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Immobilization of *kefir* and *Lactobacillus casei* on brewery spent grains for use in sourdough wheat bread making

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Abstract

New types of bread were produced employing baker's yeast, *kefir* or *Lactobacillus casei* immobilized on brewer's spent grains. Bread was produced either by the straight-dough or the sourdough method. All the studied biocatalysts and their corresponding sourdoughs were found efficient for breadmaking. Good rising was achieved and the produced breads had good overall quality and remained fresher for longer, compared to commercial type baker's yeast bread. The best results were obtained for sourdough breads, with higher moisture retention during baking, lower rates of water evaporation and staling, and maintenance of freshness for longer (4–5 days). Consumer evaluation showed bigger preference for the sourdough breads as far as aroma, taste and overall quality were concerned, justified by the GC-MS analysis of volatiles that revealed a different aroma profile, with more compounds identified than in breads produced by the straight-dough method, obviously due to variations in microbial populations.

Keywords: Bread; Sourdough; Immobilization; Baker's yeast; *Kefir*; *Lactobacillus casei*; Brewer's spent grains

1. Introduction

Research in breadmaking focuses mainly on new technologies to achieve better mechanical properties of doughs, extend preservation time, improve flavour and enhance the nutritional quality of bread. Technologies, imitating traditional processes, such as the use of sourdough, have been recently employed to satisfy the demand of consumers for natural or "*clean*" technologies (Collar Esteve, Benedito de Barber, & Martinez-Anaya, 1994; Linko, Javanainen, & Linko, 1997). Sourdough is a mixture of flour and water, containing yeasts and lactic acid bacteria (LAB), used as the starter culture to leaven bread. The use of sourdough has a number of important advantages over baker's yeast, such as the development of characteristic flavor (Czerny & Schieberle, 2002; Hansen & Hansen, 1996; Hansen & Schieberle, 2005) and texture (Meignen et al., 2001), as well as extension of preservation time through the in situ production of antimicrobial compounds (organic acids, bacteriocins etc.) (Katina, Sauri, Alakomi, & Mattila-Sandholm, 2002; Messens & De Vuyst, 2002).

Kefir is a natural mixed culture used for centuries in the areas around Caucasus for the production of the traditional fermented milk drink. Many microorganisms, sharing symbiotic relationships, have been isolated from *kefir* microflora, including yeasts (*Kluyveromyces*, *Candida*, *Torulopsis* and *Saccharomyces* sp.), lactobacilli (*Lactobacillus brevis*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus helveticus*, *Lactobacillus delbruecki* etc.), streptococci (*Streptococcus salivarius*), lactococci (*Lactococcus lactis* ssp. *thermophilus*, *Leuconostoc cremoris*, *Leuconostoc mesenteroides* etc.) and occasionally acetic acid bacteria (Simova et al., 2002). The use of *kefir*

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instead of baker's yeast for straight-dough bread making has been reported (Plessas, Pherson, Bekatorou, Nigam, & Koutinas, 2005), leading to bread of good quality, resembling traditional sourdough bread. Rising was satisfactory, and breads produced with *kefir* retained more moisture, had a firmer texture, higher acidity, better flavour (according to consumer evaluation) and retained their freshness for longer compared to baker's yeast bread. *L. casei* on the other hand, is a known and extensively studied probiotic, found in most fermented milk products and has numerous research applications for the production of probiotic dairy products. *L. casei* has also been identified in sourdough microflora of traditional baking products (De Vuyst & Neysens, 2005). However, little research has been done on the use of *L. casei* for bread making either to improve sensory characteristics or to produce probiotic bread. The survival of probiotic bacteria is a serious issue for food technologists. Immobilization techniques, such as encapsulation in alginates or chitosan, in foods like digestive juices (Koo, Cho, Huh, Baek, & Park, 2001) or yogurt (Krasaekoopt, Bhandari, & Deeth, 2006) have been found to improve their survival. *L. casei* immobilized on gluten pellets (Chronopoulos et al., 2002) or starch-milk containing matrices (Plessas, Bekatorou, Kanellaki, Psarianos, & Koutinas, 2005), has been proved effective for lactic acid production for potential use in food production. Also, *L. casei* immobilized on delignified cellulosic materials has been used successfully for malolactic fermentation in wine (Agouridis, Bekatorou, Nigam, & Kanellaki, 2005).

Cereals contain non-digestible carbohydrates that can act as prebiotics, allowing growth and survival of probiotic bacteria in the colon. Therefore, they can be used either as food additives or as fermentation substrates for the production of novel foods with probiotic properties (Charalampopoulos, Pandiella, & Webb, 2003; Charalampopoulos, Wang, Pandiella, & Webb, 2002). An alternative suggestion is to use cereals as matrices for cell inclusion to function both as biocatalysts for food fermentation processes and as probiotic food additives. Studies have verified that the use of immobilized systems in fermentation processes presents many advantages over the use of conventional free cells (Kourkoutas, Bekatorou, Banat, Marchant, & Koutinas, 2004). Among substrates for cell immobilization, brewer's spent grains (BSG) have been found to be very efficient, due to their high yeast loading capacity, easy preparation and regeneration, availability, low cost and inert nature under fermentation conditions (Almeida, Branyik, Moradas-Ferreira, & Teixeira, 2003; Branyik, Vicente, Machado-Cruz, & Teixeira, 2001). BSG is the residue left after the separation of wort during the brewing process (Santos, Jimenez, Bartolome, Gomez-Cordoves, & del Nozal, 2003). It contains most of the protein, lipids and fibre contents of the original barley grain. Due to the large continuous supply, relatively low cost and potential nutritional value, BSG has been considered as an attractive adjunct for human food (Hassona, 1993). So far, high fiber bread containing brewer's spent grain has been produced and has been shown to contribute in the prevention of the increase of both plasma total lipids and cholesterol in rats (Hassona, 1993).

The aim of this study was the production of straight-dough and sourdough bread using baker's yeast, *kefir* and *L. casei* immobilized on brewer's spent grains, and the evaluation of the quality of the new types of breads compared to commercial type baker's yeast bread.

2. Materials and methods

2.1. Microorganisms and media

Kefir, isolated from Russian kefir drink, was supplied from the Department of Chemistry, Aristotle University of Thessaloniki, Greece, where it is maintained (Athanasiadis, Boskou, Kanellaki, & Koutinas, 2001). *Kefir* biomass was obtained by successive inoculations at 30 °C, without agitation, in nutrient media containing 20 g/l glucose monohydrate, 20 g/l lactose, 1 g/l KH_2PO_4, 1 g/l $(NH_4)_2SO_4$, 10.2 g/l $MgSO_4 \cdot 7H_2O$ and 4 g/l yeast extract in distilled water. *L. casei* ssp. *casei* ATCC393 was supplied from Deutsche-Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany. It was grown in a medium containing 51 g/l MRS broth (De Man, Rogossa, & Sharpe, 1960) in distilled water, at 37 °C. *L. casei* biomass was obtained by successive inoculations at 37 °C, without agitation, in nutrient media containing 20 g/l glucose monohydrate, 7.5 g/l K_2HPO_4, 9 g/l KH_2PO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.05 g/l $MnSO_4$, 2 g/l anhydrous tri-ammonium citrate and 5 g/l yeast extract in distilled water. All media were sterilized by autoclaving at 121 °C for 15 min and cells were harvested by centrifugation at 5000 rpm for 10 min. Baker's yeast was a commercial *Saccharomyces cerevisiae* strain obtained in the form of pressed blocks (70% w/w moisture), manufactured by S.I. Lesaffre, France. Brewery spent grains (BSG) were supplied from the Athenian Brewery S.A., Greece. They were sterilized by autoclaving at 121 °C for 15 min prior to their use as immobilization support. The wheat flour used for bread making was commercial soft white flour manufactured by Hellenic Biscuit CO S.A., Greece. It contained 11.0% w/w protein, 72.0% w/w carbohydrates, 1.5% w/w fat and 2.2% w/w fibre.

2.2. Immobilization of microorganisms on BSG

Kefir and baker's yeast cells were immobilized separately on sterilized BSG. For each immobilization process, 200 g of BSG and 16 g of microorganism (wet weight) were mixed with 800 ml of sterilized culture medium, containing 132 g/l glucose monohydrate, 1 g/l KH_2PO_4, 1 g/l $(NH_4)_2SO_4$, 10.2 g/l $MgSO_4 \cdot 7H_2O$ and 4 g/l yeast extract in distilled water. Each system was allowed to ferment without agitation for 24 h at 30 °C. For the immobilization

of *L. casei* cells, 200 g of sterilized BSG and 24 g (wet weight) of microorganism were mixed with 800 ml of sterilized culture medium, containing 20 g/l glucose monohydrate, 7.5 g/l K_2HPO_4, 9 g/l KH_2PO_4, 0.2 g/l $MgSO_4 \cdot 7H_2O$, 0.05 g/l $MnSO_4$, 2 g/l anhydrous tri-ammonium citrate and 5 g/l yeast extract in distilled water. The system was allowed to ferment without agitation for 48 h at 37 °C. In all cases, the fermented liquids were, decanted and the immobilized biocatalysts were washed with 200 ml of sterilized water, and were used for bread making.

2.3. Bread making

2.3.1. Straight-dough bread making using immobilized kefir or baker's yeast

Amounts of 6%, 8% and 10% w/w (flour basis) of baker's yeast or *kefir* immobilized on BSG were mixed with 500 g wheat flour, 320 ml tap water and 4 g salt and straight-dough bread making was performed. Mixing of the ingredients was performed mechanically for 15 min and the doughs were allowed to ferment at 30 °C for 30 min, proofed at 46 °C for 30 min and baked at 230 °C for 40 min. In the same manner, bread was made by mixing 2% w/w (flour basis) free baker's yeast cells with 500 g wheat flour, 280 ml tap water and 4 g salt. Commercial white flour bread was used as control. Prior to fermentation, all doughs were moulded manually in 1.5 l baking pans.

2.3.2. Sourdough bread making using immobilized kefir

For sourdough bread making, mother sponge was prepared by mixing 300 g wheat flour, 160 ml tap water and 30% w/w (flour basis) immobilized *kefir* cells. Mother sponge was incubated at 30 °C for 24 h. Then, sourdough was prepared by mixing 250 g of fermented mother sponge with 300 g wheat flour and 160 ml tap water for 15 min. Sourdough was incubated at 30 °C for 24 h. Finally, breads containing 25%, 35% and 50% w/w (flour basis) sourdough, 400 g wheat flour, 4 g salt and tap water to 800 g, were prepared. All doughs were fermented at 30 °C for 3 h, proofed at 46 °C for 30 min and baked at 230 °C for 40 min.

2.3.3. Sourdough bread making using immobilized L. casei

Three types of bread, containing 50% w/w (flour basis) sourdough each, were produced using the sourdough bread making procedure combined with the addition of three different amounts of free baker's yeast cells. Initially, sourdough was prepared by mixing, for 15 min, 300 g wheat flour, 160 ml tap water and 90 g BSG with immobilized *L. casei*. The sourdough was incubated at 30 °C for 24 h. The final doughs contained 0.5, 1 or 1.5 g baker's yeast, respectively, 200 g sourdough, 400 g wheat flour, 200 ml tap water and 4 g salt. The ingredients were mixed for 15 min and doughs were fermented at 30 °C for 3 h, proofed at 46 °C for 30 min and baked at 230 °C for 40 min.

2.4. Assays

An amount of 15 g of breadcrumb and 100 ml of distilled water were placed in a clean dry container, which was sealed and stirred until the bread dispersed into a semi-liquid mixture. The pH was recorded using a Cyberscan 10 pH-meter. Then, an amount of 0.11 N NaOH solution was added until the pH was fixed at 6.6. The total titratable acidity (TTA) was determined by the consumed ml of NaOH, as mg of lactic acid per g of sample (Gelinas, McKinnon, & Pelletier, 1999). Loaf volume was measured by the rapeseed displacement method (Hallen, Ibanoglu, & Ainsworth, 2004). Each loaf was put in a container and covered with rapeseed to totally fill the container. Then, the loaf was removed and the volume of the rapeseed noted. Loaf volumes were calculated by deducting the rapeseed volume from the container volume. After cooling, the loaves were weighed and specific loaf volume was calculated as ml/g (Hallen et al., 2004). Moisture loss (g) was measured by deducting the weight of the bread from the initial weight of the dough before baking.

2.4.1. Determination of volatiles

Aroma volatiles were analyzed by headspace GC-MS analysis using the solid-phase microextraction technique (SPME). For each SPME analysis, 2 g of bread sample, containing both crust and crumb, were introduced into a 20-ml headspace vial stoppered with a teflon septum. The SPME syringe needle was introduced in the vial. The vial was, then, immersed in a water bath at 60 °C and the SPME fiber (50/30 um DVB/Carboxen/PDMS StableFlex for manual holder, Supelco, USA) was exposed to the headspace for 60 min. After the end of the extraction time, the fibre was inserted for 5 min into the injector port of the gas chromatograph for desorption of volatiles. The GC-MS analysis was performed on a Shimadzu model GC-17A gas chromatograph (GC) coupled to a GCMS-QP5050A mass spectrometer. A Supelco WAX-10 narrow-bore column was used (60 m × 0.32 mm i.d., 0.25 μm film thickness). The linear flow velocity for the GC column was 36 cm. The GC temperature program was: 35 °C held for 5 min; increased by 5 °C/min to 50 °C and held for 5 min; increased by 5.5 °C/min to 230 °C and held for 5 min. Total run time was 51.73 min. The carrier gas was helium with a column flow of 2 ml/min. The injector was set at 280 °C in splitless mode. The interface temperature was 230 °C. Mass spectra were recorded by electronic impact (EI) at 70 eV. The scan mode was used to detect all the compounds in the range m/z 33–200. For semi-quantification of volatile compounds, 4-methyl-2-pentanol (Sigma-Aldrich, Poole, UK) diluted in pure ethanol was used as internal standard (IS) at various concentrations (1, 4, and 40 μg/g of sample) (Ruiz, Quilez, Mestres, & Guasch, 2003). The identification of volatile compounds was performed by comparison of the mass spectra with those in

NIST107, NIST21 and SZTERP libraries. Quantitative determination was done by dividing the peak areas of the compounds of interest by the peak area of the IS and multiplying this ratio with the initial IS concentration (expressed as μg/g). The peak areas were measured from the full scan chromatograph using total ion current (TIC). Each determination was carried out in triplicate and the mean data are presented (standard deviations were ±5% in most cases).

2.5. Sensory evaluation

All breads produced, either by the straight-dough or the sourdough method, were evaluated at the bakery and compared to commercial baker's yeast bread by a number of non-trained testers (consumers) and bakers (totally 20 people). The testers were asked to evaluate the breads, using a taste-test protocol based on a 1–10 preference scale (10 = excellent to 1 = unacceptable), evaluating separately the aroma and taste of breads as well as the overall quality on the basis of loaf volume, texture, colour and flavour (Plessas et al., 2005; Vulicevic, Abdel-Aal, Mittal, & Lu, 2004). The results are given in Table 4 as average scores plus standard deviations.

3. Results and discussion

3.1. Bread making

New types of bread were produced employing baker's yeast, *kefir* and *L. casei* immobilized on brewer's spent grains. Bread was produced either directly (straight-dough) or using the sourdough method. The physical, chemical and organoleptic properties of breads were assayed and the results were evaluated by comparison with commercial type, baker's yeast leavened bread. The specific volumes of all loaves produced by the straight-dough or the sourdough method using cells immobilized on BSG, were found to be lower than those of the commercial type, yeast leavened bread (Tables 1 and 2). This is in accordance with the observations of other researchers (Autio & Laurikainen, 1997), and can be attributed to the presence of the high fibre containing BSG that did not allow maximum rising. The sourdough method resulted in breads with better rising compared to those made by the straight-dough method (20–30% and 35–40% lower specific volumes compared to commercial type bread); the highest specific volume obtained being that of breads made with BSG/*kefir* sourdough, at all the studied amounts of biocatalyst (Table 2).

Table 1
Characteristics of breads produced by the straight-dough method, using baker's yeast or *kefir* immobilized on BSG, and of commercial type bread

	Loaf weight (g)	Loaf volume (ml)	Specific loaf volume (ml/g)	Moisture loss (g)	pH	TTA (mg lactic acid/g)	Mould spoilage days
Commercial type bread	650 ± 19	2100 ± 29	3.2 ± 0.1	150 ± 16	5.7 ± 0.2	2.1 ± 0.3	3–4
Baker's yeast/BSG							
6%	726 ± 23	1395 ± 31	1.9 ± 0.2	123 ± 18	6.0 ± 0.1	1.7 ± 0.2	3–4
8%	728 ± 15	1490 ± 23	2.0 ± 0.1	125 ± 26	6.0 ± 0.2	1.7 ± 0.2	3–4
10%	743 ± 15	1545 ± 31	2.1 ± 0.1	128 ± 11	6.0 ± 0.1	1.7 ± 0.3	3–4
Kefir/BSG							
6%	710 ± 14	1330 ± 38	1.9 ± 0.1	118 ± 22	5.9 ± 0.2	2.2 ± 0.2	3–4
8%	701 ± 17	1365 ± 19	1.9 ± 0.2	120 ± 17	5.8 ± 0.1	1.9 ± 0.1	3–4
10%	693 ± 10	1425 ± 27	2.1 ± 0.1	131 ± 21	5.8 ± 0.2	1.9 ± 0.2	3–4

Table 2
Characteristics of breads produced by the sourdough method, using sourdough containing baker's yeast or *kefir* or *L. casei* immobilized on BSG, and of commercial type bread

	Loaf weight (g)	Loaf volume (ml)	Specific loaf volume (ml/g)	Moisture loss (g)	pH	TTA (mg lactic acid/g)	Mould spoilage days
BSG/*kefir* sourdough							
25%	646 ± 22	1610 ± 14	2.5 ± 0.1	124 ± 16	4.6 ± 0.1	3.1 ± 0.1	8–9
35%	664 ± 12	1652 ± 19	2.5 ± 0.2	119 ± 11	4.5 ± 0.1	3.5 ± 0.1	8–9
50%	700 ± 19	1740 ± 21	2.5 ± 0.1	89 ± 26	4.4 ± 0.1	4.1 ± 0.1	8–9
50% BSG/*L. casei* sourdough							
+0.5 g baker's yeast	714 ± 31	1580 ± 23	2.2 ± 0.1	105 ± 11	4.2 ± 0.1	5.8 ± 0.1	8–9
+1 g baker's yeast	702 ± 16	1615 ± 18	2.3 ± 0.1	110 ± 22	4.3 ± 0.1	5.3 ± 0.1	8–9
+1.5 g baker's yeast	708 ± 24	1650 ± 11	2.3 ± 0.2	100 ± 17	4.3 ± 0.1	5.3 ± 0.1	8–9

Table 3
Aroma volatiles identified in commercial type bread, in breads produced by the straight-dough method, using 10% w/w baker's yeast or *kefir* immobilized on BSG, and in breads produced by the sourdough method using 50% w/w sourdough containing *L. casei* or *kefir* immobilized on BSG

Retention index (Kovats)	Compound	Concentration (μg/g)				
		Straight-dough bread making			Sourdough bread making	
		Commercial	BSG/baker's yeast	BSG/*kefir*	BSG/*L. casei*	BSG/*kefir*
832	Ethanol	10.40[a]	9.13[a]	9.58[a]	10.57[a]	12.94[a]
1012	Isobutyl alcohol	0.42[a]	0.32[a]	0.64[a]	3.14[a]	1.67[a]
1078	1-Butanol	n.d.	n.d.	n.d.	0.67[a]	0.32[a]
1092	1-Butanol, 2-methyl	n.d.	n.d.	n.d.	0.41[a]	0.39[a]
1120	Isoamyl alcohol	1.82[a]	1.19[a]	1.39[a]	2.14[a]	1.23[a]
1159	1-Pentanol	n.d.	n.d.	0.54[a]	0.06[a]	0.31[a]
1227	2-Heptanol	n.d.	n.d.	n.d.	0.02[a]	Tr.[a]
1257	1-Hexanol	0.27[a]	n.d.	0.44[a]	0.84[a]	1.43[a]
1295	1-Heptanol	0.15[a]	n.d.	n.d.	0.05[a]	0.02[a]
1318	4-Nonanol, 4-methyl	n.d.	n.d.	0.05[a]	0.04[a]	0.03[a]
1329	1-Octen-3-ol	n.d.	n.d.	n.d.	Tr.[a]	Tr.[a]
1405	3-Octen-1-ol	n.d.	n.d.	n.d.	0.01[a]	Tr.[a]
1434	2-Nonen-1-ol	n.d.	n.d.	n.d.	n.d.	Tr.[a]
1466	1-Octanol	0.12[a]	Tr.[a]	0.03[a]	0.03[a]	Tr.[a]
1524	2-Furanmethanol	0.95[a]	0.79[a]	0.86[a]	1.23[a]	1.45[a]
1529	4-Decen-1-ol	n.d.	n.d.	0.03[b]	Tr.[b]	Tr.[b]
1670	Benzyl alcohol	0.57[a]	0.83[a]	0.69[a]	0.76[a]	0.86[a]
1812	Phenylethyl alcohol	0.25[a]	0.31[a]	0.37[a]	1.27[a]	1.43[a]
<800	Acetic acid, ethyl ester	n.d.	n.d.	0.02[a]	2.32[a]	3.80[a]
1107	Acetic acid, butyl ester	1.11[a]	0.93[a]	0.99[a]	1.12[a]	1.23[a]
1120	Hexanoic acid, ethyl ester	n.d.	n.d.	n.d.	0.05[b]	0.12[b]
1162	Acetic acid, hexyl ester	n.d.	n.d.	0.32[a]	0.09[a]	0.61[a]
1238	Heptanoic acid, ethyl ester	n.d.	Tr.[b]	Tr.[b]	0.12[b]	0.01[b]
1260	Propanoic acid, ethyl ester	0.02[b]	Tr.[b]	Tr.[b]	0.02[b]	0.06[b]
1315	Octanoic acid, ethyl ester	0.08[b]	Tr.[b]	0.02[b]	0.83[b]	1.05[b]
1463	Butanedioic acid, diethyl ester	n.d.	n.d.	n.d.	Tr.[b]	Tr.[b]
1556	Acetic acid, 2-phenyl ethyl ester	n.d.	n.d.	n.d.	0.25[b]	0.12[b]
1611	Butanoic acid, 3-hydroxy, ethyl ester	n.d.	n.d.	n.d.	Tr.[b]	Tr.[b]
<800	Acetaldehyde	n.d.	n.d.	n.d.	0.02[a]	0.09[a]
812	Butanal, 2-methyl	0.03[a]	0.05[a]	0.02[a]	0.04[a]	0.06[a]
935	Diacetyl	n.d.	n.d.	0.02[a]	0.01[a]	0.04[a]
1002	Hexanal	0.08[a]	n.d.	0.12[a]	0.11[a]	0.13[a]
1088	2,4-Decadienal	0.08[b]	Tr.[b]	0.03[b]	0.15[b]	0.21[b]
1113	2-Heptanone	0.03[a]	0.04[a]	Tr.[a]	Tr.[a]	Tr.[a]
1218	2-Heptanal	0.04[a]	Tr.[a]	0.02[a]	0.05[a]	0.02[a]
1307	2-Octanal	Tr.[b]	Tr.[b]	0.09[b]	Tr.[b]	Tr.[b]
1324	Nonanal	0.12[a]	0.09[a]	0.09[a]	0.12[a]	0.05[a]
1334	Furfural	0.73[a]	0.66[a]	0.71[a]	0.88[a]	0.45[a]
1365	2-Nonenal	0.19[b]	n.d.	0.04[b]	0.23[b]	0.29[b]
1402	2-Furancarboxaldehyde	n.d.	n.d.	n.d.	Tr.[b]	Tr.[b]
1458	Benzaldeyde	0.94[a]	0.73[a]	093[a]	0.95[a]	0.21[a]
1260	Lactic acid	n.d.	n.d.	Tr.[a]	0.54[a]	0.23[a]
1349	Isobutyric acid	Tr.[a]	Tr.[a]	Tr.[a]	Tr.[a]	Tr.[a]
1444	Hexanoic acid, 2-ethyl	n.d.	n.d.	n.d.	Tr.[b]	Tr.[b]
1615	Acetic acid	Tr.[a]	Tr.[a]	Tr.[a]	0.11[a]	0.14[a]
1713	Pentanoic acid	Tr.[a]	Tr.[a]	Tr.[a]	Tr.[a]	Tr.[a]
1900	Hexanoic acid	Tr.[a]	Tr.[a]	Tr.[a]	Tr.[a]	Tr.[a]
1021	2-Butyl furan	n.d.	n.d.	Tr.[b]	Tr.[b]	Tr.[b]
1072	D-Limonene	Tr.[b]	Tr.[b]	Tr.[b]	0.54[b]	0.34[b]
1086	2-Pentyl furan	Tr.[b]	Tr.[b]	Tr.[b]	0.54[b]	0.31[b]
1495	Azulene	n.d.	n.d.	n.d.	Tr.[b]	Tr.[b]
1655	2,3-Octahydro difuran	n.d.	n.d.	n.d.	Tr.[b]	Tr.[b]
1715	2(3H)-Furanone-dihydro-5-pentyl	n.d.	Tr.[b]	Tr.[b]	Tr.[b]	Tr.[b]

[a] Positive identification by MS data and retention times that agree with those of authentic compounds; [b]Positive identification by MS data only; Tr. = Compound present at <0.01 μg/g bread; n.d. = not detected.

All breads produced by the straight-dough method had similar pH and TTA values (Table 1). The presence of *kefir* did not lead to a significant increase of acidity as expected. This may be attributed to the predominance of yeasts instead of LAB in *kefir* which was produced aerobically, compared to sourdough which was left to mature allowing growth of LAB. Indeed, breads produced by the sourdough process had lower pH values (Table 2), as a result of the increase of LAB populations in the biocatalysts during the long time of sourdough fermentation. The pH values were lower than those found in both commercial bread and breads produced by the straight-dough method. The TTA of breads was increased when higher amounts of sourdough were incorporated. Breads containing the BSG/*L. casei* sourdough led to the highest TTA values recorded, regardless of the amount of baker's yeast cells added for leavening. This suggested that, as in the case of previous studies (Plessas et al., 2005), it would have a positive effect on the preservation of bread and contribute to longer microbiological shelf-life, therefore avoiding the addition of preservatives. The bread samples were kept at ambient temperature (20 °C) to estimate the time for appearance of mould spoilage. Indeed, the higher acidity resulted in doubling of preservation time of the sourdough breads. Macroscopic examination of the surface of breads (Barber, Ortola, Barber, & Fernandez, 1992; Esteller, Zancanaro, Palmeira, & da Silva Lannes, 2006) showed that mould spoilage (green hyphae) appeared only after the 8th day after baking, compared to commercial type bread and breads produced by the straight-dough method, where mould spoilage was obvious after the 4th day after production (Tables 1 and 2).

3.2. Volatile by-products

Table 3 shows the semi-quantitative composition of aroma volatiles in the various types of breads produced. Specifically, headspace SPME GC-MS analysis was performed on samples of commercial baker's yeast bread, in breads produced by the straight-dough method, using 10% w/w baker's yeast or *kefir* immobilized on BSG, and in breads produced by the sourdough method using 50% w/w sourdough containing *L. casei* or *kefir* immobilized on BSG. The same volatiles were identified in breads produced using baker's yeast cells (free or immobilized on BSG) by the straight-dough method (28 and 26 compounds, respectively). More volatile compounds (totally 37) were identified in breads made with *kefir* immobilized on BSG, including those identified in the baker's yeast breads plus ethyl acetate, diacetyl, lactic acid, 2-butyl furan and various alcohols. On the other hand, all breads produced by the sourdough method had a different aroma profile, with more volatiles identified (totally 53), including alcohols, esters, carbonyl compounds, organic acids and others. The volatile composition was about the same for breads produced by the sourdough method using immobilized *L. casei* or *kefir*. Additionally, almost all volatiles identified in the sourdough breads had higher concentrations compared to the straight-dough breads. These differences can be attributed to the variations of microbial populations in the different types of bread produced, i.e. the presence of LAB and yeasts in *kefir*, which obviously were in higher numbers in the sourdough breads.

3.3. Sensory evaluation

The 20 random customers and bakers, who were asked to evaluate the quality of the new types of breads at the first day of baking, showed preference for the sourdough breads as far as aroma, taste and overall quality are concerned (Table 4). They did not observe significant differences between the two types of bread produced by direct addition of immobilized baker's yeast or *kefir* cells, although the

Table 4
Sensory evaluation of breads produced by the straight-dough method, using baker's yeast or *kefir* immobilized on BSG, and of breads produced by the sourdough method, using sourdough containing baker's yeast or *kefir* or *L. casei* immobilized on BSG, and of commercial type bread

	Aroma	Taste	Overall quality
Straight-dough breads			
Commercial bread	7.4 ± 0.98	8.0 ± 0.38	8.1 ± 0.82
Baker's yeast 6%	7.1 ± 0.42	7.6 ± 0.29	7.5 ± 0.63
Baker's yeast 8%	7.3 ± 0.12	7.3 ± 0.55	7.3 ± 0.56
Baker's yeast 10%	7.4 ± 0.83	7.7 ± 0.34	7.9 ± 0.25
Kefir 6%	7.3 ± 0.45	8.0 ± 0.31	8.1 ± 0.62
Kefir 8%	7.5 ± 0.20	8.0 ± 0.68	8.1 ± 0.88
Kefir 10%	8.5 ± 0.72	8.7 ± 0.59	8.5 ± 0.43
Sourdough breads			
Kefir 25%	7.6 ± 0.73	8.2 ± 0.39	8.2 ± 0.481
Kefir 35%	7.7 ± 0.49	8.3 ± 0.61	8.3 ± 0.52
Kefir 50%	7.9 ± 0.30	8.3 ± 0.52	8.3 ± 0.79
L. casei 50% + 0.5 g baker's yeast	8.9 ± 0.81	8.8 ± 0.89	8.6 ± 0.23
L. casei 50% + 1 g baker's yeast	8.6 ± 0.26	8.8 ± 0.21	8.5 ± 0.60
L. casei 50% + 1.5 g baker's yeast	8.5 ± 0.30	8.5 ± 0.05	8.5 ± 0.62

bread made using 10% BSG/*kefir*, presented better overall quality and resembled sourdough bread. The breadcrumb moisture loss of the commercial type, baker's yeast bread, during baking was the highest recorded (Table 1). This bread retained its freshness for only 1 day and then became dry and stale. The breads made by the straight-dough method using baker's yeast or *kefir* immobilized on BSG, lost about equal amounts of moisture during baking, which were lower than those of the commercial type bread. These breads remained fresher for longer, and staling was obvious after 3 days. The best results were obtained in the case of sourdough breads with higher moisture retention during baking, lower rates of water evaporation and staling, and maintenance of freshness for longer (the breads were readily edible for 4–5 days).

4. Conclusion

The biocatalysts made by immobilization of baker's yeast, *kefir* or *L. casei* cells on brewer's spent grains were found efficient for bread making either by the straight-dough or the sourdough method, replacing the conventional pressed baker's yeast. Good rising was achieved, and breads had good overall quality, better flavour and remained fresher for longer. The higher acidities of the sourdough breads, and possible bacteriocin formation, resulted in doubling of shelf-life compared to commercial type, baker's yeast bread. Although, BSG is the main by-product of the brewing process, they have not received much attention for utilization to produce added value. Their high nutritional value, due to their composition in fibre, minerals, vitamins and amino acids, has been demonstrated by many researchers (Mussatto, Dragone, & Roberto, 2006). The differences in volatiles, flavour and shelf-life of breads produced by the straight-dough and sourdough method, reveals differences in the LAB and yeasts microflora; therefore, among other parameters (anaerobic conditions; prolonged sourdough fermentation) the potential action of BSG as prebiotics to stimulate growth of LAB should be investigated. The extension of shelf-life (reduction of staling rate and increase of microbial stability), and potential probiotic properties of bread made with immobilized *kefir* and *L. casei*, should be investigated through microbiological examinations and analysis of organic acid and bacteriocin formation.

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