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Microbial characterization of discarded breads

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ABSTRACT

Bread is one of the most wasted products in distribution systems and in households. To consider reusing it, the presence of microorganisms must be taken into account. In this study, the microbial load of crumb and crust of four kinds of bread, sold in two different types of establishments, has been compared separately. Counts of *Bacillus* spp, sulphite reducing bacteria, coliforms, *E. coli*, *Salmonella* spp, lactic acid bacteria, Aerobic plate count, molds, and yeasts were determined. The microorganisms found were identified. The microbial load of discarded breads was similar to that of cereal flours found in other studies. In general, a greater presence of bacteria in breads from small stores than in those from supermarkets has been found. Some of the least sold breads in small bakeries, such as wholemeal or candeal, presented a higher microbial load. Although their quantity or type are not a concern for people's health, good practices must be implemented to reduce health risks in products made from discarded bread.

1. Introduction

Roughly, one-third of the food produced worldwide is wasted. In the European Union (EU), this value amounts to a loss of approximately 143 billion euros per year (Tonini, Albizzati, & Astrup, 2018). In the last 14 years, the EU has been prioritizing the sustainability of food systems and circular economy to reduce the environmental impact of waste. It recommends the reuse of all waste suitable for human consumption and its reintroduction into the food chain (European Union, 2008). Bread is usually the most wasted food item, after fruits and vegetables, in the food chain distribution (FAO, 2013). Both drying and starch retrogradation are partly responsible for their changes in texture and short shelf life (Fadda, Sanguinetti, Del Caro, Collar, & Piga, 2014). Some breads rich in fats and oils, higher moisture and longer shelf life and microbial growth can also be a problem. Several modes of action focused on the use of ingredients, additives, enzymes, other processes, or the packaging material, have been proposed to reduce or minimalize this problem (Axel, Zannini, & Arendt, 2017; Dong & Karboune, 2021).

In households, stale bread is often ground and used in several culinary recipes. Considering that bread is wasted throughout the food supply chain, the industry sector has come up with several options for its reuse, such as the preparation of sourdoughs (Gélinas, McKinnon, & Pelletier, 1999), cookies (Guerra-Oliveira, Belorio, & Gómez, 2021), cakes (Guerra-Oliveira, Belorio, & Gómez, 2022), snacks (Samray, Masatcioglu, & Koksel, 2019), and beer (Brancoli, Bolton, & Eriksson, 2020).

Bread is a baked product. The bakery process destroys practically all the microorganisms present in the dough and, consequently, the microbial load is very low or null. However, bread can recontaminate with different microorganisms, after leaving the oven. Bread nutrients, such as starch and proteins, could facilitate microbial growth when conditions are adequate. Therefore, the main solutions to reuse bread are based on anaerobic digestion (Narisetty et al., 2021). Bread has different types of microorganisms according to cooling, transport, and storage conditions (Alpers et al., 2021; Smith, Daifas, El-Khoury, Koukoutsis, & El-Khoury, 2004).

Excessive contamination could be a problem for reusing wasted bread for human consumption. In addition to the number of microorganisms, their typology can greatly affect possible fermentation processes, as in the preparation of sourdoughs (De Vuyst, Comasio, & Kerrebroeck, 2021). Some researchers have analyzed the physicochemical properties of bread flours (Fernández-Peláez, Guerra, Gallego, & Gómez, 2021), others their mycotoxin content (Saladino et al., 2017), which can come from microbial contamination after or before baking, and still others the microbiological quality of cereal flours (Cardoso et al., 2019). Nevertheless, there are no studies on the microbiology quality of commercial breads.

The reuse of bread flour as a functional ingredient in food production

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depends on the type of bread, knowledge of the physicochemical and microbiological characteristics of bread is crucial to find the appropriate use for developing or creating new products with a market value, for instance, healthy, safe, and sustainable products.

In this study, four types of bread commercialized in Valladolid (Spain) have been selected: white wheat loaf, whole wheat loaf, *candeal* bread, and sliced bread. Samples were collected on two different days and from four different types of establishments, two local bakeries (artisan) and two supermarkets (industrial). Crumbs and crusts were analyzed separately. Counts of *Bacillus* spp, sulphite reducing bacteria, coliforms, *E. coli*, *Salmonella* spp, lactic acid bacteria (LAB), Aerobic plate count (APC), molds, and yeasts were determined. Finally, moisture, aw, pH and sodium chloride content were also analyzed.

2. Materials and methods

2.1. Bread samples

Fifty-eight samples from four artisan bakeries and four industrial bakeries were analyzed: white wheat loaf, whole wheat loaf, *candeal* bread, and sliced bread were picked up from two local bakeries and two supermarkets twice on two different days, from September to December 2021. The loaves were collected less than 24 h before being discarded. Samples were carried in sterile bags to the laboratory within 2 h. Crust and crumb were separated from each sample using a sterile knife. Crust and crumb samples were cut into 2 cm^2 pieces.

2.2. Microbiological analysis

Samples were tested upon receipt at the laboratory. Fifty-five grams of each sample was homogenized with 225 mL of 0.1% buffered peptone water (VWR Chemical, Leuven, Belgium) and stomached for 3 min using a Stomacher blender (Seward). Then appropriate dilutions were plated in specified media according to the type of microorganism. Appropriate dilutions were analyzed in duplicate.

Bacillus spp counts: 0.1 mL of each appropriate dilution were plated onto Polymixin-Egg Yolk-Mannitol-Bromothymol-Blue Agar (PEMBA; Merck, Darmstadt, Germany) according to Eglezos (2010) and Tallent, Knolhoff, Rhodehamel, Harmon, and Bennett (2020). Plates were incubated at 37 °C for 24 h. Count of colonies was performed according to International Organization for Standardization (ISO) 4832:2006. The limit of quantification (LOQ) was equal to 2 Log UFC/g.

Sulphite reducing bacteria counts: 5 mL of each suspension was transferred to a 50 mL, falcon sterile tube and further heat-treated in a water bath at 80 °C for 10 min. The suspensions were immediately cooled in an ice bath and 25 mL of Sulfite Iron Agar Base (Merck; ISA) and Iron (II) Sulfate hepta-hydrate (purity 98%, VWR Chemical, Darmstadt, Germany) was added. The mixture was homogenized and allowed to solidify. Afterwards, 5 mL of ISA medium was added to create anaerobiosis (LOQ = 2 UFC/g). The Falcon tubes were incubated at 30 °C for 48 h and black spots were counted, according to the ISO 15213:2003 (ISO15213, 2003).

Coliforms y *E. coli*: 1 mL of each appropriate dilution was plated onto Petrifilm plates (3MTM Petrifilm™, St. Minnesota, USA). Plates were incubated at 37 °C for 24 h and 48 h for Coliforms and *E. coli*, respectively, following the instruction of ISO 4832:2006 (ISO4832, 2006). The LOQ was equal to 1 Log UFC/g.

Salmonella spp.: The presence of *Salmonella* spp. was analyzed according to Cardoso et al. (2019). Only, purple colonies were counted.

Lactic acid bacteria (LAB): Counts of LAB were analyzed according to Caro et al. (2020). Briefly, 1 mL of appropriate dilution was plated onto a Petri dish using the two-layer method in de Man, Rogosa and Sharpe agar (MRSA; Oxoid, Hampshire, England) previously acidified at 5,5 of pH with lactic acid (Scharlab, Mas d'En Cisa, Spain); the plates were incubated at 30 °C 72 h. The LOQ was equal to 1Log UFC/g.

Aerobic plate count (APC): 1 mL of appropriate decimal dilution was pour plated in Plate Count Agar (PCA, VWR Chemicals, Leuven Belgium); LOQ = 1Log/g. The plates were incubated at 30 °C for 72 h and counted according to ISO 4832:2006 (IS ISO 4833-1, 2013).

Yeast and molds: 0.2 mL of appropriated dilution was spread onto Dichloran Glycerol Agar Base (DG18, Oxoid: CM0729, Hampshire, England), 22% of Glycerol (Scharlab, Mas d'En Cisa, Spain) and supplemented with Chloramphenicol (VWR Chemicals: 928260NL, -Leuven, Belgium). Plates were incubated at 25 °C for 72 h and 120 h to count yeast and molds, respectively (Cardoso et al., 2019). The LOQ was equal to 1,7 Log UFC/g.

2.3. Identification and isolation

2.3.1. Isolation of microorganisms

Bacteria isolation: Four colonies were randomly selected from MRSA, PEMBA, CHROM Agar, IS and PCA media. Isolates were grown in Tryptone Soy Broth (TSB: Oxoid, Hampshire, England) with 0,5% (W/V) of Yeast Extract (YE: Oxoid, Hampshire, England) (TSB-YE). Isolates from PCA, ISA, and MRSA mediums were cultured at 30 °C for 24 h, while those isolated from PEMBA and CHROM Agar media were cultured at 37 °C for 24 h. Then, 1 mL of aliquots were centrifuged (12,000 rpm, 3 min) in Eppendorf tubes. The supernatants were discarded, the pellets were re-suspended in 1 mL of TSB-YE with 50% (v/v) of glycerol (Scharlab, Mas d'En Cisa, Spain) and stored at – 80 °C until use.

2.3.2. Bacteria protein extraction

The procedure of extraction protein and identification of bacteria was analyzed following the Carballo et al. (2019) methodology, except for the recovered isolated colonies. Briefly, isolates were recovered in TSB-YE; the incubation conditions were identical to those described for bacteria isolation. Subsequently, the colonies were grown in media culture specific to each group of bacteria; the incubation time was 24 h. For the analysis, two extraction methods were used: water formic acid solution and ethanol-water extraction according to the manufacturer's instructions (Bruker Daltonik, Leipzig, Germany).

2.3.3. Mold protein extraction

Two mold protein extraction methods were used:

- The Zirconia beads method was carried out according to Lau, Drake, Calhoun, Henderson, and Celazny (2013) and Luethy and Zelazny (2018). Briefly, molds were grown in DG18 (Oxoid, Hampshire, England) at 20 °C until the mold colony size was 5 mm or more. Afterwards, 5 mm mold pieces (including the hyphal bed) were removed from de DG18 agar and placed into 1.5 mL tubes containing 500 mL of 70% ethanol and 50 mL of silica-zirconia beads (BioSpec, Bartlesville, USA). Mycelia were ground with zirconium beads and vortex for 5 min, and centrifuged for 2 min at 13,000 rpm. The ethanol was then removed and samples were dried at 37 °C using a Thermoblock (P-Selecta, Barcelona, Spain). After that, 50 μl of 70% formic acid (Sigma-Aldrich, St. Louis, USA) was added and vortex for 15 min. After 50 μl of acetonitrile (VWR Chemical, Leuven, Belgium) was also added, the tubes were vortexed once again for 5 min. After centrifugation, (13,000 rpm for 2 min), one μL of the supernatant was spotted onto a MALDI-TOF target plate. After air-drying, the sample was overlaid with 1 μL of matrix solution (HCCA).
- A large protein extraction method was carried out according to the manufacturer's instructions (Bruker Daltonik, Bremen, Germany), except for the growth recovered mold colonies. Briefly, a mold colony with approximately 5 mm of DG18 was picked and inoculated in Sabouraud broth (VWR Chemicals, Leuven, Belgium). The tubes of Sabouraud medium were incubated in agitation at 37 °C ±1 °C for 48 or 72 h (in some types of molds the incubation time was of five days) at 120 rpm using a shaking incubator (P-Selecta, Bacerlona, Spain). Subsequently, 1 mL of the growth medium, including the

hyphal bed, was placed into a sterile centrifuge tube (Eppendorf) and then the extraction protein method was similar of those described by manufacturer's before mentioned. All mold analyses were made five times.

2.3.4. Microorganism identification

For identification, each series of measurements was preceded by a calibration step with a bacterial test standard (BTS 155 255343; Bruker Daltonik, Bremen, Germany) to validate the run. Mass spectra were generated by a Flex Analysis MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (l1/4337 nm) operating in linear positive ion detection mode under the Bruker Flex Control software (Bruker Daltonik, Bremen, Germany). The Autoflex LT Speed was periodically calibrated by using the Bruker Daltonik *Escherichia coli* bacterial test standard DH5. Automated analysis of the raw spectral data was performed by the MALDI BioTyper automation (version 3.1) software (Bruker Daltonik, Bremen, Germany) using a library of 5627 main spectra (MSPs; database update of 7/15/2015). Identifications at species or genus level were considered if scores were above 2.0 and 1.7 respectively, according to the report generated by Bruker Compass (Clark, Kaleta, Arora, & Wolk, 2013; Patel, 2013) The analysis was carried out at the Laboratory for Instrumental Analysis, University of Valladolid (Valladolid, Spain).

2.4. Physicochemical analysis: water activity, pH moisture, and sodium chloride

Water activity (aw) was determined in duplicate at 25 °C using a CX-2 hygrometer (Decagon Device Inc, Pullman, WA, USA) following the manufacturer's instructions, using 5 g of each ground sample. For pH determination, 5 g of each ground sample was mixed with 20 mL of distilled water and maintained for 5 min, before measuring with a pH meter (Model 507; Crison, Barcelona, Spain). The measure was done in duplicate at 20 °C. Moisture was determinate according to ISO712:2009. Finally, sodium chloride was measured according to AACC methods (2005) and Carcea, Narducci, Turfani, and Aguzzi (2018) with a modified Volhard titration method. Briefly, 5 g of each ground sample was mixed with 45 mL of distilled water and homogenized using a T10 basic Ultraturrax (IKA-Werke, Staifen, Germany). The mixture was then centrifuged at 2000 rpm for 2 min. Afterwards, 25 mL supernatant was mixed with 25 mL 2% of nitric acid (Sigma-Aldrich, St. Louis, USA) to acidify the sample; automatic titration was made using selective electrode chloride (Metrohm, 719 S Tritino, Herisau Switzerland). Before the analysis, the calibration curve was carried out. The calculations of the amounts of chlorides were performed with the following equation:

$$\frac{V\ AgNO_3 \times C\ AgNO_3 \times 1L/1000mL \times Pm\ NaCl}{V\ sample}$$

V $AgNO_3$: volume of silver nitrate; *C $AgNO_3$*: concentration of silver nitrate (0,1 mol/L $AgNO_3$); *Pw NaCl*: molecular weight sodium chloride (58,44 g/mol); *V sample*: volume of suspension samples after homogenization (10 mL).

2.5. Statistical analysis

Statistical results were obtained using a Statgraphics Centurion XVI software (Statpoint Technologies, Warrenton, USA). All the results obtained were analyzed for variance (ANOVA). Fisher's least significant difference (LSD) was used to describe means with 95% confidence intervals.

3. Results and discussion

Results of physicochemical characteristics are shown in Table 1. The lowest moisture and water activity (aw) was found in the crust and the highest in the crumb of breads. This seems logical because the crust reaches over 100 °C at the end of the baking stage, after the water evaporated in this zone. In contrast, the crumb does not exceed 100 °C. In regard to differences between types of bread, the only significant difference is that *candeal* bread, made with the lowest moisture content, and the industrial sliced bread, with higher oil content, have a lower moisture content. This does not translate into differences in the a_w. In the case of the crumb, the higher humidity values of the sliced bread stand out and can be explained because this bread is baked inside a mold that prevents water evaporation. This fact does not translate either into differences in a_w.

Regarding pH, no significant differences were detected between crumbs and crusts, although the pH of wholemeal bread crust was somewhat higher. The salt content is higher in the crusts in comparison to the crumbs, something that can be anticipated due to greater water evaporation and subsequent concentration of the rest of the components in the crusts. In general, only a few significant differences were appreciated between the various types of bread. Furthermore, the crusts of sliced bread showed a significantly lower NaCl/moisture ratio compared to the crusts of the other types of bread.

Results on bacterial counts of each type of bread are shown in Table 2. In general, there is no significant difference between crust and crumb. On the one hand, we can think that the superficial part of the pieces may be more exposed to microbial contamination, but, on the other, the lower aw of the crusts reduces the development of microorganisms. Differences by type of bread depended on each microorganism

Table 1
Physicochemical characteristics of different types of bread.

Origin	Bread	Localization	a_w	pH	NaCl g/100 g	Moisture g/100 g	Ratio NaCl/Moisture %
Artisan	White wheat loaf	Crust	0.87 ± 0.05^{a}	5.73 ± 0.12^{ab}	1.65 ± 0.30^{def}	17.53 ± 0.63^{a}	9.46 ± 1.83^{h}
		Crumb	0.98 ± 0.00^{c}	5.82 ± 0.20^{ab}	1.20 ± 0.15^{a}	41.99 ± 3.24^{e}	2.86 ± 0.34^{a}
	Whole wheat loaf	Crust	0.85 ± 0.03^{a}	5.63 ± 0.15^{ab}	1.97 ± 0.13^{f}	23.10 ± 2.06^{b}	8.53 ± 0.28^{fgh}
		Crumb	0.97 ± 0.00^{c}	6.19 ± 0.42^{c}	1.42 ± 0.05^{abcd}	44.65 ± 2.49^{e}	3.20 ± 0.08^{ab}
	Candeal bread	Crust	0.87 ± 0.05^{a}	5.64 ± 0.11^{ab}	1.73 ± 0.17^{def}	19.24 ± 0.87^{ab}	9.04 ± 1.13^{gh}
		Crumb	0.99 ± 0.00^{c}	5.69 ± 0.13^{ab}	1.21 ± 0.11^{ab}	34.41 ± 0.44^{d}	3.53 ± 0.36^{ab}
	Tin bread loaf	Crust	0.85 ± 0.02^{a}	5.68 ± 0.33^{ab}	1.61 ± 0.37^{cdef}	31.13 ± 4.71^{cd}	5.15 ± 0.61^{cd}
		Crumb	0.97 ± 0.01^{c}	5.66 ± 0.43^{ab}	1.28 ± 0.10^{abc}	42.13 ± 6.21^{e}	3.06 ± 0.26^{a}
Industrial	White wheat loaf	Crust	0.90 ± 0.06^{ab}	5.82 ± 0.08^{ab}	1.63 ± 0.30^{cdef}	18.79 ± 0.18^{ab}	8.68 ± 1.53^{fgh}
		Crumb	0.98 ± 0.01^{c}	5.92 ± 0.22^{bc}	1.28 ± 0.25^{abc}	41.50 ± 1.89^{e}	3.08 ± 0.49^{a}
	Whole wheat loaf	Crust	0.87 ± 0.06^{a}	5.50 ± 0.09^{a}	1.71 ± 0.29^{def}	23.27 ± 4.61^{b}	7.38 ± 0.42^{ef}
		Crumb	0.96 ± 0.00^{c}	5.55 ± 0.08^{a}	1.25 ± 0.41^{ab}	43.91 ± 7.44^{e}	2.81 ± 0.75^{a}
	Candeal bread	Crust	0.88 ± 0.08^{a}	5.66 ± 0.09^{ab}	1.56 ± 0.46^{bcde}	20.47 ± 0.83^{ab}	7.71 ± 2.56^{efg}
		Crumb	0.97 ± 0.00^{c}	5.72 ± 0.13^{ab}	1.25 ± 0.22^{ab}	35.68 ± 5.06^{d}	3.49 ± 0.24^{ab}
	Tin bread loaf	Crust	0.87 ± 0.09^{a}	5.71 ± 0.32^{ab}	1.86 ± 0.23^{ef}	29.22 ± 2.91^{c}	6.36 ± 0.58^{de}
		Crumb	0.95 ± 0.00^{bc}	5.74 ± 0.41^{ab}	1.64 ± 0.06^{cdef}	35.79 ± 1.57^{d}	4.58 ± 0.13^{bc}

[a-h] Column with different letters represents significant differences ($p < 0.05$). n = 4. number of samples assessed; aw water activity.

Table 2
Counts (Log ufc/g) of main groups of microorganisms in different types of breads[a].

Origin	Bread	Place	Bacillus spp.	SRB	Coliforms	CHROMAgar spp.	LAB	APC	Yeast	Molds
Artisan	White wheat loaf	Crust	2.69 ± 1.12^{cd}	<2[a]	1.32 ± 0.43^{a}	Absent	0.40 ± 0.80^{ab}	1.74 ± 1.26^{abc}	LOQ>	1.01 ± 1.16^{a}
		Crumb	1.80 ± 2.37^{abc}	0.23 ± 0.45^{ab}	1.35 ± 0.40^{a}	0.50 ± 1.00^{ab}	0.25 ± 0.50^{ab}	1.66 ± 1.19^{ab}	LOQ>	0.91 ± 1.05^{a}
	Whole wheat loaf	Crust	2.12 ± 1.48^{abc}	0.57 ± 0.66^{b}	2.24 ± 0.35^{cd}	Absent	1.13 ± 0.85^{bc}	3.10 ± 0.80^{e}	0.79 ± 1.59^{ab}	1.20 ± 1.38^{a}
		Crumb	2.16 ± 1.58^{abc}	0.49 ± 0.57^{ab}	2.42 ± 0.39^{e}	Absent	0.71 ± 0.88^{abc}	2.93 ± 0.85^{de}	0.79 ± 1.59^{ab}	1.12 ± 1.32^{a}
	Candeal bread	Crust	4.76 ± 0.47^{d}	<2	1.68 ± 0.96^{abcd}	1.29 ± 1.82^{ab}	0.92 ± 1.30^{abc}	5.49 ± 0.57^{f}	LOQ>	1.85 ± 0.21^{a}
		Crumb	4.69 ± 1.01^{d}	<2	1.72 ± 0.60^{abcd}	1.87 ± 2.64^{b}	1.15 ± 0.21^{abc}	5.53 ± 0.52^{f}	LOQ>	0.85 ± 1.20^{a}
	Tin bread loaf	Crust	1.99 ± 1.40^{abc}	<2	1.54 ± 0.12^{ab}	1.59 ± 1.97^{b}	1.48 ± 1.19^{c}	2.76 ± 1.04^{cde}	1.63 ± 1.90^{b}	0.85 ± 0.98^{a}
		Crumb	1.76 ± 1.22^{abc}	<2	1.69 ± 0.20^{abc}	1.44 ± 1.81^{b}	1.04 ± 0.69^{abc}	2.44 ± 1.09^{bcde}	0.75 ± 1.50^{ab}	0.42 ± 0.85^{a}
Industrial	White wheat loaf	Crust	2.18 ± 0.28^{abc}	0.08 ± 0.15^{ab}	1.37 ± 0.22^{a}	Absent	0.73 ± 0.85^{abc}	1.95 ± 0.25^{abcd}	0.67 ± 1.35^{ab}	1.39 ± 0.95^{a}
		Crumb	2.30 ± 0.32^{bc}	<2	1.38 ± 0.06^{a}	0.50 ± 1.00^{ab}	LOQ>	1.64 ± 0.48^{ab}	LOQ>	0.92 ± 1.07^{a}
	Whole wheat loaf	Crust	2.26 ± 0.23^{bc}	<2	2.02 ± 0.39^{bcde}	Absent	0.25 ± 0.50^{ab}	1.84 ± 0.66^{abc}	LOQ>	1.02 ± 1.18^{a}
		Crumb	2.04 ± 0.08^{abc}	0.11 ± 0.23^{ab}	2.17 ± 0.26^{cde}	Absent	0.25 ± 0.50^{ab}	1.48 ± 0.26^{ab}	LOQ>	1.39 ± 0.96^{a}
	Candeal bread	Crust	1.00 ± 1.15^{abc}	0.27 ± 0.54^{ab}	1.73 ± 0.68^{abcd}	Absent	0.51 ± 1.03^{abc}	1.92 ± 0.16^{abcd}	0.79 ± 1.58^{ab}	0.50 ± 1.00^{a}
		Crumb	0.65 ± 1.30^{ab}	0.27 ± 0.54^{ab}	1.64 ± 0.49^{abc}	Absent	0.38 ± 0.77^{ab}	1.25 ± 0.87^{a}	0.67 ± 1.35^{ab}	0.51 ± 1.02^{a}
	Tin bread loaf	Crust	0.50 ± 1.00^{a}	0.23 ± 0.45^{ab}	1.40 ± 0.20^{a}	0.50 ± 1.00^{ab}	0.50 ± 0.58^{abc}	1.35 ± 0.22^{ab}	LOQ>	1.27 ± 0.85^{a}
		Crumb	1.08 ± 1.25^{abc}	0.13 ± 0.27^{ab}	1.35 ± 0.16^{a}	Absent	0.75 ± 0.50^{abc}	1.26 ± 0.24^{a}	LOQ>	0.92 ± 1.07^{a}

Column with different letters represents significant differences ($p < 0.05$). n = 4; number of samples assessed; APC, aerobic plate count; LAB, Lactic acid bacteria;; SRB, Sulphite reducing bacteria.

[a] LOQ (Limit of quantification) = 2 ufc/ml.

studied. In the case of *Bacillus* spp, *candeal* artisan bread, which showed the highest counts, sulphite-reducing bacteria (SRB)- counts were very low and never exceeded 0.6 LogUFC/g. Salmonella spp was absent in the bread tested. Even though two bread samples showed mauve colonies on CHROMagar, MALDI TOF identified none of the isolates from those samples as Salmonella spp. This would indicate that this microorganism was absent in all the types of bread analyzed in this study. Results on lactic acid bacteria, molds, and yeast are low and were not significantly different between types of bread. However, *candeal* bread shows a high mesophilic bacterial count while artisan sliced bread shows a lower count. Since all the samples tested had *E. coli* counts below the LOQ, these results are not shown in the tables.

Due to the baking process, most of the microorganisms present in bread are thermally destroyed (Knight & Menlove, 1961). Therefore, the microbial contamination observed must be caused by recontamination after baking. Industrial bread comes in individual bags and, thus, contact with the environment and people is minimal, while artisan bread has no individual packaging, is transported in boxes between the point of production and the point of sale, and thus, contact with the environment is elevated. This could explain the higher microbial load of some of these breads.

The levels of *Bacillus* spp. found in this study were similar to those obtained by Katsi et al. (2021) and below those associated with foodborne illnesses (Valerio et al., 2015) even in the *candeal* artisan bread. However, a deficient conservation may increase the amount of *Bacillus* spp., which can become more problematic (Kaur, 1986).

The values of sulfite-reducing bacteria were low, in agreement with the fact that no *Clostridium* spp outbreaks associated with the consumption of bakery products have been found in Western countries, although one was detected in India and was associated with an inadequate storage (Chaudhry, 1998). Regarding Salmonella counts, our results agree with those founded by Cardoso et al. (2019) who did not find *Salmonella* spp. in bread flour. However, the presence of microorganisms of the Enterobacteriaceae family in bread shows that it is necessary to handle and store bread correctly to keep it safe (Smith et al., 2004). Our study also agrees with the study by Katsi et al. (2021) in terms of LAB and mesophilic counts; besides, we both indicate that microbial load may increase with storage time. The bread analyzed in our study is usually the one discarded within 24 h of being offered for sale, except for sliced bread; yet, inadequate handling may increase the levels of microorganisms and may become a cause of health concern.

A factorial analysis was carried out using the results of 10 selected variables from bread samples: aw, humidity, NaCl, ratio NaCl/humidity, coliforms, sulfite reducing bacteria *B. cereus*, Aerobic Plate Count, Lactic acid bacteria and molds. Counts on CHROMagar Salmonella, E coli, and yeasts were not included in the factorial analysis because the growth of these microorganisms was observed only in few samples. The factor loading and score plots are shown in Fig. 1. Factor 1 explained 34,3% of the variance. This factor was directly associated with two groups of correlated variables: group one, positively highly correlated, including aw, humidity, Na Cl and ratio Na Cl/humidity; and group two (not so strongly correlated), including: mold and LAB counts (see Fig. 1A). Factor 2, explained 24.3% of the variance. This factor was directly associated to microbial counts, highly positively correlated variables: APC, *B. cereus*, SRB and coliforms, and negatively correlated to mold, but not so strongly.

Fig. 1B shows that the first component was able to separate crust and crumb. Thus, the bread crust showed two groups: one had higher salt content and mold counts. The second group was characterized by high SRB, APC and *B. cereus* counts. Crumb samples showed higher humidity content and water activity; notwithstanding, they had lower LAB or PCA counts. The high microbiological load observed on the bread crust is

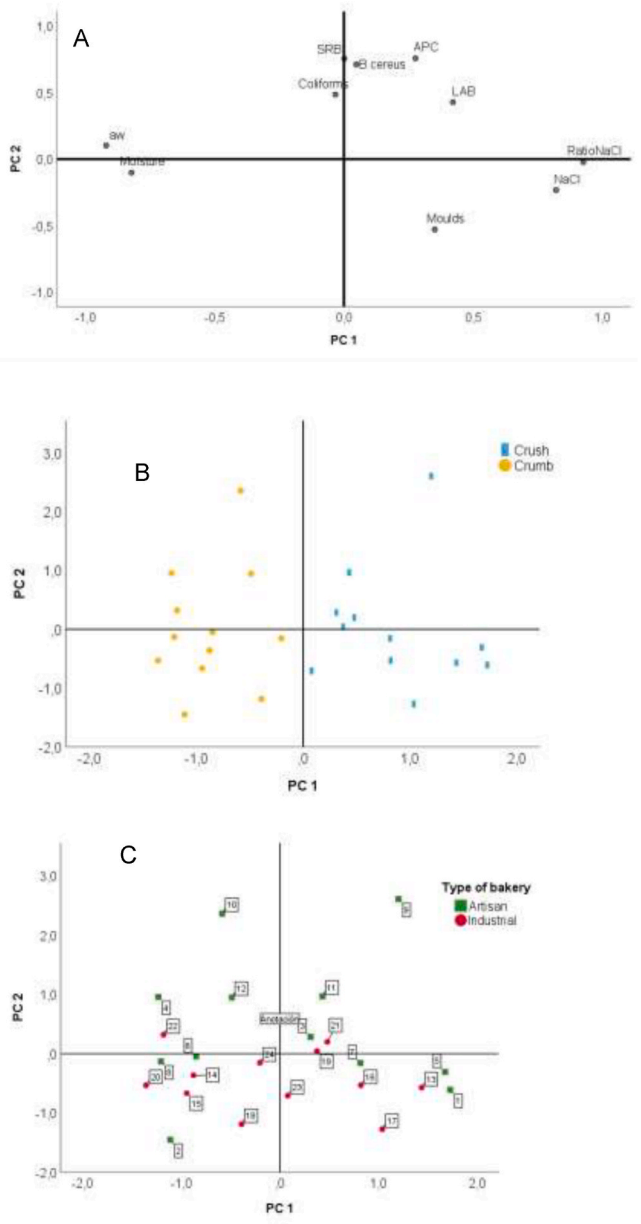

Fig. 1. Factor loadings and score plots **B** by type of bakery and **C** by localization in bread obtained from factor analysis using 10 selected characteristics: $\mathbf{a_w}$, water activity: **SRB, Sulphite reducing bacteria: APC,** Aerobic plate count: **LAB** lactic acid bacteria: **Ratio NaClH**, NaCl/Humidity.

possibly due to poor handling practices, bakery surfaces and equipment at the end of the process or during distribution Thus, the advancement of personnel personal hygiene and adequate food handling practices could be necessary to prevent the transmission of pathogens (Garayoa, Abundancia, Diez-Leturia, & Vitas, 2017).

The second component was almost able to separate artisan and industrial bread (see Fig. 1C). Artisan bread had a high PCA, SRB, B. cereus and coliforms contents, but lower mold content. This result indicates a poor hygiene during processing and a low quality of the ingredients used in this type of bread. Regarding industrial bread, some samples were placed in the bottom right quadrant and had a higher mold content. According to Smith et al. (2004) and Bernardi, Garcia, and Copetti (2019), post contamination of baked products with airborne mold spores and with raw ingredients, such as flour and sugar, is possible.

The results of this study are similar to those obtained by Cardoso et al. (2019) on wheat and rye flours, but in this research, mold and yeast counts are somewhat lower. These would suggest that the flours from these types of bread could be used for human food, such as the production of cookies (Guerra-Oliveira et al., 2021) or cakes (Guerra-Oliveira et al., 2022), and thus reduce food waste. Alpers et al. (2021) argue that the absence of visual detection is sufficient to eliminate the risk of mycotoxin growth. However, as seen in this study, some artisan breads have shown high mold counts. Therefore, the use of these types of bread for human consumption calls for caution. Also, special attention should be paid to the larger pieces of bread since, as Kaur (1986) states, the temperature reached by the bread in the central part of the piece with the heat treatment is lower and some microorganisms can survive, facilitating their subsequent growth.

In this study, the physicochemical conditions in bread allowed the growth of sporulated microorganisms, such as *B. cereus* and molds. The 5.35% of bread samples contained *B. cereus* according to MALDI results (Fig. 2). Those samples showed a pH near 6.0 and more than 0.90 water activity. According to Samapundo, Heyndrickx, Xhaferi, De Baenst, and Devlieghere (2014), a heat treatment at 80 °C for 10 min, a pH near 6.0 and water activity at 0.990 enabled the spores of *B. cereus* to germinate and grow spontaneously at 10 °C. However, Smith et al. (2014) mentioned *B. cereus* spores high heat resistance (90 °C for 10 min). Heat treatment into the bread reaches 100 °C but only for a few minutes. *B. cereus* strains can produce one or more enterotoxins in the intestine or emetic toxin in the food. Enterotoxins that cause diarrhea are heat labile and cause symptoms such as abdominal pain and diarrhea. The toxins responsible for the emetic type are heat stable and cause nausea and vomiting (Hariram & Labbé, 2015). Additionally, the *B. cereus* group is among the ten most reported causative agents of foodborne and waterborne outbreak (EFSA, 2019). In our study, counts on PEMBA (presumptive *Bacillus cereus)* were below the number necessary (more than 10^5 CFU/g cells or spores) to cause diarrhea (Cufaoglu & Deniz, 2022).

There are differences between the isolated species and between the types of bread. *Micrococcus luteus* and *Staphylococcus epidermis* were isolated in 3 types of bread (see Fig. 2). White wheat bread, whole wheat loaf bread and *candeal* bread. *Saccharomyces cerevisiae* and *Kokuria rhizophila* were also isolated in a whole wheat loaf (see Fig. 2B). In *candeal* bread, the most frequently isolated strains were *Bacillus subtilis, Saccharomyces cerevisiae,* and *Enterobacter cloacae* (see Fig. 2C). Finally, in whole wheat loaf bread *Saccharomyces cerevisiae*, Staphylococcus warneri, Micrococcus luteus, *Enterobacter cloacae*, Brevibacterium casei, and B. cereus were isolated (see Fig. 2B). This type of bread showed a larger diversity of microorganisms.

Enterobacter cloacae were isolated from 10.3% of the collected samples of artisan bread. This microorganism has been isolated in 20% of infant food formula samples (Shaker, Osaili, Al-Omary, Jaradat, & Al-Zuby, 2007). Since the Enterobacter is heat-sensitive, its presence in the bread must come from cross-contamination via surface and hand manipulations after oven treatment.

The most frequently isolated LAB was Leuconostoc mesenteroides mainly from artisan bread and, specifically, from the whole wheat loaf (Data not shown). Curiously enough, in this study *Saccharomyces cerevisiae* was isolated from MRSA mainly in artisan breads. The reason why those microorganisms showed good growth in this medium could be that MRSA was acidified at pH 5.5, and that is a near optimal pH, between 4 and 5 (Salari & Salari, 2017).

MALDI-TOF MS identified 15 out of 21 mold strains isolated (see Fig. 3). Using the protein extraction with a water-formic acid solution it was possible to identify only one isolated strain. Following the extraction with zirconia beads, 9 of 21 mold strains were identified. The extraction with ethanol-water identified 12 out 21 of the molds isolated. The extraction with zirconia beads increases the probability to identify the Penicillium spp but not Aspergillus spp. Difficulties with identifications are also reported in Aspergillus spp, where not all strains could be identified with 100% accuracy (Iriart et al., 2012; Verwer et al., 2013). In this study, the extraction with ethanol and water was the best

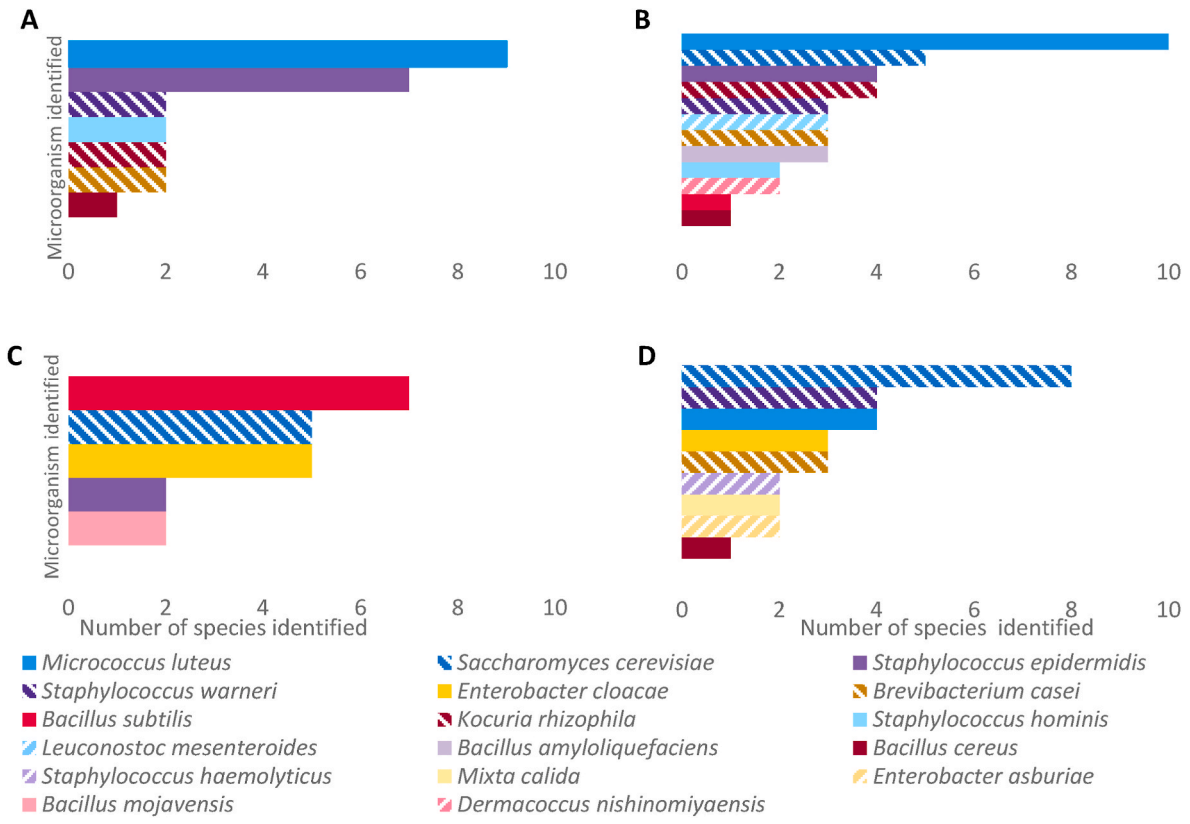

Fig. 2. Bacteria strains isolated from bread identified by MALDI-TOF. **A**: White wheat loaf bread; **B**: Whole wheat loaf; **C**: Candeal bread; **D**: Tin bread loaf.

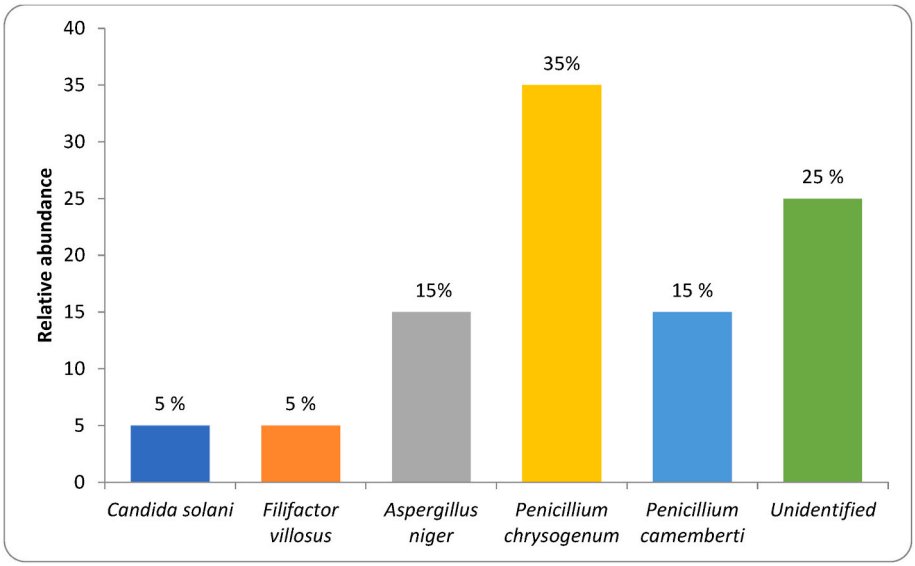

Fig. 3. Relative abundance (%) of the mold strains isolated from bread identified by MALDI-TOF.

method to identify these molds although three isolated strains could not be identified because the MALDI score was less than 1.70. Lau et al. (2013) found high accuracy when using the extraction method with zirconium beads. The difference between our study and theirs is that they have used their database and not Bruker's as in this research.

The main molds identified in bread in this study were Penicillium chrysogenum, Penicillium camemberti, Aspergillus niger and Candida solani. Both Penicillium chrysogenum and Aspergillus niger are considered producers of ochratoxin A (OTA) (Varga, Houbraken, Samson, & Frisvad, 2008). Moreover, the last mold species can also be considered as a potential producer of fumonisin (FB2) because most of the strains isolated presented fum1 genes.

Moreover, Penicillium chrysogenum produce mycotoxins such as Patulin and Cyclopiazonic acid on several fruits and vegetables (Barkai-Golan, 2008). However, according to Reib (1981), this mold produced only Citrinin, a secondary metabolite, on bread surface. Citrinin is a strong nephrotoxin and it exhibited teratogenic effects in animals. In humans, the renal system and in that system, the mitochondrial respiratory chain was identified as a possible target for this mycotoxin (Barkai-Golan, 2008) and, because of that its presence in food is

considered a potentially hazard.

According to the bibliography, Ochratoxin has not been found yet in bread (Saladino et al., 2017; Mousavi, Fakhri, Raesi, & Armoon, 2018). However, Penicillium chrysogenum and Aspergillus niger were found in bread in this study. In addition, microorganisms isolated in our breads may not produce this mycotoxin. In fact, according to Lasram et al. (2007) only 3% of *A. niger* aggregate isolates were ochratoxigenic. Although cereal products are not the main foodstuffs associated with mycotoxins, studying their presence in bread seems to be necessary. More studies are needed to establish the importance of this mycotoxin in bread.

4. Conclusions

This study shows that the breads available in the market have microorganisms. In general, the breads sold in small stores present a higher microbial contamination than those sold in supermarkets. The crusts present higher mold contamination than the crumbs. If these breads, once withdrawn from the market, were for the production of fermented products, such as sourdoughs, this microbial presence could affect them. Some of the molds found are considered as mycotoxin producers. Therefore, although in the breads studied it was not of concern, either because of their type or quantity, the level of microorganisms should be taken into account when considering the reuse of bread withdrawn from the market for human consumption. Good quality system implementation is essential to minimize health risks in products elaborated with discarded bread. Future studies should analyze the mycotoxin content in these types of breads to complete the information on their safety. These studies can serve as a basis for, or support, new studies on the use of discarded bread to be included in the human food chain.

CRediT authorship contribution statement

Irma Caro: Conceptualization, Methodology, Formal Analysis, Resources, Writing - Original Draft, Writing - Review & Editing; Sara Portales: Investigation, Writing - Original Draft; Manuel Gómez: Conceptualization, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

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

Data availability

Data will be made available on request.

References

AACC. (2005). *Approved methods of the American association of cereal chemists*, 10th ed. The Association: St. Paul,MN, USA

Alpers, T., Kerpes, R., Frioli, M., Nobis, A., Hoi, K. I., Bach, A., et al. (2021). Impact of storing condition on staling and microbial spoilage behavior of bread and their contribution to prevent food waste. *Foods, 10*, 76.

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*, 3528–3542.

Barkai-Golan, R. (2008). Penicillium mycotoxins. In R. Barkai-Golan, & N. Paster (Eds.), *Mycotoxins in fruits and vegetables* (pp. 153–183). Academic Press.

Bernardi, A. O., Garcia, M. V., & Copetti, M. V. (2019). Food industry spoilage fungi control through facility sanitization. *Current Opinion in Food Science, 29*, 28–34.

Brancoli, P., Bolton, K., & Eriksson, M. (2020). Environmental impacts of waste management and valorisation pathways for surplus bread in Sweden. *Waste Management, 117*, 136–145.

Carballo, D. E., Mateo, J., Andrés, S., Giráldez, F. J., Quinto, E. J., Khankari, A., et al. (2019). Microbial growth and biogenic amine production in a Balkan-style fresh sausage during refrigerated storage under a CO_2-containing anaerobic atmosphere: Effect of the addition of Zataria multiflora essential oil and hops Extract. *Antibiotics, 8*, 227.

Carcea, M., Narducci, V., Turfani, V., & Aguzzi, A. (2018). A survey of sodium chloride content in Italian artisanal and industrial bread. *Foods, 7*, 181.

Cardoso, R. V. C., Fernandes, A., Heleno, S. A., Rodrigues, P., Gonzalez-Paramas, A. M., Barros, L., et al. (2019). Physicochemical characterization and microbiology of wheat and rye flours. *Food Chemistry, 280*, 123–129.

Caro, I., Quinto, E. J., Fuentes, L., Alessandria, V., Cocolin, L. S., Redondo-del-Río, M. P., et al. (2020). Characterization of Lactococcus strains isolated from artisanal Oaxaca cheese. *LWT - Food Science and Technology, 122*, Article 109041. https://doi.org/10.1016/j.lwt.2020.109041

Chaudhry, R. (1998). Outbreak of suspected Clostridium butyricum botulism in India. *Emerging Infectious Diseases, 4*, 506–507.

Clark, A. E., Kaleta, E. J., Arora, A., & Wolk, D. M. (2013). Matrix-assisted laser desorption ionization-time of flight mass spectrometry: A fundamental shift in the routine practice of clinical microbiology. *Clinical Microbiology Reviews, 26*, 547–603.

Cufaoglu, G., & Deniz, A. N. (2022). Potential risk of Bacillus cereus in spices in Turkey. *Food Control, 132*, Article 108570.

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*. https://doi.org/10.1080/10408398.2021.1976100

Dong, Y., & Karboune, S. (2021). A review of bread qualities and current strategies for bread bioprotection: Flavor, sensory, rheological, and textural attributes. *Comprehensive Reviews in Food Science and Food Safety, 20*, 1937–1981.

EFSA (European Food Safety Authority). (2019). The European Union one health 2018 zoonoses report. *EFSA Journal, 17*, 5926.

Eglezos, S. (2010). Microbiological quality of wheat grain and flour from two mills in Queensland, Australia. *Journal of Food Protection, 73*, 1533–1536.

European Union. (2008). Directive 2008/98/EC of the European parliament and of the council of 19 november 2008 on waste and repealing certain directives. *Official Journal of the European Union, 51*, 3–30.

Fadda, C., Sanguinetti, A. M., Del Caro, A., Collar, C., & Piga, A. (2014). Bread staling: Updating the view. *Comprehensive Reviews in Food Science and Food Safety, 13*, 473–492.

FAO. (2013). Food wastage footprint: Impacts on natural resources - summary report. Retrieved from https://www.fao.org/3/i3347e/i3347e.pdf. (Accessed 8 July 2022).

Fernández-Peláez, J., Guerra, P., Gallego, C., & Gómez, M. (2021). Physical properties of flours obtained from wasted bread crusts and crumbs. *Foods, 10*, 282.

Garayoa, L., Abundancia, C., Diez-Leturia, M., & Vitas, A. I. (2017). Essential tools for food safety surveillance in catering services: On-site inspections and control of high risk cross-contamination surfaces. *Food Control, 75*, 48–54.

Gélinas, P., McKinnon, C. M., & Pelletier, M. (1999). Sourdough-type bread from waste bread crumb. *Food Microbiology, 16*, 37–43.

Guerra-Oliveira, P., Belorio, M., & Gómez, M. (2021). Waste bread as main ingredient for cookie elaboration. *Foods, 10*, 1759.

Guerra-Oliveira, P., Belorio, M., & Gómez, M. (2022). Wasted bread flour as a novel ingredient in cake making. *International Journal of Food Science and Technology*. https://doi.org/10.1111/ijfs.15577

Hariram, U., & Labbé, R. (2015). Spore prevalence and toxigenicity of Bacillus cereus and Bacillus thuringiensis isolates from U.S. retail spices. *Journal of Food Protection, 78*, 590–596.

Iriart, X., Lavergne, R. A., Fillaux, J., Valentin, A., Magnaval, J. F., Berry, A., et al. (2012). Routine identification of medical fungi by the new vitek MS matrix-assisted laser desorption ionization-time of flight system with a new time-effective strategy. *Journal of Clinical Microbiology, 50*, 2107–2110.

IS ISO 4833-1. (2013). *Microbiology of the food chain – horizontal method for the enumeration Colony count at 30 degrees C by the pour plate technique*. International Organization for Standardization.

ISO15213. (2003). *Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions*. International Organization for Standardization.

ISO4832. (2006). *Microbiology of food and animal feeding stuffs – horizontal method for the enumeration of coliforms – colony-count technique*.

ISO712. (2009). *Moisture Oven drying method Cereals and cereal products —determination of moisture content— reference method*. International Organization for Standardization.

Katsi, P., Kosma, I. S., Michailidou, S., Argiriou, A., Badeka, A. V., & Kontominas, M. G. (2021). Characterization of artisanal spontaneous sourdough wheat bread from central Greece: Evaluation of physico-chemical, microbiological, and sensory properties in relation to conventional yeast leavened wheat bread. *Foods, 10*, 1–23.

Kaur, P. (1986). Survival and growth of Bacillus cereus in bread. *Journal of Applied Bacteriology, 60*, 513–516.

Knight, R. A., & Menlove, E. M. (1961). Effect of the bread-baking process on destruction of certain mould spores. *Journal of the Science of Food and Agriculture, 12*, 653–656.

Lasram, S., Bellí, N., Chebil, S., Zghonda, N., Mliki, A., Sanchis, V., et al. (2007). Occurrence of ochratoxigenic fungi and ochratoxin A in grapes from a Tunisian vineyard. *International Journal of Food Microbiology, 114*, 376–379.

Lau, A. F., Drake, S. K., Calhoun, L. B., Henderson, C. M., & Celazny, A. M. (2013). Development of a clinically comprehensive database and a simple procedure for

identification of molds from solid media by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry. *Journal of Clinical Microbiology, 51*(3), 828–834.

Luethy, P. M., & Zelazny, A. M. (2018). Rapid one-step extraction method for the identification of molds usingMALDI-TOF MS. *Diagnostic Microbiology and Infectious Disease, 91*, 130–135.

Mousavi, K. A., Fakhri, Y., Raesi, S., & Armoon, B. (2018). Prevalence and concentration of ochratoxin A, zearalenone, deoxynivalenoland total aflatoxin in cereal-based products: A systematic review and meta-analysis. *Food and Chemical Toxicology, 118*, 830–848.

Narisetty, V., Cox, R., Willoughby, N., Aktas, E., Tiwari, B., Matharu, A. S., et al. (2021). Recycling bread waste into chemical building blocks using a circular biorefining approach. *Sustainable Energy Fuels, 5*, 4842–4849.

Patel, R. (2013). Matrix-assisted laser desorption ionization-time of flight mass spectrometry in clinical microbiology. *Clinical Infectious Diseases, 57*, 564–572.

Reiβ, J. (1981). Studies on the ability of mycotoxins to diffuse in bread. *European Journal of Applied Microbiology and Biotechnology, 12*, 239–241.

Saladino, F., Quiles, J. M., Manes, J., Fernandez-Franzon, M., Luciano, F. B., & Meca, G. (2017). Dietary exposure to mycotoxins through the consumption of commercial bread loaf in Valencia, Spain. *LWT–Food Science and Technology, 75*, 697–701.

Salari, R., & Salari, R. (2017). Investigation of the best Saccharomyces cerevisiae growth condition. *Electronic Physician, 9*(1), 3592–3597. Jan.

Samapundo, S., Heyndrickx, M., Xhaferi, R., De Baenst, I., & Devlieghere, F. (2014). The combined effect of pasteurization intensity, water activity, pH and incubation temperature on the survival and outgrowth of spores of Bacillus cereus and Bacillus pumilus in artificial media and food products. *International Journal of Foof Microbiology, 181*, 10–18.

Samray, M. N., Masatcioglu, T. M., & Koksel, H. (2019). Bread crumbs extrudates: A new approach for reducing bread waste. *Journal of Cereal Science, 85*, 130–136.

Shaker, R., Osaili, T., Al-Omary, W., Jaradat, Z., & Al-Zuby, M. (2007). Isolation of Enterobacter sakazakii and other Enterobacter spp. from food and food production environments. *Food Control, 18*, 1241–1245.

Smith, J. P., Daifas, D. P., El-Khoury, W., Koukoutsis, J., & El-Khoury, A. (2004). Shelf life and safety concerns of bakery products - a review. *Critical Reviews in Food Science and Nutrition, 44*, 19–55.

Tallent, S. M., Knolhoff, A., Rhodehamel, E. J., Harmon, S. M., & Bennett, R. W. (2020). BAM chapter 14: Bacillus cereus | FDA. In *Bacteriological analytical manual* (8th ed.).

Tonini, D., Albizzati, P. F., & Astrup, T. F. (2018). Environmental impacts of food waste: Learnings and challenges from a case study on UK. *Waste Management, 76*, 744–766.

Valerio, F., Di Biase, M., Huchet, V., Desriac, N., Lonigro, S. L., Lavermicocca, P., et al. (2015). Comparison of three Bacillus amyloliquefaciens strains growth behaviour and evaluation of the spoilage risk during bread shelf-life. *Food Microbiology, 45*, 2–9.

Varga, J., Houbraken, J., Samson, R. A., & Frisvad, J. C. (2008). Molecular diversity of Aspergillus and Penicillium species on fruits and vegetables. In R. Barkai-Golan, & N. Paster (Eds.), *Mycotoxins in fruits and vegetables* (pp. 208–223). Academic Press.

Verwer, P. E. B., Leeuwen, W. B., Girard, V., Monnin, V., van Belkum, A., Staaf, J. F., et al. (2013). Discrimination of Aspergillus lentulus from Aspergillus fumigatus by Raman spectroscopy and MALDITOF MS. *European Journal of Clinical Microbiology & Infectious Diseases, 33*, 245–251.