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Application of *Kluyveromyces marxianus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *L. helveticus* for sourdough bread making

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Abstract

The application of *Kluyveromyces marxianus* (IFO 288), *Lactobacillus delbrueckii* ssp. *bulgaricus* (ATCC 11842) and *Lactobacillus helveticus* (ATCC 15009) as starter cultures for sourdough bread making was examined. Production of lactic and acetic acids, bread rising, volatile composition, shelf-life and organoleptic quality of the sourdough breads were evaluated. The amount of starter culture added to the flour, the dough fermentation temperature and the amount of sourdough used were examined in order to optimise the bread making process. The use of mixed cultures led to higher total titratable acidities and lactic acid concentrations compared to traditionally made breads. Highest acidity (3.41 g lactic acid/kg of bread) and highest resistance to mould spoilage were observed when bread was made using 50% sourdough containing 1% *K. marxianus* and 4% *L. delbrueckii* ssp. *bulgaricus*. The use of these cultures also improved the aroma of sourdough breads, as shown by sensory evaluations and as revealed by GC–MS analysis.

Keywords: Bread; Sourdough; *Lactobacillus delbrueckii* ssp. *bulgaricus*; *Lactobacillus helveticus*; *Kluyveromyces marxianus*; GC–MS

1. Introduction

The use of sourdough has been established as a very important process to modern baking technology, due to the superior quality and prolonged shelf-life of the sourdough baking products (Ganzle, Ehmann, & Hammes, 1998). The advantages of sourdough over yeasted breads can be highlighted by its influence on the following features: (i) technological properties including improved dough machinability, (ii) nutritional properties, like phytate hydrolysis, (iii) organoleptic properties such as improved bread volume, crumb texture and unique flavour and (iv) extended shelf-life (De Vuyst & Neysens, 2005). Katina et al., 2005 have stated that sourdough fermentation can modify the healthiness of cereals in a number of ways, including improvement of texture and palatability of whole grain products, enrichment in fibre or reduction of gluten, stabilisation or increase of various bioactive compounds, improvement of mineral bioavailability, etc. The properties of sourdoughs depend on several factors, such as the sourdough making process and the microorganisms involved (Lonner & Preve-Akesson, 1989). Sourdough fermentation has been widely studied, however, due to its complicated nature, it is still not a well understood process. Sourdough microorganisms are traditionally allowed to develop naturally, but in order to control the sourdough making process and optimise the benefits of sourdoughs, there is great interest in using new, defined starter cultures. The current trend is to develop new and improved starter cultures for the optimisation of sourdough fermentations

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leading to better quality food products. Therefore, the choice of microorganisms used is of great importance, not only to ensure improved sourdough quality, but also for easier control and economic viability of the sourdough making process. Associations of yeasts and lactic acid bacteria (LAB) appear to be self-protecting and self-regulating. Compared to heterofermentative LAB, the homofermentative ones show greater inhibitory effects to coliforms (Ganzle et al., 1998; Hansen & Schieberle, 2005).

LAB such as *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus* and the yeast *Kluyveromyces marxianus* have not been tested as starter cultures for sourdough bread making, although they have been identified in the natural sourdough microflora. *L. delbrueckii* ssp. *bulgaricus* is an important probiotic species and is currently used in the production of fermented milk products as it plays a protective role against phagocytosis, phage attacks, antibiotics and toxic compounds (Ruas-Madiedo, Hugenholtz, & Zoon, 2001). The advantage of *L. helveticus* compared to other thermophilic *Lactobacilli* is that it can adapt to different growth conditions and it is able to utilise various carbohydrates, as well as proteins, as it possesses a powerful proteolytic system (Kenny, FitzGerald, OCuinn, Beresford, & Jordan, 2003). Moreover, *L. helveticus* shows higher ability to produce lactic acid and higher oxidative tolerance than other *Lactobacilli* (Roy, Goulet, & LeDuy, 1986). On the other hand, *K. marxianus* has been successfully used in bread making as baker's yeast (Caballero et al., 1995). It should be underlined that all these microorganisms can be grown in cheese whey, which is the main by-product of the dairy industry, and its disposal causes serious environmental problems (John, Nampoothiri, & Pandey, 2007; Kargi & Ozmihci, 2006; Siso, 1996). Therefore, the aim of this study was to evaluate the use of two mixed starter cultures for sourdough bread making, containing the yeast *K. marxianus* combined with either *L. bulgaricus* or *L. helveticus*.

2. Materials and methods

2.1. Microorganisms

The homofermentative LAB *L. delbruekii* ssp. *bulgaricus* (DSMZ, strain ATCC11842) and *L. helveticus* (DSMZ, strain ATCC 15009) were grown in MRS broth at 40 °C for 24 h and maintained at 4 °C in the broth, which was periodically refreshed. *K. marxianus* (DSMZ, strain IFO 288) was grown at 30 °C for 48 h and maintained at 4 °C in nutrient broth containing 3 g/l yeast extract, 3 g/l malt extract, 5 g/l peptone and 10 g/l glucose. The microorganisms were harvested by centrifugation at 5000 rpm for 10 min, and 4 g of each harvested biomass were added in 2 l of cheese whey and incubated at 30 °C for about 24 h. Suitable amounts of microbial biomass were then obtained by centrifugation and were used for the production of sourdoughs.

2.2. Flour

The wheat flour used for sourdough and sourdough bread production was commercial hard white flour (Hellenic Allatini CO, Greece), containing 13 g/100 g protein, 69 g/100 g carbohydrates, 1.5 g/100 g fat, and 22 g/100 g dietary fibre.

2.3. Sourdough preparation

For preparation of the sourdoughs, 400 g of flour and various amounts of microbial biomass were mixed with 200 ml tap water. The doughs were mixed for 5–10 min until the correct consistency was obtained and then sourdough fermentation was carried out at 30 °C and 40 °C, for 16 h. The amounts of microorganisms examined were: 1% (w/w on flour basis) of *K. marxianus* mixed with 1%, 2% or 4% of either *L. bulgaricus* or *L. helveticus*. Controls were carried out with sourdoughs prepared using only 1% *K. marxianus* and with traditional, wild microflora sourdoughs.

2.4. Bread making

Sourdough bread making involved mixing 400 g flour, 120 g or 200 g sourdough (30% and 50% w/w on flour basis, respectively), 6 g salt (1.5% w/w on flour basis) and 200 ml tap water and kneading of the doughs for 5 min. Dough fermentation and rising were carried out at 30 °C and 40 °C for 2 h and baking was carried out at 200–210 °C, for 1 h. The effects of the amounts of the microorganisms used for sourdough preparation, the amount of sourdough used for bread making, as well as the dough fermentation temperature, in the quality and shelf-life of the sourdough breads, were evaluated. All experiments were carried out three times.

2.5. Assays

2.5.1. pH and total titratable acidity (TTA)

For pH and acidity determination, 15 g of breadcrumbs and 100 ml of distilled water were placed in a clean dry vial, which was sealed and stirred for 10 min. The pH was then recorded using a Cyberscan 10 pH-meter. TTA was determined by the volume of 0.11 N NaOH solution consumed until the pH reached 6.6 and expressed as ml NaOH consumed (Gélinas, McKinnon, & Pelletier, 1999). The standard deviations (STD) for pH and TTA were 0.1 and 0.3, respectively.

2.5.2. Loaf volume and moisture loss

After cooling, the loaves were weighed and 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. After the removal of the loaf, the volume of the rapeseed was noted. Loaf volumes were calculated by

deducting the rapeseed volume from the container volume. Specific loaf volume was calculated as ml/g. Moisture loss (g) was measured by deducting the weight of the bread from the initial weight of the dough before baking. STD for specific volume and moisture loss were 0.1 and 15, respectively.

2.5.3. Organic acids

For organic acid extraction, 10 g of dough were mixed with 90 ml of sterile distilled water for 2 min using a Stomacher Blender 400 (Seward Laboratory, London, UK). The dough extracts were centrifuged at 20,000 rpm (Meignen et al., 2001) and organic acids (lactic and acetic) were determined by ion-exchange liquid chromatography on a Shimadzu system consisting of a Shim-pack ICA1 column, an LC-10AD pump, a CTO-10A oven and a CDD-6A conductivity detector. A solution of 2.5 mM phthalic acid and 2.4 mM tris (hydroxymethyl) aminomethane (pH 4.0) was used as mobile phase (1.2 ml/min). The column temperature was 40 °C. The sample dilution was 5% v/v and the injection volume was 60 μl. Determinations were carried out by means of standard curves. STD for lactic acid and acetic acid were 0.1.

2.5.4. Determination of volatiles

Volatile compounds were analysed by GC–MS analysis using the solid-phase microextraction method (SPME). For each SPME analysis, 2 g of bread sample, containing both crust and crumb, were introduced into a 20 ml headspace vial with a teflon-lined septum fit at its top. The SPME needle was introduced into the vial, which was then immersed in a water bath at 60 °C. The SPME fibre (2 cm–50/30 mm DVD/Carboxen/PDMS Stable Flex Supelco, Bellefonte, PA, USA) was then was exposed to the headspace for 60 min. The water bath was continuously shaken with a magnetic stirring bar during the extraction process. After the end of the extraction time, the fibre was inserted into the injector port of the gas chromatograph (GC) for thermal desorption of the volatiles for 5 min. The GC analyses were performed using a Shimadzu model GC-17A gas chromatograph coupled to a GCMS-QP5050A mass spectrometer. A Supelco CO WAX-10 column with a 0.25 μm film thickness, 60 m × 0.32 mm i.d. was used. The GC temperature program was 35 °C, held for 5 min, then increased by 5 °C/min to 50 °C, where it was held again for 5 min, then increased by 5.5 °C/min to 230 °C, where it was held again for 5 min, for a total run time of 51.73 min. The carrier gas was helium with a column flow of 2 ml/min. The injector was 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. The identification of volatile compounds was performed in comparison with standard compounds and MS data obtained with those in NIST107, NIST21 and SZTERP libraries.

2.6. Sensory evaluation

All breads produced with the mixed starter cultures and the traditional sourdough breads were left at a bakery the same day of their production, in order to be evaluated as far as their sensorial characteristics were concerned. Bread samples were judged by 15 random untrained customers who were asked to give scores on a 0 (unacceptable) to 10 (excellent) scale using locally approved protocols in our laboratories for taste and aroma and compared to commercial sourdough bread. Results obtained by sensory

Table 1
Characteristics and shelf-life of breads made with sourdough containing *K. marxianus* and *L. bulgaricus* or *L. helveticus* and with traditional, wild microflora sourdough

Bread sample	Amount of microorganism in sourdough (% w/w on flour basis)		Fermentation temperature (°C)	Amount of sourdough (% on flour basis)	Final pH	TTA (ml)	Moisture loss (g)	Specific loaf volume (ml/g)	Mould spoilage (days)	Lactic acid (g/kg bread)	Acetic acid (g/kg bread)
	K. marxianus	*L. bulgaricus*									
1	1	4	40	30	4.5	8.1	121	2.1	11	1.95	0.21
2	1	2	40	30	4.6	8.1	119	2.2	10	1.53	0.35
3	1	1	40	30	4.6	7.5	120	2.0	9	1.11	0.12
4	1	4	40	50	4.3	9.2	113	2.1	12	2.88	0.25
5	1	4	30	30	4.6	6.6	110	2.3	11	1.55	0.24
	K. marxianus	*L. helveticus*									
6	1	4	40	30	4.5	7.1	125	2.0	10	1.25	0.35
7	1	2	40	30	4.5	6.9	130	1.9	8	1.11	0.22
8	1	1	40	30	4.6	6.6	145	1.6	8	1.10	0.19
9	1	4	40	50	4.4	8.2	105	2.2	12	3.41	0.38
10	1	4	30	30	4.6	6.3	122	1.9	10	1.28	0.25
11	1	–	40	30	4.6	5.9	135	1.7	8	0.82	0.27
12	–	–	40	30	5.2	3.6	129	1.8	8	1.05	0.17
13	–	–	40	50	5.0	5.1	121	1.9	8	1.10	0.15

evaluation were further statistically studied by one-way analysis of variance. Duncan's multiple range test was used to determine significant differences among of results (coefficients and the ANOVA tables were computed using Statistica v7.0). The results were marked as average scores plus STDs for aroma, taste and overall quality (volume, texture, colour and flavour).

3. Results and discussion

Sourdough breads were made using sourdoughs containing mixed cultures of the yeast *K. marxianus* with the LAB *L. bulgaricus* or *L. helveticus*. Traditional wild microflora sourdough breads were also produced as controls, with and without the addition of *K. marxianus* as leavening culture. The results concerning quality characteristics and shelf-life of the produced breads are shown in Table 1. Rising was good in all cases, with specific loaf volumes being quite similar (2.0–2.3 ml/g) and higher than those of the control samples (1.6–1.9 ml/g). This indicated that the presence of the specific LAB may have affected dough rising. The new types of breads retained more moisture after baking, which provided loaves with better crumb texture (Table 1).

The acidities of the sourdough breads made using the above starters were significantly higher compared to the traditional sourdough breads. This variation was more evident when higher amounts of microorganisms were used to prepare the sourdoughs or when higher amounts of sourdoughs were used to make the bread. Generally, lower pH and higher TTA values were achieved when the fermentation temperature of the sourdough was higher. Moreover, the shelf-life of the new breads was higher compared to that of the traditional breads. Specifically, the best results were achieved when sourdough was made with 1% w/w of *K. marxianus* and 4% w/w of either *L. bulgaricus* or *L. helveticus* and fermented at 40 °C, and when 50% w/w (on flour basis) of sourdough was used to make bread. At these conditions the pH and TTA values of the produced breads were 4.3 and 9.2 ml, respectively, when *L. bulgaricus* was used and 4.4 and 8.2 ml, respectively, when *L. helveticus* was used. On the other hand, the highest TTA recorded in the control sample (traditional sourdough bread) was 5.1 ml. Correspondingly, these samples contained about three times more lactic acid (2.88 and 3.41 g/kg of bread, respectively), than the control samples (Table 1). The lactic acid concentrations were much higher in all the breads containing 50% sourdough compared to breads made with 30% sourdough. On the other hand, acetic acid concentrations were generally found in low levels, which are in agreement with other researchers (Robert et al., 2006). However, acetic acid concentrations in the new breads were found higher than in the traditional sourdough breads, especially when 50% sourdough was used. This is a significant observation since acetic acid is an aroma enhancer and leads to breads with a strong aroma profile (Göçmen, 2001). Macroscopic observations for appearance of mould spoilage justified the above results. Specifically, mould spoilage in the new breads appeared after 12 days of storage at 20 °C, while traditional sourdough breads were spoiled at about 8 days (Table 1).

Headspace analysis of volatiles revealed the impact of the used cultures on bread flavour. In total, 24 and 18 compounds were identified in the headspace of breads made using sourdoughs containing *K. marxianus* and *L. bulgaricus* or *K. marxianus* and *L. helveticus*, respectively. Fifteen compounds were identified in the headspace of the traditional sourdough bread. Compounds that are considered

Table 2
Volatile by-products identified in bread produced using 50% w/w sourdough containing 1% *K. marxianus* and 4% LAB (samples 4 and 9) and bread made with traditional, wild microflora sourdough (sample 13)

Kovats index	Compound	*K. marxianus* and *L. bulgaricus*	*K. marxianus* and *L. helveticus*	Wild microflora
Alcohols				
832	Ethanol	a	a	A
1012	Isobutyl alcohol	–	a	–
1120	1-Butanol, 3-methyl	–	a	–
1257	1-Hexanol	a		A
1395	1-Decanol, 2-ethyl	a	a	–
1434	2-Nonen-1-ol	a	–	–
1452	3-Pentanol,2,4-dimethyl	–	–	A
1466	1-Octanol	a	–	–
1502	Non-2-en-1-ol	a	–	A
1512	2-Undecanol	a	–	A
1600	3-Nonen-1-ol	a	–	–
1670	Benzyl alcohol	a	a	–
1812	Phenyl ethanol	a	a	A
Esters				
<800	Ethyl acetate	a	a	–
1107	Butyl acetate	a	–	–
1590	Isobutyl acetate	a	–	A
1682	3-Hydroxy butyl, acetate	a	–	–
1925	Ethyl pentadecanoate	a	–	–
Carbonyls				
1002	Hexanal	a	a	A
1067	Heptanal	a		–
1091	Butanal, 3-methyl	a		–
1324	Nonanal	–		A
1334	Furfural	a	a	–
1365	2-Nonenal	–	a	A
1448	Butyrolactone	a	a	A
1458	Benzaldehyde	a	a	A
1484	Hexadecanal	–	a	–
Organic acids				
1260	Lactic acid	a	a	A
1615	Acetic acid	a	a	A
1900	Hexanoic acid	a	a	–
1934	Octanoic acid	–	a	A
Miscellaneous compounds				
1452	2*H*-Pyran-2-one, tetrahydro-4,6 dimethyl	–	a	–

a = positive identification from MS data and retention times.

Table 3
Sensory evaluation of breads produced using traditional, wild microflora sourdoughs and sourdoughs containing *K. marxianus* and *L. bulgaricus* or *L. helveticus* as average scores plus standard deviations for aroma, taste and overall quality

Bread samples	Amount of microorganism in sourdough (% w/w on flour basis)		Fermentation temperature (°C)	Amount of sourdough (% on flour basis)	Aroma	Taste	Overall quality
	K. marxianus	*L. bulgaricus*					
1	1	4	40	30	7.6 ± 0.73	7.9 ± 0.15	8.9 ± 0.17
2	1	2	40	30	7.5 ± 0.63	7.8 ± 0.14	8.5 ± 0.13
3	1	1	40	30	7.5 ± 0.13	7.8 ± 0.11	8.1 ± 0.12
4	1	4	40	50	7.9 ± 0.20	8.7 ± 0.13	8.9 ± 0.15
5	1	4	30	30	7.8 ± 0.20	8.2 ± 0.11	8.9 ± 0.13
	K. marxianus	*L. helveticus*					
6	1	4	40	30	7.5 ± 0.13	7.9 ± 0.13	8.7 ± 0.16
7	1	2	40	30	7.7 ± 0.15	7.8 ± 0.14	8.5 ± 0.17
8	1	1	40	30	7.5 ± 0.13	7.7 ± 0.13	8.5 ± 0.20
9	1	4	40	50	8.3 ± 0.12	8.1 ± 0.12	8.9 ± 0.23
10	1	4	30	30	8.0 ± 0.20	8.0 ± 0.20	8.9 ± 0.12
11	1	–	40	30	7.7 ± 0.15	7.7 ± 0.12	7.7 ± 0.13
12	–	–	40	30	7.6 ± 0.13	7.7 ± 0.16	8.5 ± 0.13
13	–	–	40	50	7.5 ± 0.20	7.7 ± 0.19	8.5 ± 0.13

important to bread quality, such as 2-nonen-1-ol, 3-nonen-1-ol, benzyl alcohol and furfural (Hansen & Schieberle, 2005; Kirchhoff & Schieberle, 2001, 2002) and a variety of esters (Maga, 1974), were identified in the new types of breads but not in the traditional sourdough breads (see Table 2).

Differences in flavour were also revealed by the sensory evaluation (Table 3). The scores given by testers, as far as taste, aroma and overall quality are concerned, showed statistically significant ($P < 0.01$) differences among the produced breads. The highest score for aroma was noted for breads made with 50% sourdoughs, which were fermented at 40 °C and contained 1% *K. marxianus* and 4% *L. bulgaricus* or *L. helveticus*. Moreover, from these breads, the one containing *L. bulgaricus* had the best taste according to testers. Generally, testers showed preference to breads made with the sourdoughs containing the higher amounts of the studied LAB (*L. bulgaricus* or *L. helveticus*), suggesting that the presence of these bacteria affected positively the organoleptic characteristics of the breads.

4. Conclusions

The results obtained in this study showed that the proposed mixed starter cultures of *K. marxianus* and *L. bulgaricus* or *L. helveticus* can be successfully used for the production of good quality breads. The breads produced from sourdoughs containing the starter cultures had overall better sensorial qualities and longer shelf-life and were preferred by consumers, compared with the traditional sourdough breads.

It should also be underlined that all the microorganisms were produced from cheese whey, which is considered as a waste product of cheese industry. Therefore the potential utilisation of cheese whey can minimise the cost for the growth of these microorganisms.

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