Research Article

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The occurrence of spoilage yeasts in cream-filled bakery products

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Abstract

BACKGROUND: Filling creams can provide an adequate substrate for spoilage yeasts because some yeasts can tolerate the high osmotic stress in these products. To discover the source of spoilage of a cream-filled baked product, end products, raw materials, indoor air and work surfaces were subjected to microbiological and molecular analyses. The efficacy of disinfectants against spoilage yeasts was also assessed.

RESULTS: The analyses on end products revealed the presence of the closest relatives to *Zygosaccharomyces bailii* with counts ranging from 1.40 to 4.72 log cfu g^{-1}. No spoilage yeasts were found in the indoor air and work surfaces. Polymerase chain reaction-denaturing gradient gel electrophoresis analysis, carried out directly on filling creams collected from unopened cans, showed the presence of bands ascribed to the closest relatives to *Z. bailii sensu lato,* although with counts < 1 log cfu g^{-1}. Susceptibility testing of yeast isolates to disinfectants showed a significantly lower effect of 10% alkyl dimethyl benzyl ammonium chloride. Different responses of isolates to the tested disinfectants were seen.

CONCLUSION: To guarantee the quality of end products, reliable and sensitive methods must be used. Moreover, hygiene and the application of good manufacturing practices represent the most efficient way for the prevention and minimization of cross-contamination.
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Keywords: air sampling; PCR-DGGE; susceptibility test; disinfectants; bakery hygiene

INTRODUCTION

The baking industry places a wide range of different products such as bread, pastry and other bakery goods on the market daily. From the point of view of the consumer, quality parameters of bakery products can be assessed on the basis of odor, taste and appearance. The factors that can contribute to the production of good quality products are the raw materials quality, the production technology and the plant hygiene.[1] With regard to the raw materials, bakery products can be obtained by mixing basic ingredients such as flour, water or other allowed liquids and leavening agents; moreover, additional fillings, composed of various sweet or savory ingredients, including dried fruits, syrups and creams, can be used.

In particular, filling creams, made by mixing crystallized fats with sugar and other ingredients (i.e. cocoa, chocolate, vanilla, fruits, etc.), can be used as sandwich creams in many confectionery foods such as biscuits and wafers or added to leavened baked products before or after baking. Usually, filling creams include added preservatives such as alcohol, sorbic acid and benzoic acid.[2] However, as with many other foods containing high concentrations of sugar (40–70%), filling creams can provide an adequate substrate for spoilage yeasts that are able to tolerate the conditions of low water activity and high osmotic stress (osmotolerance) in these products.[3] Typical osmotolerant yeast species belong to the genera *Debaryomyces*, *Pichia* and *Zygosaccharomyces*.[4] Among these genera, *Zygosaccharomyces* is the one most commonly associated with the spoilage of sugar-rich products. Moreover, members of the genera *Brettanomyces*, *Candida*, *Kloeckera*, *Rhodotorula*, *Saccharomyces*, *Schizosaccharomyces* and *Torulopsis* are noteworthy as spoilage agents when foods are not produced in accordance with good manufacturing practices.[5]

Spoilage yeasts are able to convert sugars present in filling creams to ethanol, carbon dioxide, alcohols, glycerol, esters, aldehydes, ketones, sulfur volatiles and organic acids.[6] These latter compounds can be responsible for the changes in the acidity and flavor profile of the product, with acetic acid having the strongest sensory impact.[7]

Sources of yeasts contamination are widespread, and they can mainly be traced to the raw materials used during food processing and to the production environment (e.g. indoor air, surfaces, equipment, etc.).

Some studies have addressed yeast spoilage in products with high sugar content, including candied fruits, concentrated fruit juices, cream-filled cakes, syrups and nougat.[3,8] Among these studies, methods to control microbial contamination by the use of specific sanitizers have been evaluated, whereas other research has focused on the monitoring of spoilage yeasts during the production process.[4,9,10]

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However, yeasts contamination of products with high contents of sugar is still an unsolved problem, especially in baked products where filling creams are added after baking; contamination by yeast is particularly insidious because some yeasts are able to form biofilms on process surfaces.[5]

The present study began following customer complaints of atypical tastes in a cream-filled sweet baked product that had been produced in an industrial bakery. To track spoilage microorganisms, seven different batches of end products were analyzed by culture-dependent methods and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). To identify the contamination source, raw materials and environmental samples were also analyzed. Moreover, the efficacy of seven disinfectants commonly used in the food industry was assessed against identified causative agents of spoilage.

MATERIALS AND METHODS

Description of the bakery and sweet baked goods production

The industrial bakery plant was divided into different areas (dough mixing area, leavening room, baking area, filling area and warehouse) for the different production activities. The doughs to be leavened were prepared by mixing all the ingredients in a mixer. They were then placed in fluted paper cases using a calibrated filling machine. The doughs were kept in the leavening room at 25 °C for approximately 2 h and then transferred to the ovens, which were located in the baking area. After baking at 160 °C for 40 min, the sweet leavened goods were allowed to cool to room temperature for approximately 3 h and then filled with different creams (chocolate or vanilla filling cream) using the same machine used for the portioning of the doughs. Prior to their use in filling, the tank and the other parts of the machine were washed and sanitized; suitable filling needles (five) were then mounted on the machine. After being filled, the sweet baked goods were packaged into food-grade plastic bags, placed in paper boxes and stored at room temperature until delivery. Packaged baked goods were delivered in a period that varied from 1 to 4 weeks.

The ingredients used for the production of the sweet baked goods dough were water, wheat flour, saccharose, butter, eggs, milk, glucose syrup, sorbic acid and compressed baker's yeast. Pasteurized chocolate filling cream was composed of: water, saccharose, glucose syrup, palm oil, nut butter, ethyl alcohol, powdered milk, milk proteins, cocoa, acetylated distarch adipate (E1422), pectin (E440i), amidated pectin (E440ii), potassium sorbate (E202) and flavorings. The pasteurized vanilla filling cream was composed of: glucose syrup, water, saccharose, dextrose, powdered milk, egg yolk, butter, ethyl alcohol, acetylated distarch adipate (E1422), amidated pectin (E440ii), potassium sorbate (E202) and flavorings. The pH of the creams was 6.00.

The following batches of baked goods were subjected to microbiological and molecular analyses: 005, 038, 077, 160, 190, 203 and 208. Analyses of filled baked goods and baked goods before filling on batches 005 to 160 were carried out on samples collected from those provided by the bakery's quality control service and stored at room conditions, whereas batches 190 to 208 were analyzed as soon as they were produced.

The numbers of the batches represent their day of production in relation to the calendar year. For easier reading of the results, the original codes of the analyzed batches were renamed as follows: B1 (005), B2 (038), B3 (077), B4 (160), B5 (190), B6 (203) and B7 (208). All the batches were analyzed before the end of shelf-life, which was 10 months from the day of production.

Microbiological analyses of the bakery air

For the analysis of airborne fungal contamination a calibrated impaction sampler (SAS Super 90; VWR International-Pbi, Milan, Italy) was used; the device was placed at the height of 1 m from the floor of each production area.[11] The air sampler allows the microbial cells to be impacted on agarized culture medium in plates, which are then incubated to allow colony growth. The production areas and the air volumes subjected to the airborne yeast analysis are reported in Table 1.

Air samples were kept at +4 °C and transported to the laboratory on the same day of collection for incubation.

Yeasts were counted on modified Yeast Extract Peptone D-glucose (YEPD) medium plates (yeast extract 10 g L^{-1}; peptone 10 g L^{-1}; D-glucose 20 g L^{-1}, Rose Bengal 0.025 g L^{-1}; agar 17 g L^{-1}) containing 100 mg L^{-1} of chloramphenicol (Sigma-Aldrich, St Louis, MO, USA) to inhibit the growth of bacteria.[12] Enumeration of the yeasts was carried out after 5 days of incubation at 25 °C.

The mean value of triplicate samples was calculated, and the results were expressed as colony-forming units per cubic meter (cfu m^{-3}).

Microbiological analyses on work surfaces

The work surfaces subjected to microbiological analyses are reported in Table 2. Samples were collected with sterile swabs and sterile tubes containing 10 mL peptone solution (0.1%) (Oxoid, Basingstoke, UK) as reported by Osimani *et al.*[13] Swabs were maintained at +4 °C and analyzed on the same day of collection. The samples were tenfold diluted in a saline-peptone solution (8.5 g L^{-1} NaCl and 1 g L^{-1} peptone) and 0.1 mL aliquots of each dilution were plated on modified YEPD medium as described above.

The mean value of duplicate samples was calculated, and the results were expressed as colony-forming units per square centimeter (cfu cm^{-2}).

pH measurements

pH values of the sweet leavened goods (final product) and raw materials were measured with a pH meter equipped with an HI2031 solid electrode (Hanna Instruments, Padova, Italy). For each sample, measurements were performed in triplicate.

Microbiological analyses of the filling creams and bakery products

The samples collected were: (i) compressed baker's yeast; (ii) chocolate and vanilla filling creams from unopened cans; (iii) baked products before filling; and (iv) seven batches of filled and packaged baked goods. For each batch of filled and packaged baked products, two samples were analyzed, whereas, for batch B2, four samples were analyzed.

Baker's yeast (10 g) was diluted in sterile peptone-saline solution and then ten-fold diluted in the same solution; aliquots (0.1 mL) of each dilution were used for counting yeasts on modified YEPD agar (as described above). Moreover, 10 g of the same sample underwent enrichment in 90 mL of YEPD broth (yeast extract 10 g L^{-1}; peptone 10 g L^{-1}; D-glucose 20 g L^{-1}) with 100 mg L^{-1} of chloramphenicol,[13] incubated for 5 days at 25 °C and then streaked on modified YEPD agar and incubated again for 5 days at 25 °C.

Yeasts from the baked products (before filling) and filled products were enumerated following culture on modified YEPD agar with 100 mg L^{-1} of chloramphenicol added and incubation for 5 days at 25 °C. Aliquots (10 g) of the same matrices underwent

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Table 1. Airborne eumycetes detected in the bakery production plant

Air samples	Volume (L)	Counts, mean ± SD (cfu m^{-3})	Bands[a]	Closest relatives	Identical (%)[b]	Accession number[c]
Leavening room	500	12 ± 1.5	5	*Saccharomyces cerevisiae*	96%	HM107799
			6	*Aspergillus sydowii*	98%	JN938955
Filling area	500	50 ± 2.6	1, 3	*Penicillium chrysogenum*	98%	EU146308
			2	*Cladosporium* sp.	97%	EU715631
			4	*Penicillium solitum*	95%	JX290030
Filling area	250	48 ± 2.6	7, 8	*Penicillium solitum*	95%	JX290030
Dough mixing area	500	44 ± 0.6	9	*Cladosporium coralloides*	95%	JQ388759
			10	*Cladosporium halotolerans*	95%	KF417564
			11	*Alternaria alternata*	94%	KC445303
			12	Failed	–	–
			13	*Penicillium solitum*	93%	JX290030
			14	*Penicillium citrinum*	93%	KJ880094
Baking area	500	66 ± 1.5	15	*Cladosporium coralloides*	95%	JQ388759
			16	*Penicillium chrysogenum*	94%	EU862182
			17	*Penicillium solitum*	95%	JX290030
			18	*Penicillium citrinum*	93%	KJ880094

[a] Bands are numbered as indicated in Fig. 2.
[b] Percentage of identical nucleotides in the sequences obtained from the colonies and the sequences of the closest relative found in the GenBank database.
[c] Accession number of the sequence of the closest relative found by BLAST search tool.

enrichment in 90 mL of YEPD broth with 100 mg L^{-1} of chloramphenicol, incubated for 5 days at 25 °C and streaked on modified YEPD agar containing 100 mg L^{-1} of chloramphenicol.

Two batches of chocolate and vanilla filling creams (used to produce batches B3 to B7 of the filled and packaged baked products) were collected under sterile conditions from unopened cans (25 kg each) and analyzed for yeast counts on modified YEPD agar (as described above); enumeration of the yeasts was conducted after 5 days of incubation at 25 °C. Aliquots (10 g) of all of the cream samples were subjected to enrichment in 90 mL of YEPD broth with 100 mg L^{-1} of chloramphenicol, incubated for 5 days at 25 °C and streaked on modified YEPD agar containing 100 mg L^{-1} of chloramphenicol.

The mean value of duplicate samples was calculated, and the results were expressed as log colony-forming units per g (log cfu g^{-1}).

Formation of bulk cells

Colonies grown on the modified YEPD agar plates were suspended in 2 mL of saline solution and glycerol (0.85% NaCl, 50% glycerol), harvested with a sterile pipette and stored at −20 °C. Bulk cells were obtained from plates spiked with both the highest and lowest dilution.

DNA extraction from the bulk cells, filling creams and bakery products

The DNA from the bulk cells was extracted as described by Makimura *et al.*[14]

Microbial direct DNA extraction from the filling creams and bakery products, was carried out using the PowerFood Microbial DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA).

Optical measurements at 260, 280 and 234 nm, using a ultraviolet-visible Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan), were carried out to assess the DNA quantity and purity.

PCR-DGGE analyses

Fungal DNA (100 ng), extracted directly from the samples and from the bulk cells, was amplified via PCR in a 50-μL reaction volume using $NL1_{GC}$ and LS2 primers (Table 3), producing a fragment of 240 nucleotides near the 5′-end region of the 26S rRNA gene.[15] A GC clamp was attached to the NL1 primer as proposed by Sheffield *et al.*[16] The PCR mixture and conditions (Table 3) have been described by Osimani *et al.*[17]

The PCR product (5 μL) was checked by electrophoresis in a 1.5% (w/v) agarose gel as described by Osimani *et al.*[17]

DGGE analysis was carried out with a vertical DCode electrophoresis system (Bio-Rad Laboratories, Hercules, CA, USA) as described by Osimani *et al.*[17] Briefly, PCR products (25 μL) were applied to a 0.8-mm polyacrylamide gel, containing a denaturing gradient (30–60% urea–formamide) increasing in the direction of the electrophoresis.

The DGGE bands were cut from the gels, eluted in 50 μL sterile deionized water overnight at 4 °C and subsequently amplified under the same conditions as described above but using the primer NL1 without the GC clamp. PCR amplicons were sent to Beckman Coulter Genomics (London, UK) for purification and sequencing. The sequences were compared with those deposited in the GenBank DNA database (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool.[18]

Isolation and molecular identification of yeasts

Yeast colonies were selected on the basis of colony morphology, and an adequate number of colonies were picked, equivalent to 10% of the colonies grown on the modified YEPD agar plates at the highest sample dilution. Cell morphology was observed with a light (×100) microscope and the DNA was extracted as explained by Makimura *et al.*[14] The quantity and purity of the extracted DNA were assessed as described above. Yeast isolates were stored at −80 °C in a glycerol-YEPD (1:1) mixture.

Isolates were first subjected to molecular identification by sequencing a portion of the region of the 26S rRNA gene, using

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Table 2. Eumycetes detected on surface samples collected in the bakery production plant

Surface samples	Area (cm^2)	Counts, mean ± SD ($cfu\ cm^{-2}$)	Bands[a]	Closest relatives	Identical (%)[b]	Accession number[c]
Leavening room wall 1	100	1 ± 0.7	21, 22, 24, 25	*Clavispora lusitaniae*	96%	KC442253
			19	*Penicillium citrinum*	91%	AB284185
			20, 23	*Saccharomyces cerevisiae*	97%	HM107796
				Arthrinium pterospermum	90%	NG042786
Leavening room wall 2	100	ND		ND	ND	ND
Leavening room ventilation grid	100	1.6 ± 0.9	36	*Mucor plumbeus*	96%	JX123134
			37, 38	*Penicillium chrysogenum*	94%	EU862182
Metal filling needle 1	5	ND		ND	ND	ND
Metal filling needle 2	5	ND		ND	ND	ND
Metal filling needle 3	5	ND		ND	ND	ND
Metal filling needle 4	5	ND		ND	ND	ND
Metal filling needle 5	5	ND		ND	ND	ND
Filling needles plate 1	100	0.4 ± 0.0	26	*Cladosporium*	94%	AB572904
			27	*sphaerospermum Penicillium chrysogenum*	96%	EU862182
Filling needles plate 2	100	ND		ND	ND	ND
Filling cream tank	100	1 ± 0.4	28	failed	–	–
			29	*Saccharomyces cerevisiae*	95%	KM103041
Filling machine piston 1	25	12 ± 2.1	30	*Saccharomyces cerevisiae*	97%	HM107796
Filling machine piston 2	25	0.2 ± 0.0	31	*Candida parapsilosis*	96%	JX463229
Filling machine cylinder 1	100	ND		ND	ND	ND
Filling machine cylinder 2	100	0.2 ± 0.0	32, 35	*Penicillium citrinum*	98%	AB284185
			33	*Arthrinium pterospermum*	90%	NG042786
			34	*Penicillium chrysogenum*	95%	EU862182

[a] Bands are numbered as indicated in Fig. 2.
[b] Percentage of identical nucleotides in the sequences obtained from the colonies and the sequences of the closest relative found in the GenBank database.
[c] Accession number of the sequence of the closest relative found by BLAST search tool.
ND, not detected.

Table 3. Primers and PCR conditions

Primer	Sequence	PCR conditions: Initial denaturation	Cycle	Final extension
$NL1_{GC}$	5′-GCC ATA TCA ATA AGC GGA AAA G-3′	95 °C for 5 min	30 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 45 s and extension at 72 °C for 1 min	72 °C for 7 min
LS2	5′-ATT CCC AAA CAA CTC GAC TC-3′			
fRPB2–5F	5′-GAYGAYMGWGATCAYTTYGG-3′	95 °C for 5 min	30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 2 min and extension at 72 °C for 2 min	72 °C for 10 min
fRPB2-7cR	5′-CCCATRGCTTGYTTRCCCAT-3′			
M13	5′-GAG GGT GGC GGT TCT-3′	94 °C for 5 min	40 cycles of denaturation at 94 °C for 1 min, annealing at 45 °C for 1 min and extension at 72 °C for 2 min	72 °C for 5 min
OPA02	5′-TGC CGA GCT G-3′	94 °C for 5 min	40 cycles of denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min and extension at 72 °C for 2 min	72 °C for 10 min

primers NL1 and LS2 as described above. Because the sequencing of this region of the 26S rRNA gene did not allow the unequivocal identification, the DNA extracted from the isolates was further amplified by PCR using the degenerate primers fRPB2–5F and fRPB2-7cR (Table 3), which amplified approximately 1200 nucleotides of the 5–7 region of the second-largest subunit gene (RPB2) of RNA polymerase II.[19] Template DNA (100 ng) was amplified in a 50-μL reaction volume containing 1.25 U of Taq DNA polymerase (SibEnzyme Ltd, Novosibirsk, Russia), 1 × reaction buffer, 0.2 mmol L^{-1} dNTPs and 0.2 μmol L^{-1} of each primer, using the cycling program reported in Table 3.

The PCR products were purified and as described above. The closest relatives to the sequences obtained were determined through searches in the GenBank DNA database using the Basic Local Alignment Search Tool algorithm.[18]

Molecular typing of yeasts

Randomly Amplified Polymorphic DNA (RAPD) analysis was carried out for the molecular typing of yeasts. The primers M13 and OPA02 (Table 3) were used as described by Osimani *et al.*[17] Briefly, genomic DNA (100 ng) underwent PCR in a 25-μL reaction volume,

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Table 4. The coded commercial disinfectants tested against yeast isolates.

Disinfectants	Composition	In-use concentration
D1	20–30% isopropyl alcohol; 0.005% didecyl dimethyl ammonium chloride	100%
D2	30–50% isopropyl alcohol	100%
D3	10% alkyl dimethyl benzyl ammonium chloride	0.5%
D4	< 25% ethanol; < 0.5% didecyl dimethyl ammonium chloride; < 0.5% *N*-(3-aminopropyl)-*N*-dodecylpropane-1,3diamine	25%
D5	3% active chlorine	1%
D6	1.5% alkyl dimethyl benzyl ammonium chloride	100%
D7	0.5–0.6 benzalkonium chloride; 9-14% isopropyl alcohol	100%

The compositions and in-use concentrations are based on information from the manufacturers.

then each PCR product (10 μL) was subjected to electrophoresis in a 2.5% (w/v) agarose gel as described by Osimani *et al.*[17]

Susceptibility of yeast isolates to disinfectants

Seven commercial disinfectants (D1, D2, D3, D4, D5, D6 and D7) commonly used in the food industry were studied in the suspension test. More specifically, these disinfectants were provided by the bakery under study, a mass catering plant and a large food retailer. Disinfectant D3, composed of 10% alkyl dimethyl benzyl ammonium chloride and used at 0.5% concentration, was the disinfectant used by the bakery during the production of all the batches under study. The antimicrobial compounds tested and the in-use concentrations are listed in Table 4. The preparation of the dilutions to obtain the in-use concentrations of the disinfectants was conducted at the beginning of each test to prevent any evaporation of the active compound and the relative loss of efficacy. The susceptibility of yeast isolates to the disinfectants was assessed after exposure for 5 min at 20 °C.[5] Briefly, for each isolate, 1 mL of broth culture (16–18 h of growth) was inoculated into an aqueous solution of disinfectant formulation to obtain approximately 5–6 log cfu mL^{-1}. After 5 min, 1 mL of each sample was transferred to 9 mL of D/E (Dey/Engley) Neutralizing Broth (Becton Dickinson, Franklin Lakes, NJ, USA). The samples were diluted with peptone saline and cultured on YEPD medium within 15–30 min after the treatment. The incubation was performed at 25 °C for 5 days. The suspension test was performed in duplicate for each isolate.

The results were expressed as microbiocidal effect based on the survival relative to the appropriate controls exposed to saline solution.

Statistical analysis

RAPD profiles were subjected to cluster analysis using the Phoretix 1D, version 11.4 (Nonlinear Dynamics, Durham, NC, USA) as reported by Osimani *et al.*[17]

Regarding susceptibility of the yeast isolates to disinfectants, all microbiological data were log-transformed before statistical analysis. Analysis of variance (ANOVA) was carried out using JMP, version 11.0.0 (SAS Institute Inc., Cary, NC, USA) to test the main effects: disinfectants (D1, D2 … D7); isolate (z1, z2 … z21). $P > 0.0001$ was considered nonsignificant. Conformance to a normal distribution and identification of outliers were carried out before statistical analysis.

RESULTS AND DISCUSSION

All analyzed batches were characterized by filling cream pH values between 4.35 ± 0.02 and 5.89 ± 0.01 (Table 5). These values were not significantly different from those of the respective portion of baked doughs. The observed drop in pH of the creams was probably a result of the interaction between the filling cream (initially at 6.00) and the baked product during storage.

The analyses carried out on the baked products before filling showed yeast counts < 1 log cfu g^{-1} in all batches. Regarding the microbiological analyses carried out on the batches after filling, the viable yeast counts carried out on the cream portions ranged from 1.40 ± 0.01 to 4.72 ± 0.03 log cfu g^{-1}. The differences in the contamination of the analyzed cream portions might be explained as the result of two main causes: (i) different initial contamination levels and (ii) the time elapsed between the production and the analysis of baked products (which varied from 6 months to a few days, depending on the day of production), during which the contaminating yeast could multiply. No viable yeast counts were found in the baked products showing no spoilage (batches B1, B6 and B7) (Table 5).

From the PCR-DGGE analyses of the DNA recovered from the bulk cells (both in high and low dilution agar plates), the closest relatives to *Zygosaccharomyces bailii sensu lato* were found to be ubiquitous (Fig. 1 and Table 5). With regard to the unambiguous identification of *Z. bailii* and related yeasts to the species level using conventional taxonomic tests, some problems can occur.[20] For example, the sequencing of the region of the 26S rRNA gene with the NL1 and LS2 primers did not allow the unequivocal identification of the DNA excised from DGGE gels to the species level; indeed, the alignment of the sequences for *Z. baili/parabailii* resulted in 99% identities. Hence, the *Z. bailii sensu lato* designation was adopted.

Zygosaccharomyces spp. are major causative agents for the spoilage of beverages and foods because they are able to adapt to high glucose concentrations and to grow at low pH and in the presence of high levels of preservatives such as the potassium sorbate used in the formulation of the filling creams under investigation.[21] Three species, namely *Z. bailii*, *Zygosaccharomyces rouxii* and *Zygosaccharomyces lentus*, are usually associated with the spoilage of high-sugar products, such as honey, syrup and concentrated fruit juices, as a result of their unique physiological characteristics.[8] Recently, two new species, *Zygosaccharomyces parabailii* and *Zygosaccharomyces pseudobailii*, which are closely related to *Z. bailii*, have been reported.[21]

Many studies have categorized the spoilage effects of *Zygosaccharomyces* species, reporting (i) visible growth on the surface of the product and (ii) fermentative spoilage manifested by alcoholic, esteric or other types of odors and/or visible evidence of gas production, leading to bubbling of the product and/or expansion of flexible packaging.[8,22,23] In the present study, no visible alteration of the products was noted; moreover, none of the samples of the batches investigated showed packaging alterations or gas formation. This finding is probably a result of the small amount (approximately 40 g) of the cream filling in the baked products.

The results of the microbiological and PCR-DGGE analyses carried out on the commercial baker's yeast highlighted the presence

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Table 5. Microbial counts and sequencing results of the bands excised from the fungal DGGE gel from the creams of filled baked products.

Analyzed batches	Filling cream	Days of storage	Spoilage[a]	pH of the cream, mean ± SD	Counts (log cfu g^{-1}, mean ± SD)	Closest relatives	Identical (%)[b]	Accession number[c]
B1	Vanilla	210	–	5.08 ± 0.02	< 1	ND	ND	ND
B1	Vanilla	210	–	5.10 ± 0.01	< 1	ND	ND	ND
B2	Chocolate	150	+	4.37 ± 0.03	3.48 ± 0.02	*Zygosaccharomyces bailii sensu lato*	99%	FJ914902[T]
B2	Chocolate	150	+	4.40 ± 0.03	3.07 ± 0.01	*Zygosaccharomyces bailii sensu lato*	99%	FJ914902[T]
B2	Chocolate	150	+	4.37 ± 0.02	3.64 ± 0.01	*Zygosaccharomyces bailii sensu lato*	99%	FJ914902[T]
B2	Chocolate	150	+	4.41 ± 0.02	3.28 ± 0.05	*Zygosaccharomyces bailii sensu lato*	99%	FJ914902[T]
B3	Vanilla	120	+	5.17 ± 0.01	3.46 ± 0.02	*Zygosaccharomyces bailii sensu lato*	99%	FJ914902[T]
B3	Vanilla	120	+	5.15 ± 0.01	3.11 ± 0.02	*Zygosaccharomyces bailii sensu lato*	99%	FJ914902[T]
B4	Chocolate	60	+	4.35 ± 0.02	4.72 ± 0.03	*Zygosaccharomyces bailii sensu lato*	99%	FJ914902[T]
B4	Chocolate	60	+	4.38 ± 0.02	4.23 ± 0.05	*Zygosaccharomyces bailii sensu lato*	99%	FJ914902[T]
B5	Chocolate	0	+	5.30 ± 0.01	2.11 ± 0.02	*Zygosaccharomyces bailii sensu lato*	99%	FJ914902[T]
B5	Chocolate	0	+	5.32 ± 0.01	1.40 ± 0.01	*Zygosaccharomyces bailii sensu lato*	99%	FJ914902[T]
B6	Vanilla	0	–	5.50 ± 0.01	< 1	ND	ND	ND
B6	Vanilla	0	–	5.48 ± 0.02	< 1	ND	ND	ND
B7	Chocolate	0	–	5.89 ± 0.01	< 1	ND	ND	ND
B7	Chocolate	0	–	5.87 ± 0.01	< 1	ND	ND	ND

For batches B1, B3, B4, B5, B6 and B7, two samples were analyzed, whereas, for batch B2, four samples were analyzed.
ND, not determined.
[a] +, cream showing spoilage; −, cream showing no spoilage.
[b] Percentage of identical nucleotides in the sequences obtained from the isolates and the sequences of the closest relatives found in the GenBank database.
[c] Accession number of the sequence of the closest relative found by BLAST search .
[T]Type strain.

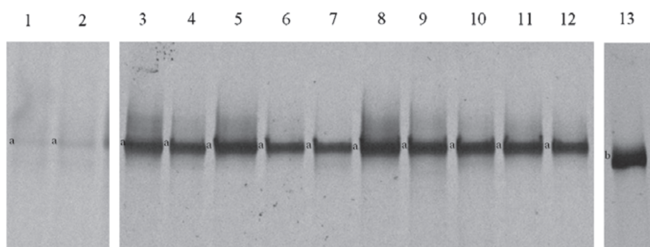

Figure 1. PCR-DGGE profiles of the yeasts present in the samples of filling creams and baker's yeast, obtained with primers $NL1_{GC}$ and LS2, which produced a fragment spanning approximately 240 nucleotides near the 5′-end region of the 26S rRNA gene. The bands indicated by letters were excised, reamplified and subjected to sequencing: [a]*Zygosaccharomyces sensu lato* and [b]*Saccharomyces cerevisiae*. Lanes 1 and 2 represent yeast DGGE profiles of the DNA extracted directly from chocolate and vanilla filling creams, respectively. Lanes 3 (batch B2), 4 (batch B2), 5 (batch B2), 6 (batch B2), 7 (batch B3), 8 (batch B3), 9 (batch B4), 10 (batch B4), 11 (batch B5) and 12 (batch B5) represent yeast DGGE profiles of the DNA extracted from bulk cells obtained from viable counts. Lane 13 represents yeast DGGE profile of the DNA extracted directly from the baker's yeast bulk cells obtained from viable counts.

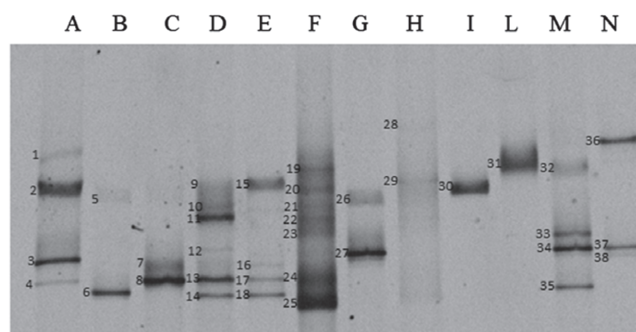

Figure 2. PCR-DGGE profiles of yeasts present in the bulk cells collected from the air and surface samples, obtained with primers $NL1_{GC}$ and LS2, which produced a fragment spanning approximately 240 nucleotides of the 5′-end region of the 26S rRNA gene. Air samples: A, filling area (500 L); B, leavening room; C, filling area (250 L); D, dough mixing area; E, baking area. Surface samples: F, leavening room wall 1; G, filling needles plate 1; H, filling cream tank; I, filling machine piston 1; L, filling machine piston 2; M, filling machine cylinder 2; N, leavening room ventilation grid. Bands indicated by numbers were excised, reamplified and subjected to sequencing. Identification of the bands is reported in Tables 1 and 2.

of *Saccharomyces cerevisiae* as the sole yeast species present (Fig. 1).

Concerning the two batches of filling creams (chocolate and vanilla) collected from unopened cans used for the production of batches from B3 to B7, yeast counts were < 1 log cfu g^{-1} in all of the samples; moreover, no yeast growth was observed after enrichment. Despite this, the PCR-DGGE analyses showed the presence of bands ascribed to the closest relatives to *Z. bailii sensu lato* (Fig. 1). This finding can probably be attributed to the presence of DNA from dead yeast cells after the pasteurization treatment of the creams. No batches of filling creams used for the production of baked goods batches B1 and B2 were available at the time of the investigation.

The results of the microbiological analyses on the indoor air are reported in Table 1. The viable eumycetes counts were generally low. The colonies grown on plates were collected in bulk, and the extracted DNA was analyzed with PCR-DGGE (Fig. 2). The closest relatives, the percent identities and the accession numbers of the obtained sequences are given in Table 1. The closest relatives to the genus *Penicillium* were found in all of the environments,

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with the exception of the leavening room, which showed the presence of the closest relatives to *Aspergillus* and *S. cerevisiae*. The presence of the closest relatives to the genera *Cladosporium* and *Alternaria* was also detected. *Cladosporium*, *Penicillium* and *Aspergillus* represent the most common fungi in the atmosphere; these genera produce high quantities of small and light spores, and this certainly allows their dominance in bioaerosols.[24] By contrast, the genus *Alternaria* produces a low quantity of bigger and heavier spores, which tend to settle faster.[25] Many species of *Alternaria* and *Penicillium*, which are phylogenetically related, are associated with the same habitats or substrates such as soil, indoor environments, cereals, fruits and vegetables. The presence of *S. cerevisiae* in the indoor air of the leavening room was likely to have been favored by the leavening process itself.

The results of the microbiological analyses performed on the selected work surfaces are reported in Table 2. In detail, yeast colonies were not found on the modified YEPD agar plates from the filling needles and were occasionally found on the leavening room wall and filling needle plate. The PCR-DGGE analyses of the DNA extracted from the bulk cells revealed the presence of the closest relatives to molds and *S. cerevisiae* (Fig. 2).

The presence of *Z. bailii sensu lato* was never detected in the bakery environment, thus indicating that, during the study period, *Z. bailii sensu lato* was not widespread. Hence, the source of contamination remained uncertain.

According to the results of the analyses, the source of contamination of the filling creams can only be hypothesized. Indeed, at the time of the analysis, the production facilities had already undergone a thorough sanitizing with bleach. Nevertheless, the presence of fungal DNA related to *Z. bailii sensu lato* in the unopened cans of filling creams throws a shadow on the quality of the same, suggesting that the contamination of the products and the environment could be a result of the possibility that previous batches of filling creams were contaminated consequent to a failure in pasteurization.

To better characterize the spoilage causative yeasts, 21 yeast isolates were subjected to molecular identification and typing (RAPD analysis). The fingerprints obtained were subjected to UPGMA cluster analysis, and the 21 isolates formed one cluster with a similarity of 100%, thereby indicating no presence of different biotypes (data not shown).

The results of sequencing of a portion of the region of the 26S rRNA gene, using primers NL1 and LS2, although confirming the results of the PCR-DGGE analyses on the presence of *Zygosaccharomyces* in the filling creams, did not allow the unequivocal identification of the isolates at the species level. Hence, a new set of primer was used (fRPB2-5F and fRPB2-7cR). The alignment of the sequences of the 5–7 region of the second-largest subunit (RPB2) of the RNA polymerase II gene amplicons the 21 isolates with those published for the species *Z. bailii* (Accession number HG316458) resulted in identities of 99% for all isolates.[19]

The economic importance of *Zygosaccharomyces* spp. as spoilage organisms makes their rapid identification a high priority because an informed decision on cleaning procedures can be made only after the causative agent has been defined.[26]

To optimize cleaning and sanitation procedures, the 21 isolates were further tested for their susceptibility to seven commercial disinfectants and cleaning agents commonly used in the food industry.

The standardized suspension tests require a 4-log-unit reduction of the yeast cells for the agent tested to be recognized as effective.[5]

Among the seven tested disinfectants, ANOVA showed a significantly lower effect of the disinfectant used by the bakery (D3) on the isolates compared to the other disinfectants, with an average reduction to 3.9 ± 1.51 log cfu mL^{-1}. However, all tested disinfectants reached the threshold of the 4-log-unit reduction (Fig. 3a).

Among the 21 isolates, ANOVA showed different responses to the disinfectants. Specifically, isolate z1 showed the highest log cfu mL^{-1} values of surviving cells after treatments with respect to the other isolates. Conversely, isolates z5, z11, z12, z13, z15, z16, z17 and z19 showed the highest susceptibility to the disinfectants (Fig. 3b).

To our knowledge, few recent studies report data on the resistance of *Z. bailii* or other yeasts to disinfectants. The results of the susceptibility test of the *Z. bailii* isolates to the disinfectants confirmed the efficacy of the alcohol-based compounds (D1, D2, D4 and D7) against the suspended vegetative yeast cells, as reported previously by Salo and Wirtanen.[5] Regarding chlorine-based disinfectants, Salo and Wirtanen[5] found that the disinfectants containing chlorine were ineffective in destroying yeast cells in suspensions. However, the chlorine-based disinfectants (D3, D5 and D6) tested in the present study were found to be effective against planktonic yeast cells, although the disinfectant D3 was the least-effective compound among all of the tested substances, partially confirming the results of Salo and Wirtanen.[5]

The prevention of spoilage is mainly based on the application of hygiene and good manufacturing practices, which are often the most effective methods for managing microbial contamination.[6]

To prevent plant contamination, raw materials and ingredients (e.g. creams and syrups) should be free of yeasts through the adoption of appropriate specifications. Regarding environmental contamination, effective cleaning and sanitation of the equipment and process lines is another basic requirement, together with the use of appropriate detergents and disinfectants. Regarding the sanitizing of the surfaces under study, given the proven efficacy of the disinfectant, the suspicion exists that the contamination of the batches of bakery products might be a result of noncompliance of the contact times for the disinfectant used by the bakery staff, which would lead to cross-contamination between raw material (filling creams) and improperly sanitized surfaces. Therefore, the adequate training of staff is of fundamental importance and may be the key factor that can positively or negatively affect the production of good quality foods.

CONCLUSIONS

To guarantee the quality of the end product, routine monitoring must be part of an overall strategy to manage yeast spoilage, and therefore reliable and sensitive yeast detection methods need to be used. In the last decade, newly developed sequencing strategies, commonly referred to as next-generation DNA sequencing techniques, have successfully been applied in food microbiology; nevertheless, surprisingly, the PCR-DGGE technique still proves to be a valid tool for the profiling of spoilage microbial communities that can occur in the food industry. Indeed, the PCR-DGGE technique, coupled with a culture-dependent method, was found to be a useful tool for identifying *Z. baili* as the spoilage agent in filling creams. Unfortunately, the source of contamination was not clarified because the yeast was not detected in either the environment or the raw materials. Despite this, PCR-DGGE showed the presence of *Z. bailii sensu lato* DNA in the filling creams collected from unopened cans. To prevent food spoilage, the application of good manufacturing practices is pivotal. In the food industry,

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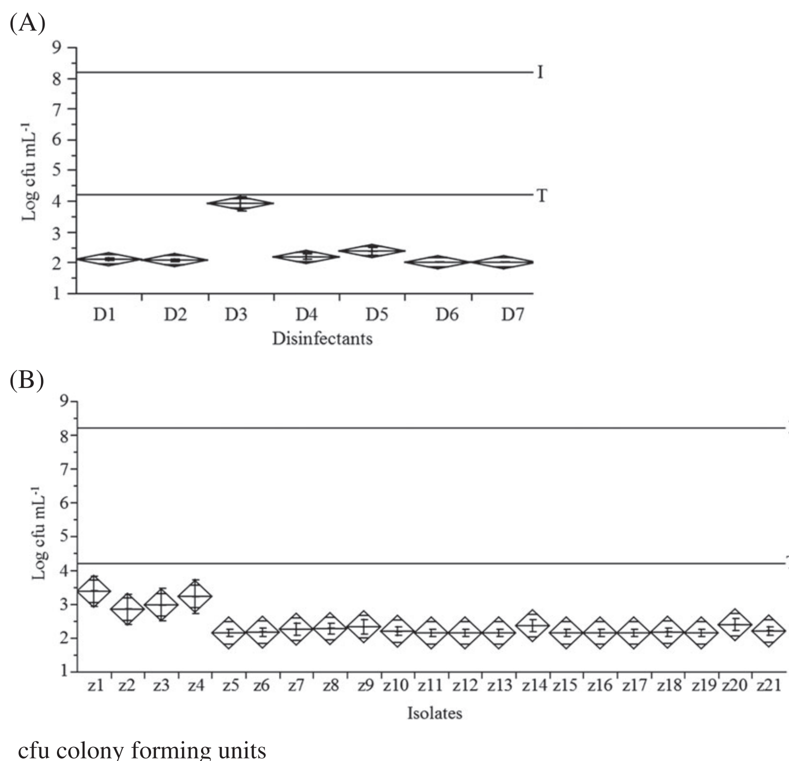

Figure 3. ANOVA performed to test the following main effects: disinfectants (a) and *Zygosaccharomyces bailii* isolates (b). Disinfectants were coded as reported in Table 3. Differences were considered nonsignificant at $P > 0.0001$. I, Initial average value of yeasts count (8.2 log cfu mL^{-1}). T, Threshold of the microbicidal effect (4.2 log cfu mL^{-1}). The threshold of the microbicidal effect was set at a 4-log-unit reduction in the yeast cells. Each diamond represents the mean of each count; the line across each diamond represents the count mean; the height of each diamond represents the 95% confidence interval for each count; the diamond width represents the sample size

effective cleaning and sanitation of the equipment and process lines represents a basic requirement for the prevention and minimization of cross-contamination. Interestingly, different responses of the *Z. bailii* isolates to the disinfectants were observed; furthermore, the lowest effect of a disinfectant was observed with the 10% alkyl dimethyl benzyl ammonium chloride used at a 0.5% concentration on the isolates.

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