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Occurrence and enumeration of rope-producing spore forming bacteria in flour and their spoilage potential in different bread formulations

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ABSTRACT

This study aimed to investigate the prevalence of spore forming rope-producing microorganisms in different types of flour and to evaluate the potential for spoilage of the isolates through the simulation of the bread manufacturing process. Mesophilic and thermophilic aerobic spore forming bacteria were enumerated in 100 flour samples. Strains isolates were evaluated for their ability to produce amylase and cause rope spoilage in different bread formulations. Amylase-producing strains were identified through 16S rRNA sequencing. The wholemeal flour from industry presented the highest aerobic mesophilic spore forming bacteria counts (3.1 log spores/g). A total of 327 strains were isolated from flours, among which 45 produced amylase. These isolates were identified as *Bacillus licheniformis* (62%), *B. sonorensis* (20%) *B. cereus* (11%), *B. pumilus* (2%), and *Paenibacillus polymyxa* (2%). All 45 strains were able to cause spoilage in bread with and without preservatives. The addition of calcium propionate and reduction of water activity and pH were able to prevent the development of rope for 7 days. The development of new formulations can help to assure the microbiological quality and safety of baked products.

1. Introduction

Bakery products, such as loaves of bread and cakes, can be spoiled by different microorganisms, including filamentous fungi and bacteria (Garcia, Da Pia, Freire, Copetti, & Sant'Ana, 2019; Mantzourani et al., 2014; Morassi et al., 2018). Among them, species of *Bacillus*, such as *B. subtilis, B. amyloliquefaciens, B. licheniformis, B. pumilus, B. megaterium* and *B. cereus* stand out as the main agents responsible for a spoilage process known as ropiness or rope (Fangio, Roura, & Fritz, 2010; Valerio et al., 2012).

Rope spoilage is characterized by bread crumb discoloration and a sweet fruit odor that resembles ripe melon or pineapple. Bread crumb contaminated with rope-producing *Bacillus* becomes soft and sticky to touch and in more advanced stages can almost liquify. However, the dominant feature of this spoilage process is the formation of strings or threads in bread crumb when pulling two ends (Valerio et al., 2015).

Rope spoilage occurs due to *Bacillus* survival to thermal processing of bakery products. Even though bake temperature may reach 180–200 °C, in the center of the crumb the maximum temperature reached is around 97–101 °C for a few minutes (Valerio et al., 2012). Once spores withstand baking, they may further germinate and grow under favorable conditions (temperature above 25 °C, water activity $\geq$ 0.95 and pH > 5.0) (Valerio et al., 2012, 2015; Viedma, Abriouel, Omar, López, & Gálvez, 2011).

Rope takes place even when low counts of *Bacillus* spores are found in the flour, such as 10^3 spores/g if intrinsic and extrinsic parameters are favorable to the germination and outgrowth of these spore forming microorganisms (Vaičiulytė-Funk, Žvirdauskienė; Šalomskienė, & Šarkinas, 2015). The bread texture is modified, becoming slimy due to the action of proteolytic and amylolytic enzymes released into the environment by *Bacillus* (Valerio et al., 2012; Viedma et al., 2011).

Raw materials, especially flours, are widely known as the main source of contamination of rope-producing *Bacillus* in bakery products (Vaičiulytė-Funk, Žvirdauskienė, Šalomskienė, & Šarkinas, 2015). Moreover, processing the environment or other raw materials such as yeast or additives may also be contaminated with rope-producing *Bacillus* (Valerio et al., 2012). Besides, nowadays, most of the bread has been produced with wholemeal flour and without preservatives, favoring the deterioration of bread caused by *Bacillus* (Vaičiulytė-Funk et al., 2015).

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Despite being a significant challenge for bakeries and baking industries (Plessas, Mantzourani, & Bekatorou, 2020), the economic losses associated with rope seem to be underestimated, as they are often confused with changes in bread structure due to both insufficient cooking and yeast amounts in the dough (De Bellis et al., 2015). Also, rope-producing microorganisms (*B. cereus; B. subtilis; B. licheniformis*) can also pose safety risks for consumers and have been implicated in foodborne outbreaks (De Bellis et al., 2015; Viedma et al., 2011).

The elimination of *Bacillus* spores from raw materials is difficult considering the ubiquitous occurrence of these microorganisms in the environment, technological and economic aspects (Carlin, 2011; Húngaro, Alvarenga, Peña, & Sant'Ana, 2013; Oteiza, Alvarenga, Sant'Ana, & Giannuzzi, 2014). Thus, modifications in the formulation of bakery products combined with the use of raw materials containing low levels of *Bacillus* spores seem to be the most tangible strategy to reduce the occurrence of rope spoilage. Thus, this study aimed to investigate the prevalence of spore forming rope-producing microorganisms in different types of flour and to evaluate the potential for rope spoilage of the isolates through the simulation of the bread manufacturing process.

2. Material and methods

2.1. Samples collection

A total of 100 samples of wheat flour from different lots were obtained from a bakery and a baking industry, located in São Paulo State, Brazil. From the bakery, 40 samples were collected, namely 20 of white cake flour (F1) and 20 of white bread flour (F2). From the baking industry, 60 samples were obtained, to know: 20 of white cake flour (F3), 20 of white bread flour (F4), and 20 of wholemeal flour (F5). "All samples were collected during their shelf-life and were free from insect infestation."

2.2. Enumeration and prevalence of spore forming microorganisms in flour samples

Samples were submitted to microbiological analysis for the enumeration of mesophilic and thermophilic aerobic spore forming microorganisms. For the enumeration of thermophilic aerobic microorganisms, 20 g of each sample was dissolved in 100 mL of sterilized water, followed by a thermal shock performed at 100 °C for 5 min. Then, an aliquot (2 mL) was distributed into five Petri dishes, following the addition of Dextrose Tryptone Agar (DTA, Oxoid, Basingstoke, UK) and incubation at 55 °C for 48 h (Olson & Sorrells, 2001). For enumeration of mesophilic aerobic microorganisms, 20 g of each sample was dissolved in 100 mL of sterilized water, and an aliquot (10 mL) was transferred to 100 mL of Tryptone Glucose Extract Agar (TGE, Oxoid, Basingstoke, UK). After heat shock (80 °C for 30 min), the homogenate containing the diluted sample and the TGE agar was distributed homogeneously into five Petri dishes, followed by incubation at 37 °C for 48 h (Stevenson & Lembke, 2001). Microbial counts were expressed as log spores/g. (Olson & Sorrells, 2001; Stevenson & Lembke, 2001). Among 3–5 colonies from each sample were isolated in nutrient agar (NA, Oxoid, Basingstoke, UK) under incubation at 37 °C for 24 h (mesophiles) and 55 °C for 48h (thermophiles) and subsequently submitted to Gram staining. Rod-shaped, Gram-positive, and spore forming isolates were tested for the ability to produce amylase.

2.3. Amylase production by spore forming microorganisms

For the evaluation of amylase production, drops of an iodine solution were added to purified colonies of spore forming isolates previously grown on starch agar [beef extract: 3 g/L (Acumedia, Lansing, USA), starch: 10 g/L (Ecibra, Santo Amaro, Brazil), bacteriological agar: 12 g/L (Oxoid, Basingstoke, UK)] at 30 °C for 18–24 h. Unhydrolyzed starch exhibits a deep blue color in the presence of iodine; on the other hand, zones, where starch has been hydrolyzed, become clear due to the formation of a halo as a result of amylase activity. Thus, strains were differentiated based on amylase production and halo diameter (+++, >10 mm; ++, 5–10 mm) (Pepe, Blaiotta, Moschetti, Greco, & Villani, 2003).

2.4. Identification of amylase-producing strains by 16S rRNA sequencing

Amylase-producing strains were identified by 16S rRNA sequencing. The partial 16S rRNA gene in DNA isolated from *Bacillus* was amplified by PCR using primers designed in a previous study (Goto, Omura, Hara, & Sadaie, 2000), which amplify a hyper variant region (HV), highly specific for several *Bacillus* strains. For amplification of the HV region, two primers were used; a forward primer (5′-TGT AAA ACG ACG GCC AGT GCC TAA TAC ATG CAA GTCGAGCG-3′) and a reverse primer (5′-CAGGAAACAGCT ATG ACC ACT GCT GCC TCCCGT AGG AGT-3′). Each PCR mixture contained 2 μL of DNA sample, 1.50 U of Taq DNA polymerase (Vivantis Technologies Sdn. Bhd., Malaysia), 0.25 mM dNTPs, 0.1 mM of each primer, 1 × viBuffer A from Taq DNA polymerase kit, and 2.5 mM of $MgCl_2$ in a total volume of 25 μL. PCR was performed in a PTC-200 programmable thermal cycler (MJ Research, USA), using the following program: 95 °C for 6 min, 30 cycles at 94 °C for 1 min, 66 °C for 1 min, and 72 °C for 30 s, with a final extension performed at 72 °C for 3 min. The PCR products (5 μL) were subjected to electrophoresis on a 1% agarose gel in 1 × TAE buffer (pH 8.0), and DNA amplicons were visualized under UV illumination after staining with ethidium bromide. The remaining PCR products (~20 μL) were purified with Wizard® SV Gel and PCR Clean-up System (Promega, WI, USA) according to the manufacturer's instructions.

DNA sequencing was performed at the Life Sciences Core Facility (LaCTAD) from State University of Campinas (UNICAMP, SP, Brazil) using Sanger sequencing in a 3730xL DNA Analyzer (Applied Biosystems®, CA, USA), a BigDye® terminator v3.1 Cycle Sequencing kit (Applied Biosystems®, CA, USA), a forward primer 5′- TGTAAAACGACGGCCAGT-3′ and a reverse primer 5′- CAGGAAACA GCTATGACC-3'. Sequences obtained were edited and aligned using BioEdit and blasted against NCBI non-redundant nucleotide database nt (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The best results presenting an E-value of $<10^{-5}$ were used for the identification of the microorganisms.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987) Evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei, & Kumar, 2004) and expressed as the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were performed in MEGA6 (Tamura, Stecher, Peterson, Peterson, & Kumar, 2013).

2.5. Preliminary assessment of "rope" spoilage potential by Bacillus spp. in bread

Bread made with the addition of the calcium propionate preservative and breads made without preservatives were acquired from a bakery industry and used in this step of the study. Bread slices were transferred to Petri dishes and sterilized in an autoclave (121 °C/15 min) to inactivate any spore forming bacteria potentially present. Preliminary experiments were performed to ensure that moisture content, aw, and pH did not change during this treatment. For determination of moisture content, 10 g of samples were weighed into ceramic crucibles and kept at 130 °C for 90 min using the equipment OVEN EM 10 NG (Chopin Technologie, Villeneuve-la-Garenne, France). For aw determination, the temperature was kept at 25 °C, and the equipment 4 TE Dewpoint Water Activity Meter (AquaLab Decagon, Washington, USA) was used. The pH was determined according to the method described by Adolfo Lutz Institute (ALI, 2008).

Amylase-producing isolates were inoculated in 20 mL of Bread Extract Broth (BEB) and kept overnight at 30 °C. BEB was formulated

and consisted of a homogenized solution containing 100 g of white bread and 350 mL of distilled water. The solution was filtered through a paper filter, pH was adjusted to 6.8 with 1M NaOH solution, following sterilization at 121 °C/15 min. The inoculated broth was divided into four aliquots of 5 mL each. Two of them were heat-treated at 96 °C for 10 min and distributed individually into two slices of sterilized bread. The two other aliquots that have not undergone heat treatment were distributed on the surface of two slices of bread. The final concentration of spores per slice of bread was approximately 10^3 spores/g. An aliquot of 5 mL of sterilized distilled water was dispensed on the surface of bread slices (control). The inoculated slices were incubated at 30 °C, and "rope" formation was evaluated daily (Pepe et al., 2003). The patterns shown in Fig. 1 were used as criteria to determine the potential for bread spoilage ("rope") of each tested strain. "Rope" development scale was set up as follows: (−) no "rope" development; (+) slight "rope"; (++) moderate "rope"; and (+++) strong "rope" (Thompson, Waites, & Dodd, 1998).

2.6. Influence of formulation and bread size on the survival of "rope"-producing bacillus

2.6.1. Preparation of suspensions of spores

Four *Bacillus* strains capable of causing "rope" spoilage in bread with preservative (strong "rope": #414, #462, #516, and slight "rope" #069) were used in this experiment. Suspensions of spores were prepared using nutrient broth (Kasvi, Roseto degli Abruzzi, Italy) added 5 mg/L of manganese sulfate as previously described (Peña et al., 2014).

2.6.2. Bread formulations and inoculation with "rope"-producing Bacillus

Large (500g) and small (20g) bread were prepared for each type of formulation studied, namely standard (pH 6.0, a_w 0.95), low aw (0.90), and low pH (5.6) (Table 1). Suspension of spores was individually inoculated on bread doughs to obtain a final concentration of 10^8 spores/g. Bread doughs were then baked at 180 °C for 20 min (industrial oven 8–4000 W, Imequi, São Paulo, Brazil). "Rope"-producing *Bacillus* strains inoculated on bread doughs were counted before (N0) and after baking (Nf). The counts of these microorganisms were performed after heat shock at 80 °C for 15 min and plating on Trypticase Soy Agar (Kasvi, Roseto degli Abruzzi, Italy), followed by incubation at 37 °C for 48 h. The survival of spores was expressed as log spores/g.

2.7. Rope spoilage potential of B. licheniformis #414 in different bread formulations

Suspension of spores of *B. licheniformis* #414 was prepared, as described in section 2.6.1. *B. licheniformis* #414 was chosen for this study as this strain presented the high spoilage potential of bread. Four different formulations of bread were prepared (standard, low aw, low pH, and containing preservative) as described in section 2.6.2 (Table 1).

Bread dough of the different formulations studied was inoculated with 10^6 spores/g of *B. licheniformis* #414 during the mixing of ingredients. For all formulations, a negative control (no spores inoculated) was included. Bread of 140g was placed on the baking tins and further baked at 180 °C for 20 min (industrial oven 8–4000 W, Imequi, São Paulo, Brazil). Then, bread was cooled for 15 min at laminar hood, the following disposal in sterilized bags, and storage at 37 °C for 7 days. The bread was checked daily for the appearance of rope spoilage.

Table 1
Different types of bread formulation.

Ingredients (%) (w/w)	Formulations[a]			
	Standard	Low a_w	Low pH	Containing preservative
Whole wheat flour	28.6	28.4	28	28.6
White wheat flour	26.4	26.2	25.9	26.4
Biological yeast	3.8	3.8	3.8	3.8
Water	34.1	33.9	33.4	34.1
Crystal sugar	3.3	3.3	3.2	3.3
Gluten	0.5	0.5	0.5	0.5
Salt	1.1	1.6	1.1	1.1
Soybean oil	2.2	2.2	2.2	2.2
Vinegar	–	–	1.9	–
Calcium propionate	–	–	–	0.5

[a] Formulations: Standard (pH 6.0, a_w 0.95); low a_w (0.90); low pH (5.6).

2.8. Statistical analysis

The analysis of variance (ANOVA), followed by the Scott-Knott test, was used to evaluate microbial counts of mesophilic and thermophilic aerobic spore forming microorganisms. The significance level was set at 5% ($p < 0.05$) for all analyses performed.

3. Results

3.1. Enumeration and prevalence of spore forming microorganisms in flour samples

The range of counts of spore forming microorganisms in flour samples analyzed is shown in Table 2. The wholemeal flour from industry (F5) presented the highest counts of aerobic mesophilic spore forming bacteria with a mean value of 3.1 log spores/g, while white cake flour (F1) and white bread flour from the bakery (F2) exhibited the lowest counts: 1.2 and 1.8 log spores/g, respectively. On the other hand, the average count of thermophilic spore forming bacteria was not significantly different for any flour. Overall, flour samples obtained from industry presented higher counts of spore forming bacteria than samples collected at the bakery.

From 100 flour samples analyzed, a total of 327 Gram-positive mesophilic and thermophilic spore forming microorganisms were isolated. The white cake flour from industry (F3) showed the highest occurrence percentage of spore forming microorganisms (27.2%). On the other hand, white cake flour - bakery (F1) showed the lowest occurrence percentage of spore forming microorganisms (12.2%)

Fig. 1. Different levels of rope production in breads. No rope (−), slight rope (+), moderate rope (++) and advanced rope (+++).

Table 2
Enumeration (log spores/g) and prevalence (%) of mesophilic and thermophilic sporeforming bacteria in flour samples.

Type of flour[a]	Range mesophilic counts (log spores/g)	Average mesophilic counts (log spores/g) [b]	Range thermophilic counts (log spores/g)	Average thermophilic counts (log spores/g)[b]	Prevalence (n = 327) (%)
F1	0.5–1.5	1.2[a] ±0.3	1.0–2.4	1.9[a] ±1.2	12.2
F2	0.9–2.7	1.8[a] ±1.4	1.3–2.3	1.9[a] ±1.0	21.7
F3	0.8–3.2	2.7[b] ± 2.1	1.1–3.4	2.4[a] ±2.1	27.2
F4	1.3–3.0	2.5[b] ± 1.8	1.3–2.7	2.1[a] ±1.4	17.1
F5	1.0–3.3	3.1[c] ±2.2	1.1–2.4	1.9[a] ±1.0	21.7

[a] White cake flour - bakery (F1); White bread flour - bakery (F2); White cake flour - industry (F3); White bread flour - industry (F4); Wholemeal flour - industry (F5).
[b] Different letters show statistically significant difference at $p < 0.05$.

(Table 2).

3.2. Amylase production by spore forming microorganisms

Among 327 Gram-positive spore forming isolates strains, 13.8% (45 strains) exhibited amylase enzyme production (T1S). Of these 45 strains, 11 (24.4%) presented strong amylase production potential, forming a large halo in the starch agar after the addition of iodine solution (+++, >10 mm). The other 34 strains (75,6%) showed medium halo formation (++, 5–10 mm). Most of the amylase-producing strains were isolated from wholemeal flour from industry (F5) (36%) (Fig. 2).

3.3. Identification of amylase-producing strains by 16S rRNA sequencing

The amylase-producing strains were identified as: *Bacillus licheniformis* (62.3%), *B. sonorensis* (20.0%), *B. cereus* (11.1%), *B. subtilis* (2.2%), *B. pumilus* (2%) and *B. polymyxa* (2.2%) (Fig. 3). The species *B. licheniformis* was found in all types of flour. Although the wholemeal flour from industry (F5) showed the highest number of strains (16 strains), the white bread flour from industry (F4) showed the greatest diversity of species: *B. licheniformis*, *B. sonorensis*, *B. cereus* and *B. pumilus* (Fig. 4).

3.4. Rope spoilage potential by Bacillus spp. in bread

All 45 strains tested, both: heat-treated (96 °C/10min) and those that did not receive any treatment (untreated), caused rope spoilage in bread not added of calcium propionate as observed on the third day of incubation. In bread added of calcium propionate, 34 strains (75,6%) caused rope without the use of thermal shock, compared to only four (8,9%) that were heat-treated, assessed on the third day (Table 3). Only on the ninth day, all strains (45) produced rope with and without heat treatment in bread added of calcium propionate. Initially, bread showed a slight viscosity in the center, which spread toward the sides, in some cases changing the food color to pink, yellow, and even brown. The acid odor was detected. In a more advanced stage of spoilage, bread has liquefied.

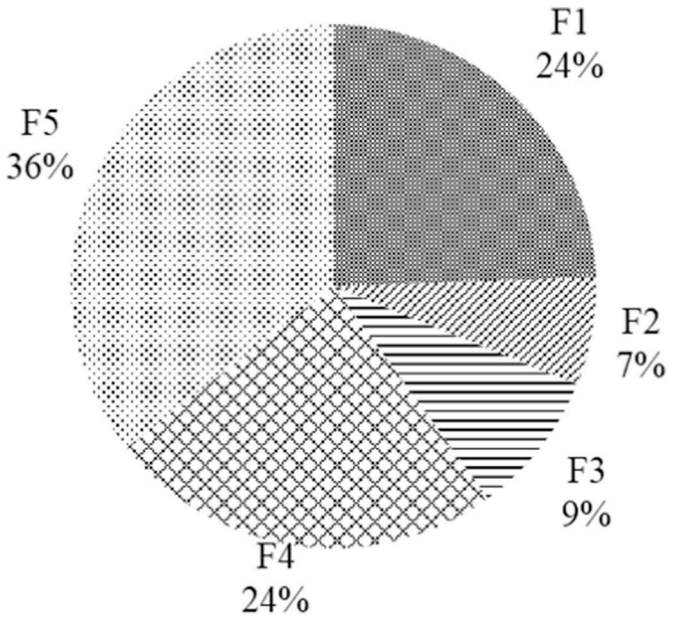

Fig. 2. Distribution of strains with amylase activity according to origin. White cake flour - bakery (F1); White bread flour - bakery (F2); White cake flour - industry (F3); White bread flour - industry (F4); Wholemeal flour - industry (F5).

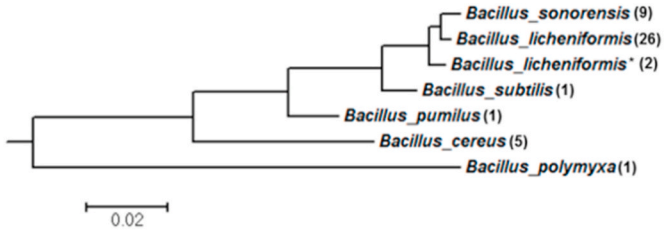

Fig. 3. Evolutionary relationships of taxa obtained based on 16S rRNA gene sequencing. Numbers between brackets indicate the number of isolates for each species identified.

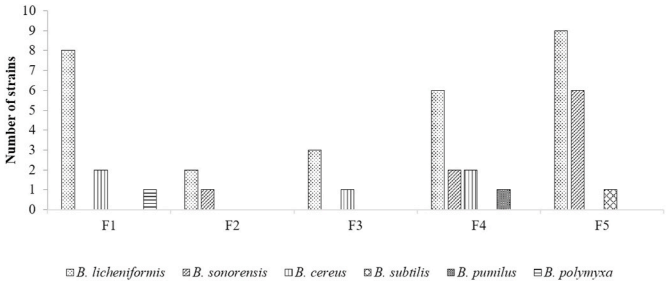

Fig. 4. Occurrence of *B. licheniformis*, *B. sonorensis*, *B. cereus*, *B. subtilis*, *B. pumilus*, and *B. polymyxa* in different types of flour. White cake flour - bakery (F1); White bread flour - bakery (F2); White cake flour - industry (F3); White bread flour - industry (F4); Wholemeal flour - industry (F5).

3.5. Influence of formulation and bread size on the survival of rope-producing bacillus

All strains survived heat treatment used (180 °C for 20 min) (Table 4). An average reduction of 4 log spores/g was observed. No statistically significant difference among strains, formulations, and size of bread was observed ($p > 0.05$).

3.6. Rope spoilage potential of B. licheniformis #414 in different bread formulations

The presence of rope in the standard formulation of bread inoculated

Table 3
"Rope" spoilage potential by *Bacillus* spp. in breads containing or not preservative.

Strain code	Species	Type of flour[b]	Rope spoilage[a]																			
			Breads without preservative						Breads with preservative													
			1st day		2nd day		3rd day		3rd day		4th day		5th day		6th day		7th day		8th day		9th day	
			HT[c]	U	HT	U	HT	U	HT	U	HT	U	HT	U	HT	U	HT	U	HT	U	HT	U
002	*B. licheniformis*	F1	–	+++	+++	+++	+++	+++	–	+	–	++	–	++	–	++	+	++	+	++	+	++
007	*B. licheniformis*	F1	+++	+++	+++	+++	+++	+++	–	++	–	+++	–	+++	–	+++	–	+++	+	+++	+	+++
008	*Paenibacillus polymyxa*	F1	+++	+++	+++	+++	+++	+++	–	++	–	+++	+	+++	+	+++	+	+++	+	+++	+	+++
009	*B. licheniformis*	F1	–	+++	+++	+++	+++	+++	–	+++	–	+++	–	+++	+	+++	+	+++	+	+++	+	+++
013	*B. licheniformis*	F1	++	+++	+++	+++	+++	+++	–	–	–	–	–	–	+	+	+	+	+	+	+	+
019	*B. licheniformis*	F1	–	–	+	–	+	+	–	–	–	–	–	–	–	+	–	+	+	+	+	+
029	*B. licheniformis*	F1	+++	–	+++	–	+++	+	–	–	–	–	–	–	+	–	+	+	+	+	+	+
043	*B. licheniformis*	F1	++	+++	+++	+++	+++	+++	–	–	–	–	–	–	+	+	+	+	+	+	+	+
069	*B. licheniformis*	F1	+++	+++	+++	+++	+++	+++	–	–	–	–	–	–	–	–	+	+	+	+	+	+
073	*B. cereus*	F1	++	+++	++	+++	++	+++	+	+	+	+	+	+	+	+	+	+	+	+	+	+
074	*B. cereus*	F1	+++	+++	+++	+++	+++	+++	–	+++	–	+++	–	+++	–	+++	–	+++	+	+++	+	+++
149	*B. sonorensis*	F2	+++	+++	+++	+++	+++	+++	–	+++	–	+++	–	+++	–	+++	+	+++	+	+++	+	+++
169	*B. licheniformis*	F2	++	+++	++	+++	++	+++	–	+	–	++	+	++	+	++	+	++	+	++	+	++
180	*B. licheniformis*	F2	–	+++	++	+++	++	+++	–	+	–	+	–	+	+	+	+	+	+	+	+	+
207	*B. licheniformis*	F3	+	+++	++	+++	++	+++	–	+	–	+	–	+	+	+	+	+	+	+	+	+
226	*B. cereus*	F3	+++	+++	+++	+++	+++	+++	–	+	–	+	–	+	+	+	+	+	+	+	+	+
238	*B. licheniformis*	F3	–	+++	++	+++	++	+++	–	+++	–	+++	–	+++	–	+++	+	+++	+	+++	+	+++
248	*B. licheniformis*	F3	–	+++	++	+++	++	+++	–	+++	–	+++	–	+++	–	+++	–	+++	+	+++	+	+++
279	*B. licheniformis*	F4	–	+++	++	+++	++	+++	–	++	–	++	–	++	+	++	+	++	+	++	+	++
280	*B. licheniformis*	F4	++	+++	++	+++	++	+++	–	–	–	+	–	+	+	+	+	+	+	+	+	+
451	*B. pumilus*	F4	–	+++	+	+++	+	+++	–	++	–	++	–	++	+	++	+	++	+	++	+	++
456	*B. cereus*	F4	–	+++	++	+++	++	+++	+	++	+	++	+	++	+	++	+	++	+	++	+	++
458	*B. licheniformis*	F4	+	+++	++	+++	++	+++	–	+++	–	+++	–	+++	–	+++	+	+++	+	+++	+	+++
462	*B. licheniformis*	F4	+++	+++	+++	+++	+++	+++	–	+++	–	+++	–	+++	+	+++	+	+++	+	+++	++	+++
472	*B. cereus*	F4	+	+++	++	+++	++	+++	–	–	–	+	–	+	+	+	+	+	+	+	+	+
473	*B. sonorensis*	F4	–	+	+	++	+	++	–	++	–	++	–	++	+	++	+	++	+	++	+	++
484	*B. licheniformis*	F4	++	+++	++	+++	++	+++	–	+	–	+	–	+	–	+	+	+	+	+	+	+
497	*B. sonorensis*	F4	++	+++	++	+++	++	+++	–	++	–	++	–	++	–	++	+	++	+	++	+	++
516	*B. licheniformis*	F4	++	+++	++	+++	++	+++	–	+++	–	+++	–	+++	+	+++	+	+++	+	+++	+	+++
320	*B. sonorensis*	F5	+	+++	+	+++	+	+++	–	++	–	++	–	++	–	++	+	++	+	++	+	++
321	*B. sonorensis*	F5	+	+++	++	+++	++	+++	–	++	–	++	–	++	–	++	+	++	+	++	+	++
336	*B. licheniformis*	F5	+++	+++	+++	+++	+++	+++	–	+	–	+	–	+	–	+	+	+	+	+	+	+
343	*B. licheniformis*	F5	+++	++	+++	++	+++	++	–	–	–	+	–	+	+	+	+	+	+	+	+	+
348	*B. sonorensis*	F5	+	+++	+++	+++	+++	+++	–	+++	–	+++	–	+++	+	+++	+	+++	+	+++	+	+++
354	*B. licheniformis*	F5	+	+++	++	+++	++	+++	+	+	+	++	+	++	+	++	+	++	+	++	+	++
359	*B. sonorensis*	F5	++	+++	+++	+++	+++	+++	–	+++	–	+++	–	+++	+	+++	+	+++	+	+++	+	+++
363	*B. subtilis*	F5	–	++	–	++	+	++	–	+	–	+++	–	+++	–	+++	–	+++	–	+++	+	+++
385	*B. licheniformis*	F5	–	++	+	+++	+	+++	–	–	–	+	–	+	–	+	–	+	+	+	+	+
389	*B. licheniformis*	F5	+	+++	++	+++	++	+++	–	+	–	+	–	+	+	+	+	+	+	+	+	+
396	*B. licheniformis*	F5	+	+++	++	+++	++	+++	–	–	–	+	–	+	+	+	+	+	+	+	+	+
397	*B. licheniformis*	F5	+++	+++	+++	+++	+++	+++	–	+++	–	+++	–	+++	+	+++	+	+++	+	+++	+	+++
414	*B. licheniformis*	F5	–	+++	++	+++	+++	+++	++	+++	++	+++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++
415	*B. sonorensis*	F5	+++	+++	+++	+++	+++	+++	–	+++	–	+++	–	+++	+	++	+	+++	+	+++	+	+++
416	*B. sonorensis*	F5	++	+++	++	+++	++	+++	–	++	–	++	–	++	+	++	+	++	+	++	+	++
418	*B. licheniformis*	F5	+	+++	++	+++	++	+++	–	–	–	–	–	–	+	–	+	–	+	–	+	+

[a] (–) no "rope" development; (+) slight "rope"; (++) moderate "rope"; and (+++) strong "rope".
[b] White cake flour - bakery (F1); White bread flour - bakery (F2); White cake flour - industry (F3); White bread flour - industry (F4); Wholemeal flour - industry (F5).
[c] Heat treated at 96 °C/10min (HT), Untreated (U).

Table 4
Survival of spores (log spores/g) in different bread formulations[b] and sizes[c].

Strain code	Species	Survival of spores (log spores/g)[a]					
		Standard		Low aw		Low pH	
		Small	Large	Small	Large	Small	Large
69	*B. licheniformis*	4.9	4.9	5.3	4.7	5.2	5.0
414	*B. licheniformis*	6.7	6.3	5.9	6.3	5.9	5.6
462	*B. licheniformis*	5.2	5.1	6.3	6.1	5.4	5.6
516	*B. licheniformis*	6.1	6.0	4.7	4.7	5.6	5.5

[a] Mean values of two independent experiments are reported. No statistically significant difference was observed ($p > 0.05$).
[b] Formulations: Standard (pH 6.0, a_w 0.95); low a_w (0.90); low pH (5.6).
[c] Small bread (20 g) and large bread (500 g).

was observed from the fifth day onwards and increased on the subsequent days (Table 5). On the other hand, no rope development was detected in bread with a variation of formulation parameters: low aw, low pH, and containing preservative (calcium propionate) throughout product shelf-life (seven days).

4. Discussion

Wholemeal flour obtained from the industry showed the highest counts for aerobic mesophilic. Wholemeal flour, an unrefined food product, is the result of the grinding of whole grains and, therefore, contains wheat germ and fiber. Thus, it is believed that such flour contains a considerably high microbial load (Saranraj & Geetha, 2012; Vaičiulytė-Funk et al., 2015). On the other hand, even though white flour is a refined food product, if hygienic procedures are not taken during processing and storage steps, it may present a high percentage of microbial contamination (Valerio et al., 2015). According to our results, in general terms, counts of spore forming microorganisms obtained in flour from industry were higher than those determined in bakery samples. This finding is most likely because, in bakeries, flours are obtained in small packages packed after grinding. Nonetheless, in industries, flours are obtained in large amounts and then carried by pipeline to flour barrels, which feed the processing lines and make Good Manufacturing Practices (GMP) challenging to control and contribute to a higher level of contamination of raw materials.

Among 327 Gram-positive SB strains, 45 showed the ability to produce amylase. The relationship between rope formation and starch hydrolysis by microbial amylases has been previously reported (Pepe et al., 2003). The enzymes produced by the strains will act on the bread carbohydrates, thus changing its texture (Valerio et al., 2012; Viedma et al., 2011).

Although *B. subtilis* is considered the most common causative agent of rope (Erem, Inan, Karakaş Budak, & Certel, 2020), in the present study, the genetic diversity of *Bacillus* species in flours was evaluated, and the prevalence of *B. licheniformis* was observed. The species *B. sonorensis*, *B. cereus*, *B. subtilis*, *B. pumilus,* and *B. polymyxa* were also identified. *B. subtilis*, *B. licheniformis*, *B. cereus*, *B. clausii*, *B. firmus*, and *B. sonorensis* were identified in ropy bread (Pepe et al., 2003).

Table 5
Rope spoilage by *B. licheniformis* #414 during shelf-life of different bread formulations.

Formulation[a]	Rope spoilage during shelf-life[b]						
	1st day	2nd day	3rd day	4th day	5th day	6th day	7th day
Standard	–	–	–	–	+	++	+++
Containing preservative	–	–	–	–	–	–	–
Low pH	–	–	–	–	–	–	–
Low a_w	–	–	–	–	–	–	–

[a] Formulations: Standard (pH 6.0, a_w 0.95); low a_w (0.90); low pH (5.6).
[b] No rope (−), slight rope (+), moderate rope (++), advanced rope (+++).

B. amyloliquefaciens was the most frequent species associated with rope in raw materials used in Italian bakery products (Valerio et al., 2012). The high counts of *B. subtilis* and *B. licheniformis* in foods may cause mild symptoms of foodborne diseases (diarrhea/vomiting). *B. cereus* is a major spore forming that can cause food poisoning in humans by producing toxins (Granum & Lund, 1997). This risk tends to increase with the consumption of bread base of whole flour and without preservatives (Saranraj & Geetha, 2012). Therefore, controlling the growth of *Bacillus* species in bakery products is necessary to avoid health risks.

All amylase-producing strains produced rope with and without heat treatment in bread with or without preservative. In the evaluation of rope production, it can be observed that heat treatment (96 °C/10min) caused cell injury, and the presence of calcium propionate worked as a barrier, reducing the speed of rope development even if it did not completely prevent such spoilage.

Although a reduction in the count of rope-producing *Bacillus* was observed after heat treatment (180 °C for 20 min), all strains survived after baking, regardless of bread size and formulation. Strains of *B. amyloliquefaciens*, *B. subtilis* and *B. pumilus* also demonstrated maintenance of the ability to spoilage and amylase activity after heat treatment (100 °C, 10 min) (Valerio et al., 2012). Heat treatment is a crucial method to inactivate microorganisms (non-spore forming bacteria) in food. However, excessive heat treatment can reduce its nutritional values (Park & Yoon, 2018; 2019). Other microorganisms (spore forming bacteria), like *Bacillus subtilis*, *B. licheniformis*, and *B. cereus,* can survive (Sudha, Viswanath, Siddappa, Rajarathnam, & Shashirekha, 2016). In this sense, change product formulation can prolong the life of bakery products.

The addition of calcium propionate and reduction of water activity and pH were able to prevent the development of rope by *B. licheniformis* #414. The pH reduction decreased the thermal resistance of *B. cereus* and helped in the inactivation of the microorganism through heat treatment (Park & Yoon, 2019). The use of LAB-based bioingredients aided in acidification of dough, resulting in greater control of rope spoilage (Mantzourani et al., 2019; Plessas et al., 2020; Valerio, De Bellis, Lonigro, Visconti, & Lavermicocca, 2008).

Results obtained in this study show a variety of *Bacillus* species involved in rope spoilage and the influence of bread formulation on the control of these microorganisms, avoiding both economic losses and potential sources of foodborne diseases. Although currently the use of additives has been discouraged, they are still of extreme importance to assure the quality and safety microbiological of baked products. The use of sourdough and natural substances can also be an alternative to replace chemical preservatives, as long as they can maintain bakery products' safety. In addition to the use of good quality raw materials and the adoption of hygienic practices in the entire production line, new strategies should be implemented to prevent food spoilage, considering the resistance characteristics of spores from bacterial strains to guarantee the quality of bakery products.

CRediT authorship contribution statement

Ana Paula M. Pereira: Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Graziele C. Stradiotto:** Formal analysis, Visualization, Formal analysis, Writing - original draft, Writing - review & editing. **Luísa Freire:** Visualization, Formal analysis, Writing - original draft, Writing - review & editing. **Verônica O. Alvarenga:** Visualization, Formal analysis, Writing - original draft, Writing - review & editing. **Aline Crucello:** Investigation, Visualization, Formal analysis, Writing - original draft. **Letícia L.P. Morassi:** Methodology, Formal analysis, Writing - original draft. **Fabiana P. Silva:** Investigation, Formal analysis, Writing - original draft. **Anderson S. Sant'Ana:** Conceptualization, Methodology, Resources, Data curation, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.110108.

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