* IAL 4533.
Santa Maria, March 04th, 2024.

for *A. montevidensis* and *A. pseudoglaucus*. Regarding propionic acid, it was between 35 and 45 mM for the tested Penicillia and between 18 and 22 mM for the tested Aspergilli and *H. burtonii*. Similarly, Debonne et al. (2020) also evaluated the activity of phenyllactic acid and acetic acid, two of the most common organic acids found in our study, against some bread-spoiling fungal species (*P. paneum* and *Aspergillus niger*). The authors concluded that the MIC of the undissociated form of phenyllactic acid was 39–84 mmol/L, while for acetic acid, the MIC was 110–169 mmol/L. The inhibitory values reported by both authors (Debonne et al., 2020; Moro et al., 2022) are much higher than those detected in the different stages of production of our panettones and alone cannot explain differences in their shelf life. Other factors acting together, such as possible metabolites also produced by yeasts, could explain the stability obtained by sourdough bread.

4. Conclusions

In all treatments, LAB and yeast counts demonstrated a mechanism of proto-cooperation with a significant correlation between both counts in the treatments containing at least *L. fermentum* and *S. cerevisiae*. Panettones containing L. *fermentum* or *W. anomalus* in their composition (treatments II, IV, and V) had longer shelf life, which could indicate their effect as potential biopreservatives to be applied in the bakery industry. The genetic sequencing data demonstrated the dominance of the lactobacilli along sourdough and final dough fermentations, corroborating the data obtained by cultivable methods. High amounts of volatile organic compounds (VOCs) were found, primarily acids (7.7%), alcohols (23.1%), aldehydes and ketones (34.6%), and esters (34.6%). The treatments containing *L. fermentum* have shown a greater diversity of VOCs. This study demonstrates the feasibility of applying endogenous starter cultures from sourdoughs, with great biopreservative activity, enabling the production of healthier and more natural panettones. In-depth studies of sourdough biodiversity and population dynamics occurring during sourdough fermentation are fundamental for controlling the leavening process and manufacturing standardized, natural, and high-quality products, as found in this study.

CRediT authorship contribution statement

Raquel F. Stefanello: Writing – original draft, Investigation, Formal analysis, Data curation. **Leonardo F. Vilela:** Methodology, Investigation, Formal analysis. **Larissa P. Margalho:** Writing – original draft, Conceptualization. **Elizabeth H. Nabeshima:** Writing – review & editing, Supervision, Resources, Methodology, Investigation. **Cleverson C. Matiolli:** Formal analysis. **Dariane Trivisiol da Silva:** Formal analysis, Data curation. **Rosane F. Schwan:** Supervision. **Tatiana Emanuelli:** Supervision. **Melline F. Noronha:** Software, Methodology. **Lucélia Cabral:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Anderson S. Sant'Ana:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Marina V. Copetti:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

I wish to confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

I confirm that all named authors have approved the manuscript and that no other persons satisfied the authorship criteria but are not listed.

I understand that the Corresponding Author is the sole contact for the Editorial process (including the Editorial Manager and direct communications with the office).

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.fbio.2024.104279.

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