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Evolution of aroma volatiles during storage of sourdough breads made by mixed cultures of *Kluyveromyces marxianus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* or *Lactobacillus helveticus*

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Abstract

Two mixed starter cultures were used for sourdough bread making to evaluate their ability to improve quality and increase bread shelf-life: *Lactobacillus delbrueckii* ssp. *bulgaricus* or *Lactobacillus helveticus* mixed with the lactose fermenting yeast *Kluyveromyces marxianus* as alternative baker's yeast. Control sourdough breads (*K. marxianus*) without the addition of bacteria, were also prepared. The changes on the headspace aroma volatiles during storage were assessed using solid-phase microextraction (SPME) GC–MS analysis. The effect of these changes on bread flavour was evaluated by consumer preference evaluations and the results were co-evaluated with those from the GC–MS analysis. The obtained results showed differences in the volatile composition of the different types of breads examined, as well as dramatic decreases of the number and the amount of volatiles after five days of storage. The sourdough breads made with *K. marxianus* and *L. bulgaricus*, had a more complex aroma profile, longer shelf-life and achieved the highest scores in the sensory tests.

Keywords: Bread; Sourdough; *Lactobacillus helveticus*; *Lactobacillus delbrueckii* ssp. *bulgaricus*; *Kluyveromyces marxianus*; Volatiles; Shelf-life

1. Introduction

The extension of shelf-life is one of the biggest challenges for the baking industry today. The shelf-life of bread and other baking products is small, mainly as a result of staling, which is a number of physicochemical alterations that occur after baking and during storage. Staling is characterised by crumb firming mainly due to retrogradation of the starch polymers and interactions between starch and proteins, crust softening due to transfer of moisture from the crumb to crust and finally flavour changes. Consequently, these changes are responsible for the disposal of large quantities of bread (8–10%), therefore resulting in economical losses (Corsetti et al., 2000; Guarda, Rosell, Benedito, & Galotto, 2004; Inagaki & Seib, 1992; Katina, Salmenkallio-Marttila, Partanen, Forssell, & Autio, 2006; Lorenz & Maga, 1972). The main ways for delaying staling and extending shelf-life are the use of hydrocolloids, emulsifiers, exogenous enzymes, etc. (Corsetti et al., 2000; Guarda et al., 2004; Katina et al., 2006). Recently, return to traditional processes like sourdough bread making, employing pure cultures of lactic acid bacteria (LAB), has been proposed as a significant means for improving bread quality in terms of delaying staling, improving taste, texture, aroma and generally increasing shelf-life (Gobbetti, 1998; Hansen & Schieberle, 2005; Messens & De Vuyst, 2002).

Defined mixed starter cultures containing both LAB and yeasts, in free cell suspensions, or even immobilised in suitable matrices, such as kefir (Plessas, Pherson, Bekatorou, Nigam, & Koutinas, 2005), baker's yeast, kefir and *Lactobacillus casei* (Plessas et al., 2007), baker's yeast and

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milk LAB immobilised on a flour/milk matrix or kefir immobilised on orange peel (Plessas et al., 2007, 2008b), *Kluyveromyces marxianus, Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus helveticus* (Plessas et al., 2008a), etc., was proposed for the production of sourdough bread making. The LAB and the lactose fermenting yeasts employed in these studies, can be grown on whey, therefore their use in baking applications was proposed as an alternative for the utilisation of this seriously polluting waste. These starter cultures proved efficient as leavening agents, and lead to products of longer shelf-life (delayed staling and resistance to mould spoilage), as well as improved organoleptic properties, which was justified by GC–MS assays of volatiles revealing more complex aroma profiles. The effect of sourdough on the aroma profile of breads has been widely studied and reviewed (Ganzle, Vermeulen, & Vogel, 2007; Gobbetti, 1998; Hansen & Hansen, 1994; Hansen & Schieberle, 2005; Kirchhoff & Schieberle, 2001, 2002; Maga, 1974; Rehman, Paterson, & Piggott, 2006).

Even though extensive research has been carried out regarding bread shelf-life, proximate analysis of quality features and aroma composition after baking, there are few studies examining the changes of the aromatic composition of bread, during storage, which may reflect the effects of staling, evaporation and microbial activities in the stored product. Therefore, the aim of this study was to monitor the quality degradation during storage of breads produced using mixed starter cultures of yeast and LAB, through the qualitative and quantitative determinations of aroma volatiles, combined with respective consumer evaluations.

2. Materials and methods

2.1. Microorganisms and media

The homofermentative LAB *L. delbruekii* ssp. *bulgaricus* (*L. bulgaricus*) (ATCC 11842) isolated from Bulgarian yoghurt and *L. helveticus* (ATCC 15009) isolated from cheese, were obtained from Deutsche-Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Germany. The cultures were grown and maintained at MRS broth (De Man, Rogosa and Sharpe), they were incubated at 40 °C for 24 h and then stored at 4 °C. The broth was refreshed regularly during the course of the experiment. *K. marxianus* (IFO 0288) was also obtained from DSMZ. The culture was grown and maintained on nutrient broth consisting of 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone and 10 g/L glucose. It was incubated at 30 °C for 48 h and then the produced culture was stored at 4 °C. For further production, an initial amount of 4 g of each microorganism was added to 2 L of cheese whey and incubated at 30 °C for about 24 h. In all cases cells were harvested by centrifugation (Sigma 3K12, Rotor No. 11133, 5000*g*, 10 min). All media were sterilised by autoclaving at 120 °C for 15 min.

Commercial hard type wheat flour manufactured by Allatini S.A. (Thessaloniki, Greece), containing 13% protein, 69% carbohydrates, 1.5% fat and 22% dietary fibre was used for bread making. Cheese whey was prepared from cow's milk, after rennin coagulation, filtration to separate casein proteins and subsequent heat treatment at 90 °C for 15 min for removal of whey proteins.

2.2. Preparation of sourdough

For preparation of the sourdoughs, 400 g of flour and various amounts of starter cultures were mixed with 200 mL tap water. The doughs were mixed manually for 5–10 min until the correct consistency was obtained and then sourdough fermentation was allowed to occur at 30 and 40 °C, for 16 h. The amounts of microorganisms examined (% w/w on flour basis) were 1% of *K. marxianus* mixed with 4% of either *L. bulgaricus* or *L. helveticus*. Control breads were also made using sourdoughs prepared with the addition of 1% *K. marxianus* as the leavening agent and no addition of pure LAB culture (Plessas et al., 2007, 2008a).

2.3. Bread making

Sourdough breads were prepared according to the sourdough method. The general process involved mixing 400 g flour, 200 g (50% w/w on flour basis) of sourdoughs prepared as describe above, addition of 1.5% salt (on flour basis) and 200 mL tap water. The doughs were kneaded mechanically for 5 min (BBA 2866 Automatic Bead Machine Clatronic International GmbH, Germany) and then allowed to ferment at 37 °C for 2 h. Baking was carried out at 200–210 °C for 1 h. The process was repeated three times.

2.4. Determination of volatiles

Headspace analysis of headspace aroma volatiles was carried out by gas chromatography mass spectrometry (GC–MS) using the solid-phase microextraction technique (SPME). For each SPME analysis, 2 g of bread sample were introduced into a 20 mL vial and the SPME needle was introduced through the vial septum. The vial was then immersed in a water bath at 60 °C and the SPME fibre (2 cm–50/30 mm DVD/Carboxen/PDMS Stable Flex Supelco, Bellefonte, PA, USA) was exposed to the headspace for 60 min. When the extraction process was completed, the fibre was inserted into the injector port (set at 280 °C) of the gas chromatograph (GC) for thermal desorption of volatiles for 5 min in splitless mode. The GC–MS instrumentation included a Shimadzu model GC-17A gas chromatograph coupled to a GC–MS-QP5050A mass spectrometer. A Supelcowax-10 column (60 m, 0.32 mm i.d., 0.25 μm film thickness) was used. The GC temperature program was set as follows: 35 °C for 5 min, increased by 5 °C/min to 50 °C (held for 5 min), increased by 5.5 °C/min to 230 °C (held for

5 min). Total run time was 51.73 min. The carrier gas was helium with a flow rate of 2 mL/min. The interface temperature was 230 °C. Mass spectra were recorded by electronic impact at 70 eV, in the mass range *m/z* 33–200. The identification of volatile compounds was performed by comparison of the MS data with those of standard compounds and those in NIST107, NIST21 and SZTERP libraries. For semi-quantitative analysis of volatiles, 4-methyl-2-pentanol (Sigma–Aldrich, UK) diluted in pure ethanol was used as the internal standard (IS) at various concentrations (4, 40 and 400 μg/g of sample). The volatile compounds were quantified by dividing the peak areas of the compounds of interest by the peak area of the IS and multiplying this ratio by the initial concentration of the IS (expressed as μg/g). Each determination was carried out in triplicate and the mean data are presented.

2.5. Preliminary sensory evaluations

A consumer blind sensory evaluation (preference) test was carried out to assess bread quality. The breads produced using the mixed starter cultures and the traditionally made sourdough breads were placed in a bakery the same day they were produced. A total of 15 random customers were asked to evaluate the breads giving scores ranging between 0 (unacceptable) and 10 (excellent) using preference protocols for bread staling and overall quality of the bread. Results were calculated as average scores plus standard deviations (Plessas et al., 2008a, 2008b).

3. Results and discussion

In a previous study, the use of sourdough containing mixed cultures of *K. marxianus* with either *L. bulgaricus* or *L. helveticus* was proposed, in order to improve the quality of sourdough bread in terms of shelf-life extension (delay staling and resist spoilage) and improvement of aroma (Plessas et al., 2008a). The use of these cultures led to higher acidity, higher amounts of lactic acid and extended product shelf-life, as well as improved aroma, compared to yeast-leavened breads. The best results were obtained in the case of breads made using 50% (w/w on

Table 1
Effect of storage time on the composition of headspace volatiles identified in breads made with 50% sourdough containing 1% *K. marxianus* and 4% *L. bulgaricus*

Kovats index	Compound	Concentration (μg/g)					
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Alcohols							
832	Ethanol	4.97[a]	4.56[a]	4.37[a]	4.02[a]	3.24[a]	0.14[a]
1012	Isobutyl alcohol	0.03[a]	0.03[a]	Tr[a]	Tr[a]	Tr[a]	Tr[a]
1257	1-Hexanol	0.05[a]	0.02[a]	0.02[a]	0.01[a]	Tr[a]	nd
1395	1-Decanol, 2-ethyl	0.01[b]	Tr[b]	nd	nd	nd	nd
1434	2-Nonen-1-ol	0.28[a]	0.25[a]	0.34[a]	0.08[a]	nd	nd
1466	1-Octanol	0.69[a]	0.71[a]	nd	0.10[a]	nd	nd
1502	Non-2-en-1-ol	0.34[b]	0.32[b]	0.22[b]	0.08[b]	nd	nd
1512	2-Undecanol	0.06[b]	Tr[b]	nd	nd	nd	nd
1600	3-Nonen-1-ol	0.01[b]	Tr[b]	nd	nd	nd	nd
1670	Benzyl alcohol	0.01[a]	Tr[a]	Tr[a]	nd	nd	nd
1812	Phenyl ethanol	0.29[a]	0.25[a]	Tr[a]	nd	nd	nd
Esters							
<800	Ethyl acetate	0.29[a]	0.28[a]	0.18[a]	0.09[a]	nd	nd
1107	Butyl acetate	0.01[a]	Tr[a]	nd	nd	nd	nd
1590	Isobutyl acetate	0.03[a]	Tr[a]	nd	nd	nd	nd
1682	3-Hydroxy butyl, acetate	0.04[b]	Tr[b]	nd	nd	nd	nd
1925	Ethyl pentadecanoate	0.03[b]	Tr[b]	nd	nd	nd	nd
Carbonyl compounds							
<800	Acetaldehyde	0.17[a]	0.18[a]	0.21[a]	0.16[a]	0.01[a]	nd
1002	Hexanal	0.05[a]	0.03[a]	0.02[a]	nd	nd	nd
1067	Heptanal	0.03[a]	Tr[a]	nd	nd	nd	nd
1091	Butanal, 3-methyl	0.16[b]	0.18[b]	0.20[b]	nd	nd	nd
1334	Furfural	0.16[a]	0.14[a]	0.03[a]	0.02[a]	Tr[a]	Tr[a]
1448	Butyrolactone	3.58[a]	2.89[a]	2.63[a]	0.57[a]	0.45[a]	nd
1458	Benzaldehyde	0.24[a]	0.25[a]	0.26[a]	0.16[a]	0.01[a]	Tr[a]
1484	Hexadecanal	0.02[b]	0.01[b]	nd	nd	nd	nd
Organic acids							
1260	Lactic acid	0.02[a]	Tr[a]	nd	nd	nd	nd
1615	Acetic acid	0.01[a]	Tr[a]	nd	nd	nd	nd
1900	Hexanoic acid	0.01[a]	Tr[a]	nd	nd	nd	nd

Tr – Compound present at <0.01 μg/g bread (traces); nd – not detected.
[a] Positive identification by MS data and retention times and those of standard compounds.
[b] Positive identification by MS data only.

Table 2
Effect of storage time on the composition of headspace volatiles identified in breads made with 50% sourdough containing 1% *K. marxianus* and 4% *L. helveticus*

Kovats index	Compound	Concentration (μg/g)					
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Alcohols							
832	Ethanol	5.86[a]	5.45[a]	4.12[a]	0.95[a]	0.08[a]	nd
1012	Isobutyl alcohol	0.04[a]	0.02[a]	0.01[a]	nd	nd	nd
1120	1-Butanol, 3-methyl	0.02[a]	0.01[a]	0.01[a]	nd	nd	nd
1395	1-Decanol, 2-ethyl	0.02[b]	0.01[b]	Tr[b]	Tr[b]	nd	nd
1670	Benzyl alcohol	0.03[a]	0.02[a]	Tr[a]	nd	nd	nd
1812	Phenyl ethanol	0.30[a]	0.25[a]	0.02[a]	Tr[a]	nd	nd
Esters							
<800	Ethyl acetate	0.31[a]	0.22[a]	0.02[a]	Tr[a]	nd	nd
Carbonyl compounds							
1002	Hexanal	0.03[a]	0.01[a]	0.01[a]	Tr[a]	nd	nd
1334	Furfural	0.19[a]	0.04[a]	0.04[a]	Tr[a]	nd	nd
1365	2-Nonenal	0.34[a]	0.01[a]	nd	nd	nd	nd
1448	Butyrolactone	0.03[a]	Tr[a]	nd	nd	nd	nd
1458	Benzaldehyde	0.34[a]	0.25[a]	0.18[a]	0.01[a]	nd	nd
1484	Hexadecanal	0.34[a]	0.01[a]	nd	nd	nd	nd
Organic acids							
1260	Lactic acid	0.03[a]	Tr[a]	nd	nd	nd	nd
1615	Acetic acid	0.02[a]	Tr[a]	nd	nd	nd	nd
1900	Hexanoic acid	0.03[a]	Tr[a]	nd	nd	nd	nd
1934	Octanoic acid	0.01[b]	Tr[b]	nd	nd	nd	nd

Tr – compound present at <0.01 μg/g bread (traces); nd – not detected.
[a] Positive identification by MS data and retention times and those of standard compounds.
[b] Positive identification by MS data only.

Table 3
Effect of storage time on the composition of headspace volatiles identified in breads made with 50% sourdough containing 1% *K. marxianus* (control)

Kovats index	Compound	Concentration (μg/g)					
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
Alcohols							
832	Ethanol	4.14[a]	4.00[a]	1.25[a]	0.22[a]	Tr[a]	Tr[a]
1257	1-Hexanol	0.01[a]	Tr[a]	nd	nd	nd	nd
1452	3-Pentanol,2,4-dimethyl	0.02[b]	0.01[b]	nd	nd	nd	nd
1502	Non-2-en-1-ol	0.29[a]	0.25[a]	0.21[a]	0.22[a]	Tr[a]	nd
1512	2-Undecanol	0.03[a]	0.01[a]	nd	nd	Tr[a]	nd
1812	Phenyl ethanol	0.05[a]	0.01[a]	0.01[a]	0.01[a]	nd	nd
Esters							
<800	Ethyl acetate	0.08[a]	0.08[a]	nd	nd	nd	nd
1590	Isobutyl acetate	0.02[a]	Tr[a]	nd	nd	nd	nd
Carbonyl compounds							
1002	Hexanal	0.07[a]	0.01[a]	Tr[a]	Tr[a]	nd	nd
1324	Nonanal	0.07[a]	0.01[a]	nd	nd	nd	nd
1334	Furfural	0.08[a]	0.05[a]	0.01[a]	nd	nd	nd
1365	2-Nonenal	0.04[b]	0.01[b]	Tr[b]	Tr[b]	nd	nd
1448	Butyrolactone	0.14[a]	0.11[a]	0.01[a]	0.01[a]	nd	nd
1458	Benzaldehyde	0.33[a]	0.25[a]	Tr[a]	Tr[a]	nd	nd
Organic acids							
1260	Lactic acid	0.01[a]	Tr[a]	nd	nd	nd	nd
1615	Acetic acid	Tr[a]	Tr[a]	nd	nd	nd	nd
1934	Octanoic acid	Tr[b]	Tr[b]	nd	nd	nd	nd

Tr – compound present at <0.01 μg/g bread (traces); nd – not detected.
[a] Positive identification by MS data and retention times and those of standard compounds.
[b] Positive identification by MS data only.

flour basis) of sourdough containing 1% *K. marxianus* and 4% *L. bulgaricus*. In this study, sourdough breads made using 50% sourdough, containing 1% *K. marxianus* with 4% *L. bulgaricus* or 4% *L. helveticus* were further examined as far as the evolution of aroma volatiles during storage is concerned. *K. marxianus* has been used successfully in bread making, although it exhibits slightly lower leavening activity compared to conventional baker's yeast (Caballero et al., 1995; Plessas et al., 2008a). Despite that, *K. marxianus* is a lactose fermenting yeast which can grow on whey, therefore its production cost can be lower and competitive compared to baker's yeast. *L. bulgaricus* on the other hand, is a common dairy probiotic, while *L. helveticus* can easily adapt to different growth conditions and shows higher lactic acid formation ability and higher oxidative tolerance compared to other LAB (Plessas et al., 2008a).

Tables 1–3 summarise the results of the GC–MS analysis for the breads produced with sourdoughs containing *K. marxianus* and *L. bulgaricus* or *L. helveticus*, and breads made with *K. marxianus* with no addition of LAB. The analysis of headspace aroma volatiles was carried out every day for a total of five days of storage, at room temperature. The volatile compounds determined with reliability were alcohols, esters, organic acids and carbonyl compounds. Most of these compounds are well known to affect bread flavour and their origin is either associated with yeast or LAB metabolism, or they are produced during the baking process. Their contribution to bread aroma has been widely reviewed (Ganzle et al., 2007; Gobbetti, 1998; Hansen & Hansen, 1994; Hansen & Schieberle, 2005; Kirchhoff & Schieberle, 2001, 2002; Maga, 1974; Rehman et al., 2006). The obtained results showed a dramatic decrease of the number and the amount of volatile compounds after five days of storage. Comparing the sourdough breads produced, it is obvious that the aroma profile of both breads made with sourdough containing *K. marxianus* and *L. bulgaricus* or *K. marxianus* and *L. helveticus* was more complex, and was maintained longer compared to the control bread (*K. marxianus*). These results are further verified by Figs. 1–3, which clearly show the daily evolution of changes in the aroma composition. Comparing the breads made either with sourdough containing *K. marxianus* and *L. bulgaricus* or *K. marxianus* and *L. helveticus*, more volatile compounds were identified in the first case, at the same day of analysis, while their losses were slower. It should also be underlined that in these bread samples more esters at higher concentrations were determined, which is considered to have a positive impact on the bread aroma (Maga, 1974). Additionally, the carbonyl compounds content seemed to play a major role for the sensory preference of breads. As presented in Fig. 1, the breads made with

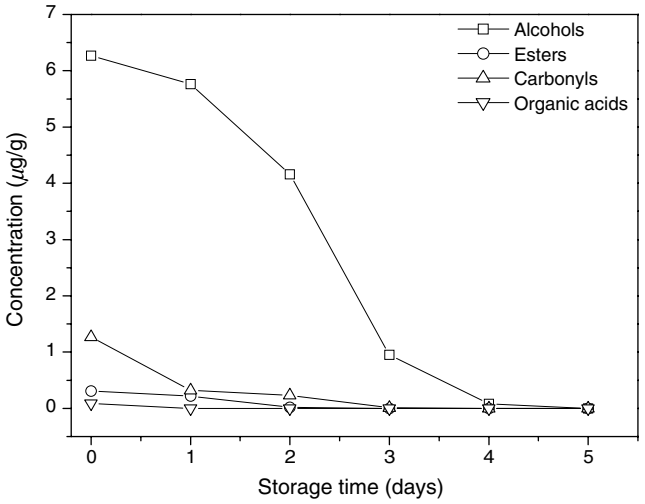

Fig. 2. Effect of storage time on the amounts of volatiles identified in the headspace aroma of breads made with 50% sourdough containing 1% *K. marxianus* and 4% *L. helveticus*.

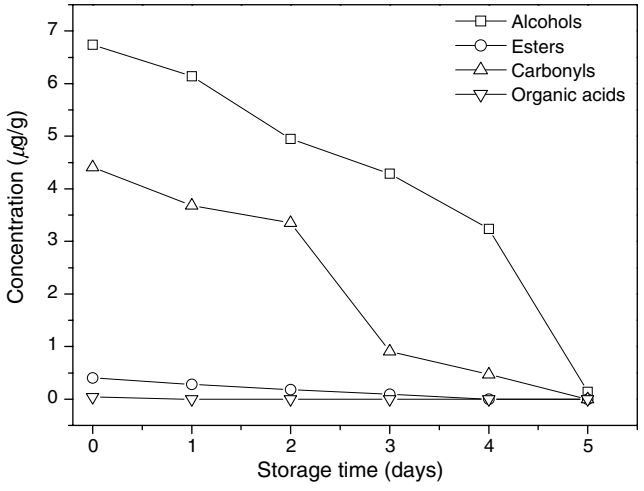

Fig. 1. Effect of storage time on the amounts of volatiles identified in the headspace aroma of breads made with 50% sourdough containing 1% *K. marxianus* and 4% *L. bulgaricus*.

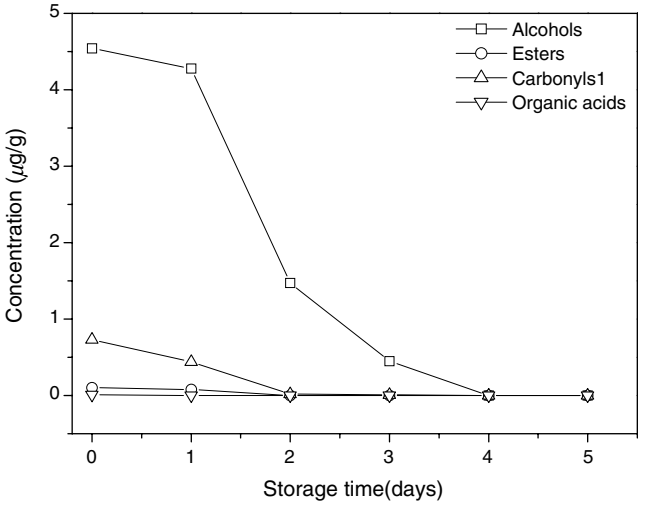

Fig. 3. Effect of storage time on the amounts of volatiles identified in the headspace aroma of breads made with 50% sourdough containing 1% *K. marxianus* (control).

Table 4
Effect of storage time on consumer preference for breads made with sourdoughs containing co-cultures of *K. marxianus* with *L. bulgaricus* or *L. helveticus*, as well as sourdough bread made using only *K. marxianus* (control)

Sourdough culture	Storage time (days)					
	0	1	2	3	4	5
K. marxianus and L. bulgaricus	8.5 ± 0.23	8.1 ± 0.14	7.9 ± 0.36	7.2 ± 0.15	6.9 ± 0.33	5.3 ± 0.07
K. marxianus and L. helveticus	7.9 ± 0.45	7.3 ± 0.11	6.9 ± 0.12	6.7 ± 0.13	6.1 ± 0.14	5.2 ± 0.23
K. marxianus	7.5 ± 0.14	7.1 ± 0.11	6.9 ± 0.16	5.4 ± 0.14	5.1 ± 0.12	4.8 ± 0.21

sourdough containing *K. marxianus* and *L. bulgaricus* maintained higher contents of alcohols and carbonyl compounds during storage, compared to the other types of breads examined, which may also impart a positive effect on bread aroma as suggested by other researchers (Lorenz & Maga, 1972).

To estimate how these changes of volatile composition during storage generally reflect on bread flavour, consumer preference evaluations were organised, and the results are shown in Table 4. These results show that the sourdough breads containing *K. marxianus* and *L. bulgaricus* achieved the best scores, the lowest being those of the control samples. These results are justified by the higher number of volatile compounds identified in this type of bread and particularly the higher total amounts of alcohols and carbonyl compounds, which were maintained at good levels after two days of production (Table 1). Consumer preference fell as storage time increased and staling/evaporation changes evolved, as expected. As far as the volatiles concentration reduction patterns are concerned, they were similar for all the examined bread samples.

4. Conclusion

The changes on headspace aroma volatiles of sourdough breads during storage was effectively assessed using SPME GC–MS analysis. The obtained results revealed differences in the volatile composition of the different types of breads examined, as well as big losses of volatiles during storage, mainly due to staling and evaporation changes since no microbial spoilage was observed within the tested time period. The sourdough bread, made with *K. marxianus* and *L. bulgaricus*, had a more complex aroma profile, longer shelf-life and achieved the highest scores during consumer evaluation (preference) tests. The longer shelf-life of this bread (especially delayed staling) compared to the other types of breads examined (Plessas et al., 2008a), indicates a direct correlation between the physicochemical changes occurring during storage and the evolution of the volatile composition of aroma. Finally, it should be noted that cheese whey was proposed as growth medium for all the microorganisms used, as a cheap by-product of the dairy industry, in order to reduce the cost of starter culture production and give at the same time an alternative solution to whey disposal, which causes serious environmental problems worldwide.

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