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ORIGINAL PAPER

A large diversity of lactic acid bacteria species is involved in the fermentation of wheat used for the manufacture of *lemzeiet*

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Abstract Algerian couscous named *lemzeiet* is manufactured from fermented wheat. Historically performed in underground silos called *matmor*, the fermentation of wheat is now generally carried out in plastic jerrycans with or without addition of vinegar at the beginning of the fermentation. Culture-dependent and culture-independent methods (PCR-TTGE) were used to characterize lactic acid bacteria and to determine their dynamic and diversity over a two-year period, with and without the addition of vinegar. Fungi, physicochemical characteristics, and volatile compound profiles were also monitored. The isolates obtained from different stages of fermentation and from both processes were characterized by coupling different molecular methods (16SrRNA/*pheS*/*rpoA* gene sequencing, species-specific PCR, RAPD and PFGE). PCR-TTGE revealed very similar profiles for both processes. Sixty-nine isolates were identified as belonging to six genera of 16 species (*Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Weissella*, and *Streptococcus*). The profiles of volatile aroma compounds showed a marked effect of the fermentation process, compared to non-fermented wheat, with 35 of the 40 volatiles detected at amounts 20- to 30-fold higher in fermented wheat samples. This study gives the first insight into lactic acid bacteria population diversity and activity in fermented wheat and will contribute to a better control of the fermentation process.

Keywords Lactic acid bacteria · Fermentation · Cereal · Vinegar · Volatile

Abbreviations

ANOVA	Analysis of variance
BHYE	Brain heart infusion yeast extract
CFU	Colony forming unit
DMS	Dimethyl sulfide
DMDS	Dimethyl disulfide
FA	Fat acidity
GC–MS	Gas chromatography–mass spectrometry
HS	Headspace
LAB	Lactic acid bacteria
LSD	Least significant difference
MA	Malt agar
MRS	Man Rogosa and Sharpe
OAIC	Office algérien interprofessionnel des céréales
PCA	Principal component analysis
pheS	Phenylalanyl-t-RNA synthetase alpha-subunit
PFGE	Pulsed-field gel electrophoresis
RAPD	Random amplified polymorphic DNA
rpoA	RNA polymerase alpha-subunit
TTA	Titratable acidity

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TTGE	Temporal temperature gradient gel electrophoresis
UPGMA	Unweighted pair group method with arithmetic mean
VBNC	Viable but non-culturable

Introduction

Fermentation is an ancestral process and one of the most economical methods of conservation and transformation of raw food materials. It improves the quality of food products not only by prolonging their shelf life, removing toxic and anti-nutritional factors but also by improving flavor development and increasing the nutritional value of food [1]. In addition, microbial fermentation processes can generate bioactive peptides during food processing or ripening [2]. Moreover, some strains of lactic acid bacteria (LAB) isolated from fermented foods, mainly belonging to the genus *Lactobacillus*, have been described as health-promoting cultures or probiotics [3, 4].

Fermented foods are staples for many populations, particularly in developing and emerging countries where fermentation is often the only way to preserve food from microbial contaminations [5]. They also partake in cultural identity since they are related to very ancient traditional food habits and practices [6].

Conventional fermented foods and beverages that are widely produced in the Western world (dairy products, sourdough, meat, and wine) have received a significant scientific attention related to the existence of strong economic markets for these products. This is not the case in developing countries, particularly in Africa [7], where the preparation of many indigenous or traditional fermented foods in certain areas still remains a house art. Fermented foods are often produced at homes, villages, or small-scale industries [7, 8].

Cereal-based fermented foods have received a particular attention because they contribute to human energy intakes. Cereal grains including sorghum, maize, and millet are common substrates for the production of lactic fermented gruels and beverages such as *poto poto* in Congo, *dégué* in Burkina Faso [9], *ben-saalga* [10], *ogi* in Nigeria [11], *koko* in Ghana [12], and *togwa* in Tanzania [13].

LAB are responsible for acid and flavor development of many fermented cereal foods. Most of the cereal fermented foods involve a lactic fermentation step, which can or cannot be associated with an alcoholic fermentation step, depending on the process used [14]. Heterofermentative and homofermentative LAB species are commonly found associated, and most of them belong to the genera *Lactobacillus*, *Leuconostoc*, *Weissella*, *Lactococcus*, *Pediococcus*, *Enterococcus*, and *Streptococcus* [15].

Most research on traditional cereal-based fermented foods was devoted to characterize the microbial community involved. The ultimate goal is to acquire the necessary knowledge to improve the quality and the attractiveness of these fermented foods, warranty their safety, and allow their production on a larger scale. In Algeria, fermented wheat is used to manufacture the couscous *lemzeiet*. It is obtained after the storage of grains of durum wheat for several years in underground silos called *matmor*, used historically for the storage of wheat. Following rainwater seepage inside *matmor*, flooded wheat with a spontaneous fermentation, offers new features with a typical flavor, texture changes, and a dark brown color [16]. Traditionally performed inside *matmor*, the fermentation of wheat is now generally carried out in plastic jerrycans or drums for around 2 years with or without the addition of vinegar at the beginning of the fermentation. In fact, the ancient process has been gradually abandoned due to rural exodus and the widespread modern methods of wheat storage. The microbiota present in fermented wheat used to prepare *lemzeiet* (and its composition) has never been investigated. The aim of this work was to characterize LAB populations, verify product safety regarding fungi contamination, and determine volatile profiles of fermented wheat with and without initial addition of vinegar over a 2-year period of storage.

Materials and methods

Wheat origin

Eighty kilograms of durum wheat (a local variety "Mhamed Ben Bachir from OAIC," produced in 2010, Sétif- Algeria) was used. The fermentation started in February 2011 and finished in February 2013.

Fermented wheat technology and sampling procedures

Two uncontrolled processes, i.e., with and without vinegar addition prior to the fermentation step, were reproduced, exactly, like the traditional artisans' method at laboratory scale in ten plastic jerrycans of 10 L. Six kilograms of wheat introduced in each jerrycan. In five of them, wheat was mixed with 4 L of spring water. For the other five, spring water was previously mixed with 150 mL of vinegar solution (Nassah, 5°). The jerrycans were sealed and incubated at room temperature in the dark, and samples were collected aseptically in plastic bags after 3, 6, 9, 12, and 24 months. Ambient temperature could vary from 0 °C

in winter to more than 40 °C in summer, and wheat was fermented at different seasons until the desirable taste and aroma were produced.

Physicochemical analyses

Fat acidity (FA) and titratable acidity (TTA) of fermented wheat were determined according to ISO 7305 and AACC international method 02-31, respectively. pH was determined using a pH meter (Jenway 3505, England) as follows: 20 g of each grinded sample was suspended into 80 mL of distilled water and homogenized using a Warring Blender for 2 min at maximal speed, and the pH was measured in the supernatant.

LAB enumeration and isolation

Ten grams of each sample was mixed with 90 mL of sterile tryptone water and homogenized in a Stomacher lab-blender for 2 min at maximal speed. Aliquot of serially diluted suspension was pour-plated with the following media: MRS agar, MRS agar (pH 5.4), and BHIYE agar (BHI agar with 1 % (w/v) Yeast Extract), (Biokar Diagnostics) at 30 °C in aerobic and anaerobic conditions (Anaerocult®AMerck, Germany) for 48–72 h. In order to collect the most diverse bacteria possible, colonies were picked from the highest dilutions based on their different morphological characteristics. The purity of the isolates was checked by streaking again and subculturing on fresh broth and agar plates of the isolation media, and by microscopic observations (Nikon Optiphot phase-contrast equipment). Non-motile rods and cocci isolates from MRS, MRS (pH 5.4), and BHIYE media were considered as presumptive LAB. Purified isolates were stored at −80 °C in the isolation broth media (MRS or BHIYE) added to (1/1) with 30 % (v/v) glycerol.

Yeasts and molds enumeration

For molds and yeast enumeration, 20 g of each sample was suspended into 180 mL sterile Tween 80 solution 0.5 % (v/v), homogenized using a Stomacher lab-blender for 2 min at maximal speed, and serially diluted from the initial suspension. Aliquots of 100 µL of each dilution were spread into MA plates (Biokar Diagnostics) and incubated at 25 °C for 2–10 days.

DNA extraction from pure cultures

Total DNA was extracted by DNeasy Bloodtissue kit (Qiagen ref 69504; Hilden, Germany) using manufacture protocol for Gram positive with two modifications: Pellet of 1 mL of pure culture was treated with lysis buffer (20 mM Tris- HCl, pH 8.0, 2 mM EDTA, 1 % TritonX100, 20 mg/mL lysozyme and 10 µL/mL mutanolysine), and the proteinase K step was achieved at 56 °C for 30 min. DNA concentrations were quantified at a wavelength of 260 nm with Nanodrop DN-1000 spectrophotometer (Labtech, Palaiseau, France).

16S rDNA sequencing

The 16S rDNA gene was amplified by w001 and w002 primers according to Godon et al. [17] using TM thermal cycler C1000 (Bio-Rad, Australia). Amplified PCR products were sequenced by the Sanger method. Sequences were assembled using VectorNTI (Invitrogen) and analyzed by Blast on NCBI.

Housekeeping gene sequencing and multiplex species-specific PCR for *Enterococcus* sp. and *Pediococcus* sp.

To discriminate the closely related species of enterococci, the housekeeping genes *rpoA* (encoding the RNA polymerase, alpha-subunit) and *pheS* (encoding the phenylalanyl-t-RNA synthetase alpha-subunit) were sequenced. The amplification was performed as previously described [18]. Amplified PCR products were sequenced and analyzed as described above in 16S rDNA sequencing section. To validate the identification of *Pediococcus* species, multiplex PCR with species-specific primers were used according to Petri et al. [19].

Nucleotide sequence accession numbers

DNA sequences were submitted to the GenBank database and were given the following nucleotide sequence accession numbers: KP189205 to KP189234, KP221609 to KP221642, KP062948, KP062949, KP233799, and KP233800.

Random amplified polymorphic DNA (RAPD) analysis

RAPD–PCR was performed with five RAPD primers previously described [20]. The PCR mix (25 µL) consisted of Taq buffer with $MgCl_2$ (Q-Biogene/EPTQA100), dNTPs (2 mM/µL, Q-Biogene/NTPMX050), random primer (100 mM/µl, RAPD Analysis Kit GE Healthcare), Taq DNA polymerase (2.5 U/µL), and 1 µL of the extracted DNA (25 ng/µL). DNA amplification was carried out in a C1000TM thermal cycler (Bio-Rad) using the following conditions: 94 °C for 2 min; 40 cycles of 94 °C for 1 min, 42 °C for 20 s and 72 °C for 2 min; and a final extension step at 72 °C for 10 min. The amplified products were

resolved by electrophoresis (100 V, 1 h) on 1.5 % agarose gel (w/v) in 0.5 × TBE buffer.

Pulse-field gel electrophoresis (PFGE)

The PFGE molecular fingerprints of LAB isolates were obtained using a method adapted from Smith et al. [21]. Macrorestriction of genomic DNA was performed with endonuclease *SmaI* (20 U/mL) and *AscI* (10 U/mL) (New England Biolabs, Hitchin, United Kingdom), according to the manufacturer's recommendations. *SmaI* was used for enterococci and pediococci or *AscI* for lactobacilli species. PFGE was carried out with a CHEF-DR II apparatus (Bio-Rad, Australia) in a 1 % agarose gel (w/v) in 0.5 × TBE at 14 °C. Running parameters were as follows: for pediococci: 6 V/cm, 14 °C, initial switch 5 s, final switch 50 s, length 20 h; for lactobacilli: 6 V/cm, 14 °C, initial switch 2 s, final switch 20 s, length 21 h; and for enterococci: 6 V/cm, 14 °C, initial switch 5 s, final switch 35 s, length 20 h (1st cycle), and initial switch 5 s, final switch 10 s, length 4 h (2nd cycle).

DNA extraction for temporal temperature gel electrophoresis (TTGE)

Wheat samples (10 g each) were mixed with 90 mL 0.9 % NaCl solution, incubated at 46 °C for 15 min, and homogenized in a Stomacher lab-blender for 5 min at maximal speed. Bacterial cells were recovered from homogenates by differential centrifugation first at 2000×*g* for 5 min and then at 8000×*g* for 15 min. DNA extraction and quantification were performed as described above in DNA extraction from pure cultures.

PCR-TTGE

The V3 region of the 16S rRNA gene was amplified by using the primers V3P3-GC-Clamp and V3P2 previously described [22]. For TTGE analysis, a DCode universal mutation detection system (Bio-Rad Laboratories, Hercules, CA) was used to separate the V3 region PCR products, and 25 µL of PCR product was added to 10 µL of loading buffer (0.05 % bromophenol blue, 0.05 % xylene cyanol, 70 % glycerol). Gels were prepared with 10 % (w/v) bisacrylamide/Bis (37.5:1), 7 M urea and run with 1.25 × TAE buffer diluted from 50 × TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 mM EDTA, pH 8). Migration was performed at 41 V for 16 h with a temperature gradient of 63 °C to 70 °C (rate of 0.4 °C h^{-1}) adapted for bacteria of low GC content. The bands were analyzed by using a database previously developed by Parayre et al. [23] for presumptive species identification.

Gel treatment and analysis

All gels were stained in GelRed (3× in 0.1 M NaCl solution) (FluoProbes®, Interchim, France) for 15–30 min. The images were acquired with GBox equipment (Syngene, Cambridge, UK) and analyzed by Bionumerics version 6.5 software (Applied Math, Belgium). The similarities of the profiles were calculated using the Dice coefficient, and the dendrograms were obtained by means of the Unweighted Pair Group Method using an Arithmetic Average (UPGMA) clustering algorithm.

Analysis of volatile compounds using GC–MS

Volatile compounds were extracted using headspace (HS) Trap and analyzed by gas chromatography–mass spectrometry (GC–MS), as previously described [24], using a Turbomatrix HS-40 automatic HS sampler with trap enrichment, a Clarus 680 GC, and a Clarus 600T quadrupole MS (Perkin-Elmer, Courtaboeuf, France). A 4 g aliquot of wheat sample (performed in triplicate) was placed in a 20 mL Perkin-Elmer vial. Briefly, the volatiles were separated on an Elite 5MS capillary column (60 m × 0.25 mm × 1 µm; Perkin-Elmer), with helium as the mobile phase. The temperature of the oven was initially 35 °C, maintained for 5 min. The temperature was increased at 7 °C min^{-1} to 140 °C and then at 13 °C/min to 280 °C. The mass spectrometer was operated in the scan mode (scan time 0.3 s, interscan delay 0.03 s) within a mass range of *m/z* 29–206. Ionization was done by electronic impact at 70 eV. Standards (a 20 ng/g aqueous solution of a mixture of 11 volatile compounds, prepared as previously described [24]) were regularly injected to verify the instrumental drift of the GC–MS system. Blank samples (boiled deionized water) were injected to control the absence of carryover.

GC–MS data were processed by converting the raw data to time- and mass-aligned chromatographic peak areas using the open source XCMS package implemented with the R statistical language [25]. Compounds were identified by comparison with mass spectra and retention times with those of authentic standards, or on the basis of their retention index and mass spectral data from the NIST 2008 Mass Spectral Library (Scientific Instrument Services, Ringoes, NJ, USA). From all the signals generated by XCMS, 40 **c**ompounds were identified and quantified from the abundance of one specific ion (*m/z*). Non-odorant compounds (e.g., alkanes, alkenes) and some contaminants, such as trichloromethane and toluene, were not retained in the dataset.

The data areas obtained were subjected to an analysis of variance (ANOVA) using R statistical software to

determine whether the process and the ripening time influenced the abundance of each volatile. Means were compared using the least significant difference (LSD) test. A principal component analysis (PCA) was performed by means of triplicates for 40 volatiles compounds, after a log(x) transformation and Pareto scaling, using the package FactomineR of the R software.

Results

Microbial counts and physicochemical analysis

Mesophilic aero-anaerobic LAB counts on MRS, MRS (pH 5.4), and BHIYE agar media did not differ by more than 0.5 log cfu g^{-1}. They were below 10 cfu g^{-1} in non-fermented wheat and reached maximum values of 4.2 and 5.1 log cfu g^{-1} in fermented wheat with and without vinegar, respectively, during the ripening process. At 3 months of fermentation, they reached similar populations for both processes (4.2 and 4.6 log cfu g^{-1} in samples with and without vinegar, respectively). After 12 months, culturable LAB population decreased to 2.1 log cfu g^{-1} in fermented wheat with vinegar, while in the process without vinegar they were still present at 5.1 log cfu g^{-1}. LAB counts markedly decreased after long ripening period, with culturable populations below 2.5 log cfu g^{-1}. Yeasts and molds initially present in wheat at 3.5 and 2.5 log cfu g^{-1}, respectively, were no more detected after 3 months of fermentation.

Fermentation process decreased the pH and increased both TTA and FA of the wheat regardless the addition of vinegar. pH, TTA, and FA ranged from 4.39 to 4.95, 0.16 to 0.24 %, and 51.2 to 122.9 mg/100 g (w/w, on dry matter basis), respectively, for fermented wheat with vinegar, and from 4.23 to 4.74, 0.15 to 0.25 %, and 79.16 to 114.4 mg/100 g for fermented wheat without vinegar (Table 1).

Table 1 Changes in physical and chemical composition of fermented wheat during fermentation (w/w or %, on dry matter basis): A with vinegar, B without vinegar

Time (months)	pH	TTA[a]	FA[b]
A			
0	4.02 ± 0.01	0.01 ± 0.00	27.63 ± 1.41
3	4.51 ± 0.01	0.16 ± 0.01	51.96 ± 0.93
6	4.95 ± 0.01	0.23 ± 0.01	66.61 ± 0.93
9	4.61 ± 0.02	0.24 ± 0.01	65.17 ± 0.93
12	4.50 ± 0.01	0.18 ± 0.01	80.16 ± 0.94
24	4.39 ± 0.03	0.24 ± 0.01	122.90 ± 2.81
B			
0	8.30 ± 0.01	0.01 ± 0.00	27.63 ± 1.41
3	4.54 ± 0.01	0.15 ± 0.01	80.24 ± 1.86
6	4.27 ± 0.01	0.21 ± 0.01	96.26 ± 1.86
9	4.36 ± 0.01	0.21 ± 0.01	80.03 ± 0.93
12	4.74 ± 0.01	0.20 ± 0.01	79.16 ± 0.94
24	4.23 ± 0.02	0.25 ± 0.01	114.40 ± 2.77

Values are means and standard deviation of three analysis. The standard deviation is given in ±

[a] Titratable acidity (%)

[b] Fat acidity (mg/100 g)

Identification of LAB fermented wheat isolates

A total of 69 LAB isolates were selected from the two processes based on cell morphology similarity using phase-contrast microscope observations, and retained for molecular characterization. Based on their 16S rRNA gene sequences, they were identified as belonging to six genera: *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Weissella*, and *Streptococcus.* The isolates that could not be identified at the species level on the basis of the 16S rRNA gene sequences, because of their high levels of similarity with closely related species, were further analyzed using *rpoA* or *pheS* genes (for enterococci) or multiplex species-specific PCR (for pediococci). A total of 16 species were detected during wheat fermentation, with nine and ten species in samples fermented without vinegar and with vinegar, respectively (Table 2). Three species were detected in both processes: *Enterococccus faecium*, *Pediococcus pentosaceus*, and *Pediococcus acidilactici*. Only *E. faecium* was identified in the non-fermented wheat (three isolates) (Table 2).

LAB clonal diversity

In order to investigate the clonal diversity of the predominant genera *Enterococcus*, *Lactobacillus*, and *Pediococcus*, a total of 57 selected isolates from all samples and from both processes were analyzed by RAPD and PFGE. LAB isolates were clustered according to the duration of fermentation and to the process used. Among the five primers used for the RAPD method, the most discriminant primers were primer nos. 1 and 5 (data not shown). Using these primers, the 57 isolates were classified into three and four distinct clusters, respectively, unrelated with the species. Compared with PFGE, RAPD showed relatively similar results for lactobacilli isolates. Only three distinct strains were identified from the 13 analyzed isolates, two for *Lactobacillus brevis* and one for *Lactobacillus coryniformis*. However, for the 24 *E. faecium* and the 18 *P. pentosaceus* isolates, PFGE was more discriminant than RAPD with 21 and 11 distinct strains,

Table 2 Identities of lactic acid bacteria isolates from fermented wheat in plastic jerrycans with or without initial addition of vinegar, over a 2-year period of storage

Number of isolates	Detected at stage (months)						Closest relative	Sequence similarity (%)
	0	3	6	9	12	24		
Wheat								
3	x						*E. faecium*	99[a]/99[b]
Without vinegar								
9			x		x	x	*L. brevis*	99[a]
4		x				x	*P. pentosaceus*	99[c]
3		x					*E. faecium*	99[a]/99[b]
1		x					*E. faecalis*	100[a]/99[b]
1					x		*L. buchneri*	99[a]
1						x	*L. namurensis*	99[a]
1		x					*Leu. mesenteroides*	100[d]
1		x					*P. acidilactici*	99[c]
1		x					*L. rapi*	99[a]
With vinegar								
18		x	x	x	x		*E. faecium*	99[a]/99[b]
14			x	x	x		*P. pentosaceus*	99[c]
4				x			*L. coryniformis*	100[a]
2						x	*S. mitis*	99[a]
1					x		*P. acidilactici*	99[c]
1		x					*E. hirae*	99[a]/99[b]
1		x					*W. paramesenteroides*	99[a]
1		x					*Leu. fallax*	99[a]
1		x					*L. curvatus*	99[a]
1				x			*L. pentosus*	99[a]

[a] Percent similarity based on rRNA sequencing

[b] Percent similarity based on *rpoA* and *pheS* genes sequencing

[c] Percent similarity based on 16S rRNA sequencing and confirmed by multiplex species-specific PCR

[d] Percent similarity based on species-specific PCR

E, *Enterococcus*; L, *Lactobacillus*; Leu, *Leuconostoc*; P, *Pediococcus*; S, *Streptococcus*; W, *Weissella*

respectively, compared with 12 and four strains with primer no.1 (Fig. 1).

TTGE

PCR-TTGE revealed very similar profiles for both processes, characterized by two phases (Fig. 2). In the first one (3–12 months), TTGE profiles showed low complexity, mainly consisting of three bands (a, b, and c). The comparison with database showed that the dominant or main species (band b) could be assigned to *P. pentosaceus*, *Lactobacillus fermentum,* or *Leuconostoc fallax* species. The second band (a) could be assigned to *Lactobacillus plantarum* or *Lactobacillus pentosus* species. Band c was also present in the majority of samples and at all stages of fermentation and was assigned to *P. pentosaceus* species. From the 24th month, samples from both processes were characterized by a more complex TTGE pattern constituted of 5 to 6 bands including the three main bands (a), (b) and (c). The three new bands (d), (e), and (f) were presumably assigned to *Lactobacillus buchneri* or *Lactobacillus rapi*, to *E. faecium* or *L. brevis,* and to *L. coryniformis*, *Lactobacillus helveticus,* or *Lactobacillus acidophilus,* respectively.

Profiles of volatile compounds

A total of 1778 signals (one *m/z* at one retention time) were generated by XCMS, corresponding to about 60 different compounds, since multiple signals are generated per compound. Non-odorant compounds (alkanes, alkenes, among others) and some contaminants, such as trichloromethane, were not retained in the dataset. Forty aroma compounds were identified, including ten alcohols, nine aldehydes, eight esters, six acids, four ketones, and three sulfur compounds. The profile of volatile aroma compounds was markedly impacted by the fermentation process, with most (35 of 40) volatiles present at abundances 30-fold higher, on average, in fermented wheat samples compared to the non-fermented wheat.

The analysis of variance (ANOVA) performed for all these compounds showed that the amount of most of them was significantly affected by the ripening time (for 38 of 40), by the process (20 compounds), and by the interactions between both factors (28 compounds) (Table 3). These results mean that the effect of the process differed depending on the sampling time for the 28 compounds showing a significant Time × Process interaction. In other words, it is not possible to conclude on a global effect of the process.

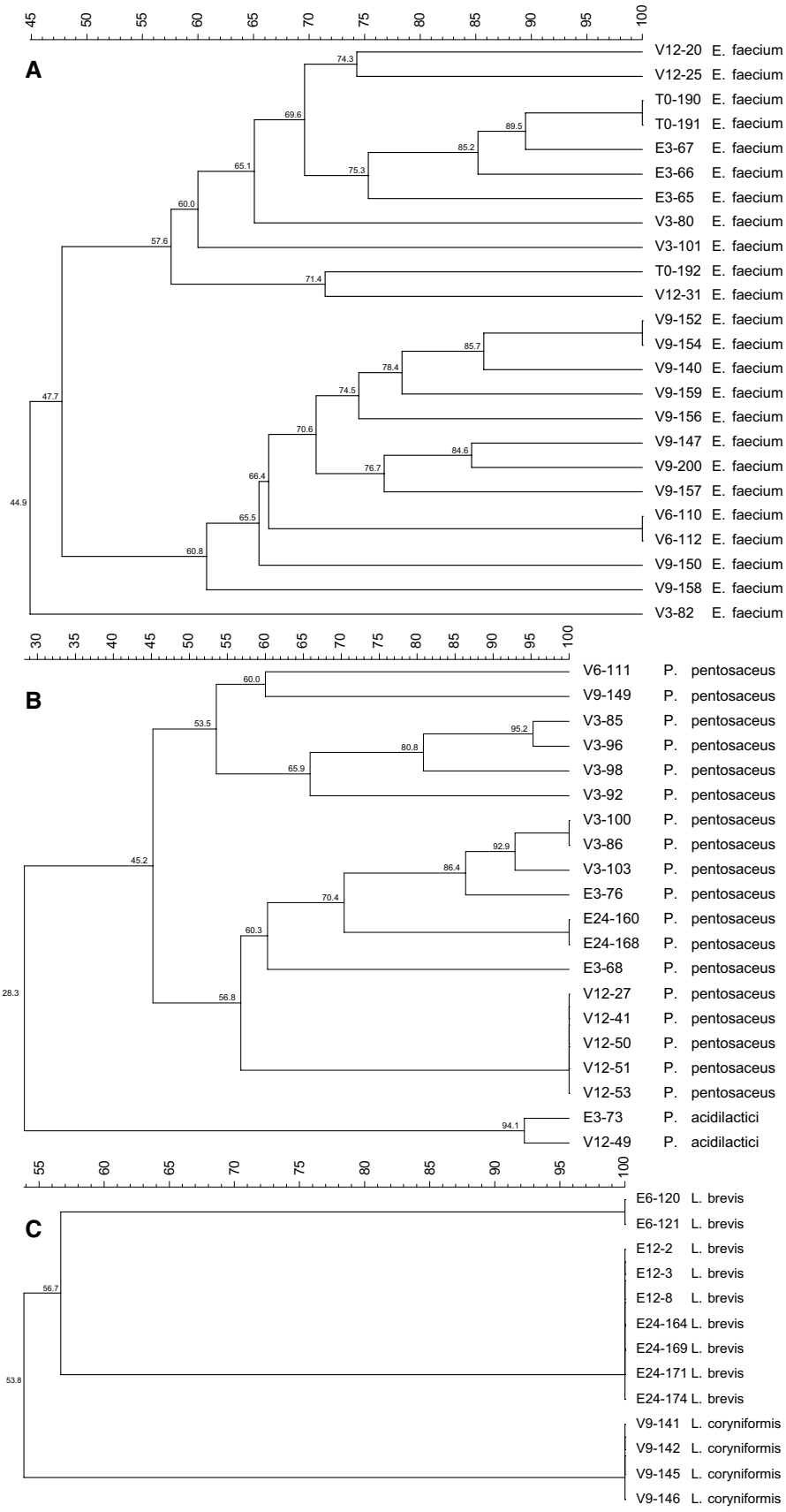

Fig. 1 Dendrograms of PFGE patterns similarity of *E. faecium* (**a**), *Pediococcus* sp. (**b**), and *Lactobacillus* sp. (**c**) strains isolated from wheat with (V) or without (E) initial addition of vinegar fermented for 0, 3, 6, 9, 12, and 24 months. **a** and **b** were obtained with endonuclease *SmaI*, C with endonuclease *AscI*. The similarities of the profiles were calculated using the Dice coefficient, and the dendrograms were obtained by UPGMA clustering algorithm. E: fermented wheat without vinegar, V: fermented wheat with vinegar, T: non-fermented wheat, 0, 3, 6, 9, 12, 24: time of fermentation or ripening in months

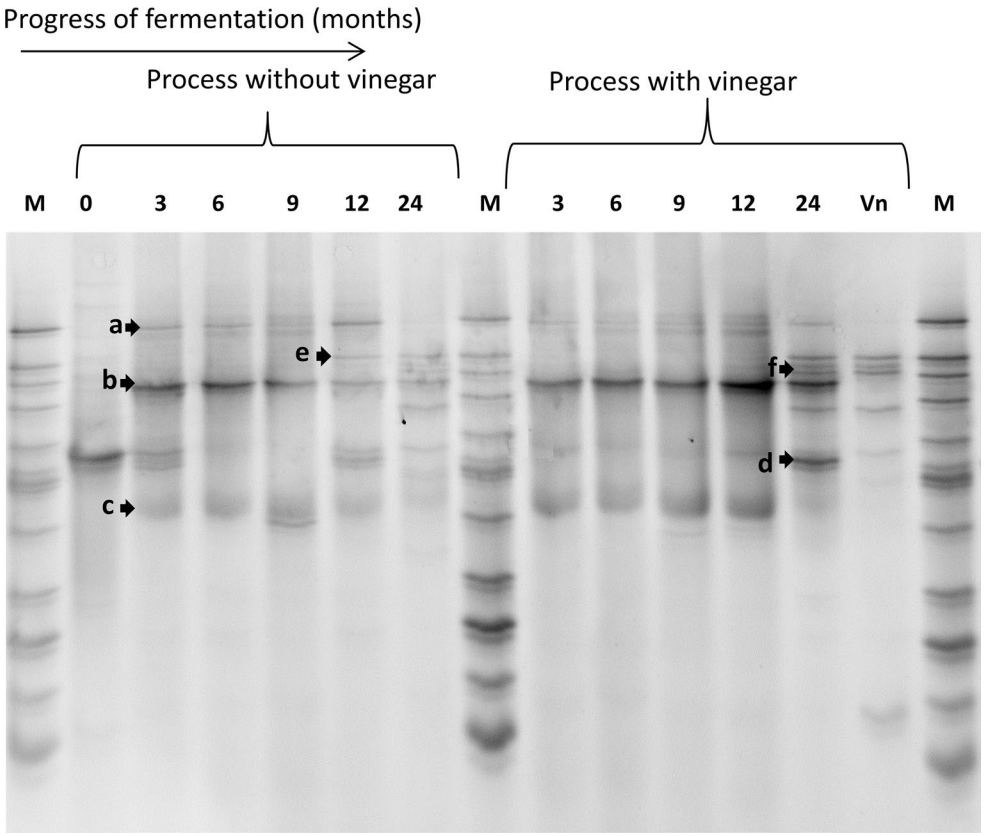

Fig. 2 PCR-TTGE analysis of V3 16S rRNA gene fragments from samples of fermented wheat collected from both processes and at different time of fermentation. *Lanes*: M, genomic DNA marker (*Lactobacillus plantarum*, *Enterococcus faecium*, *Lactobacillus helveticus*, *Lactococcuslactis*, *Streptococcus thermophilus*, *Corynebacterium moorparkense*, *Lactobacillus paracasei*, *Arthrobacterium nicotianae,* and *Brevibacterium casei*); 0, non-fermented wheat; Vn, vinegar 5°. PCR-TTGE bands obtained from samples are as follows: *a Lactobacillus plantarum/Lactobacillus pentosus; b Pediococcus pentosaceus/Lactobacillus fermentum/Leuconostoc fallax*, *c Pediococcus pentosaceus*, *d Lactobacillus buchneri/Lactobacillus rapi*, *e Enterococcus faecium/Lactobacillus brevis*, *f Lactobacillus coryniformis/Lactobacillus helveticus/Lactobacillus acidophilus*

Very probably, the variations of these aroma resulted from change of the microbial community over time. In contrast, a significant effect of the process was observed in the absence of Time × Process interactions for one compound only, ethanol (Table 3). Ethanol was detected at a higher amount (1.7-fold, on average) in wheat fermented in the presence of vinegar. More than half of the compounds exhibited a marked (>tenfold) increase during the ripening. Straight-chain aldehydes (pentanal, hexanal, and nonanal) showed their maximum amount in 3- and 6-month-fermented samples, and then, their concentration decreased until the end of the ripening period. On the opposite, some acids, such as butanoic, 2-methylbutanoic, and 3-methylbutanoic acids, reached a maximum concentration only after 12 months of ripening, regardless of the process used (data not shown).

A PCA was performed to illustrate the main differences in volatile profiles of all wheat samples (Fig. 3). The first two axes accounted for 70 % of the total variability. PC1, describing 55 % of the variability, was positively associated with high concentrations of most volatiles, including acids, esters, sulfur compounds, and diacetyl, while two alcohols (1-pentanol and 1-hexanol) and hexanoic acid were negatively associated with PC1. PC1 separated the non-fermented wheat sample (T0), appearing in the left, from all samples, positively associated with most variables. PC2, accounting for 14 % of the variability, was positively related to many esters and branched-chain alcohols and negatively associated with butanoic acid, branched-chain acids, and 2,3-pentanedione. PC2 separated samples fermented for 6 months at least, regardless of the process of fermentation. For example, the samples of 9-month-fermented wheat without vinegar (E9) and of 24 months-fermented wheat with vinegar (V24) were both situated in the upper right quartet. These two samples contained significantly more 3-methylbutanol, an ester of this alcohol 3-methylbutyl acetate and diacetyl compared to other samples. The sample of 12-month-fermented wheat without vinegar (E12) contained significantly more propanol and two corresponding esters (propyl acetate and propyl butanoate). PC3, accounting for 12 % of the variability, was positively related to straight-chain aldehydes (pentanal, hexanal, and nonanal) and to the 3- and 6-month-fermented samples (data not shown).

Discussion

To determine the diversity of microorganisms in natural ecosystems and to follow the evolution of microbial populations over space and time, culture-dependent and culture-independent methods are recommended. Both methods

Table 3 Volatile compounds identified, ion (*m/z*) of quantification, and *P* value of the ANOVA showing the effect of the time or of the process on the amounts of the compounds

RI[a]	Compounds (abbreviated code or trivial name)	*m/z*	Identification[b]	*P*, time[c]	*P*, process[c]	*P*, time and process[c]
471	Ethanol	31	S, RI, DB	**	**	ns
517	Dimethylsulfide (DMS)	62	S, RI, DB	***	***	***
554	Propanol	59	RI, DB	***	***	***
556	2-Methylpropanal	72	S, RI, DB	***	ns	***
589	2,3-Butanedione (diacetyl)	86	S, RI, DB	***	ns	***
597	2-Butanone	–	S, RI, DB	*	ns	ns
613	Ethylacetate (ethyl C2)	70	S, RI, DB	***	ns	***
622	Acetic acid (C2)	60	S, RI, DB	**	ns	ns
659	3-Methylbutanal	58	S, RI, DB	***	ns	ns
666	2-Methylbutanal	41	S, RI, DB	***	ns	ns
666	1-Butanol	55	RI, DB	***	**	***
695	2,3-Pentanedione	100	RI, DB	***	**	***
701	Pentanal	58	RI, DB	***	ns	ns
712	Propylacetate(propyl C2)	61	RI, DB	***	***	***
712	Propanoicacid (C3)	74	S, RI, DB	***	***	***
712	2-Butanone-3-hydroxy (acetoin)	45	S, RI, DB	***	**	***
739	3-Methylbutanol	70	S, RI, DB	***	**	***
745	2-Methylbutanol	57	S, RI, DB	***	***	***
752	Dimethyldisulfide (DMDS)	94	S, RI, DB	***	ns	*
766	1-Pentanol	55	RI, DB	***	***	***
781	Butanoicacid (C4)	60	S, RI, DB	***	*	***
800	Ethyl butanoate (ethyl C4)	116	S, RI, DB	***	***	***
803	Hexanal	56	RI, DB	**	ns	**
806	2,3-Butanediol	90	RI, DB	ns	ns	ns
811	Ethyl2-hydroxypropanoate (ethyl lactate)	75	RI, DB	***	***	***
816	Butylacetate (butyl C2)	56	RI, DB	***	ns	***
831	Furfural	95	S, RI, DB	***	***	***
836	3-Methylbutanoic acid	87	S, RI, DB	***	***	***
845	2-Methylbutanoic acid	74	S, RI, DB	***	***	***
859	Furanmethanol	98	RI, DB	***	ns	**
871	1-Hexanol	69	S, RI, DB	***	ns	ns
876	3-Methylbutyl acetate (3-methylbutyl C2)	87	RI, DB	***	ns	***
880	Propyl butanoate (propylC4)	71	RI, DB	***	***	***
911	3-Methylthiopropanal (methional)	48	RI, DB	***	ns	ns
961	Benzaldehyde	105	S, RI, DB	***	ns	**
962	Hexanoic acid (C6)	87	S, RI, DB	**	ns	**
993	Butyl butanoate (butyl C4)	89	RI, DB	***	***	***
1056	Benzeneacetaldehyde	91	RI, DB	**	ns	ns
1104	Nonanal	82	RI, DB	***	***	**
1118	Phenylethanol	92	RI, DB	ns	ns	ns

[a] RI, Kovatsretention index

[b] Compounds identified on the basis of: S, retention time and mass spectrum from standard, *RI* retention index, *DB* mass spectral data Library NIST

[c] *P* value of ANOVA: $***P < 0.001$, $**P < 0.01$, $*P < 0.05$, $^{ns}P > 0.05$

can get a biased view of microbial diversity, mainly if they are used independently [26, 27]. In the present study, the characterization of diversity of LAB in fermented wheat was investigated by combining the results of TTGE-PCR, species-specific PCR, and 16SrDNA, *pheS*, *rpoA* genes sequencing of representative strains.

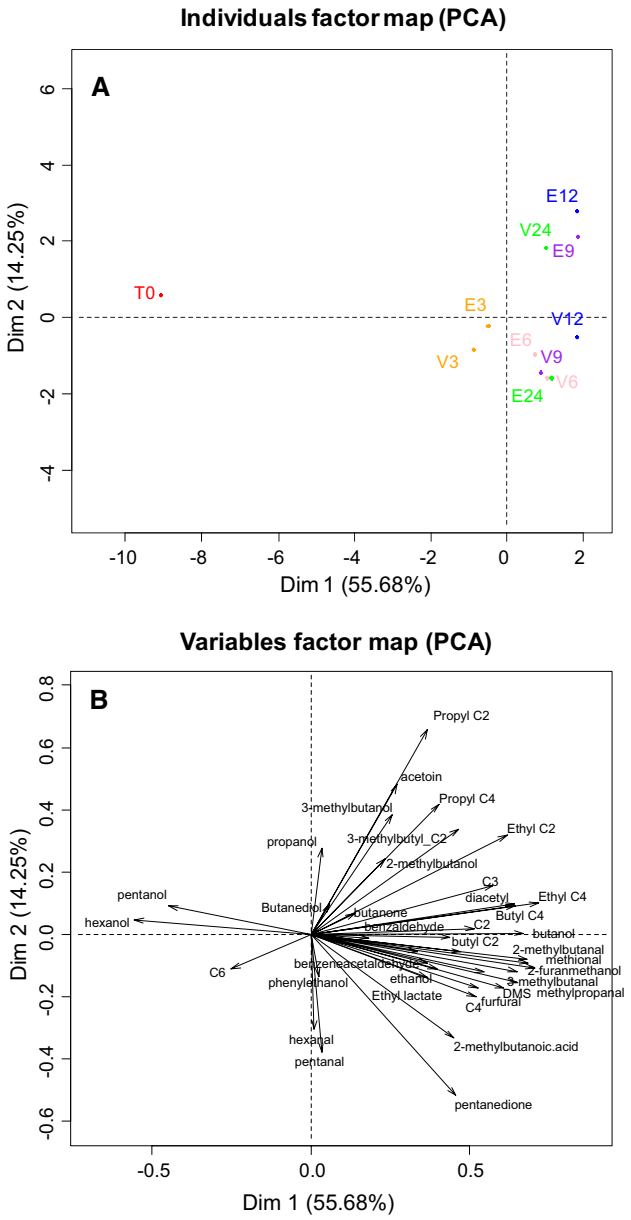

Fig. 3 Individuals factor map (**a**) and variables factor map (**b**) of principal component analysis on 40 volatile aroma compounds analyzed using head space gas chromatography–mass spectrometry produced in fermented wheat with (V) or without (E) initial addition of vinegar for 3, 6, 9, 12, and 24 months. The non-fermented wheat is represented by T0. The names of some volatiles are simplified according to Table 3

Culturable LAB counts in fermented wheat with and without vinegar addition were lower compared to other cereal-based fermented foods and beverages. LAB counts in products like *hussuwa*, *ben-saalga*, *togwa*, and *koko* ranged from 7 to 11 log cfu g^{-1}, which largely exceed our results [10, 28–30]. This difference between the fermented wheat characterized in the present study and other cereal-based fermented foods could be explained by the very long fermentation period and long intervals of sampling. In other products, LAB counts were usually monitored for hours, days, or a maximum of 1 month. Besides, natural stress like the acidity generated by LAB growth through long periods could also have resulted in a dramatic decrease in culturable LAB populations after the third month.

The fermentation process allows the disappearance of fungi flora over time and consequently making the product safer for consumption. This was probably due to the reduced oxygen atmosphere where molds evolved. For yeasts, the significant accumulation of CO_2 in jerrycans could have a considerable impact on their viability and metabolic activity as reported by Haas et al. [31], and Jones and Greenfiel [32]. Moreover, some of the LAB strains present may have exerted an antifungal activity, since some LAB such as *P.acidilactici*, present in *lemzeiet*, have been reported to produce antifungal compounds [33, 34].

TTA increased and the pH dropped during fermentation (Table 1), which is in accordance with the results reported on cereal-based fermentations [28–30]. FA also increased significantly during fermentation and in both processes. LAB are considered as weakly lipolytic compared with other bacteria [35]. However, their presence in fermented foods during long periods can bring them to release appreciable amounts of free fatty acids [36].

Comparing LAB population profiles of fermented wheat with and without addition of vinegar, using both methods, we can state that the two processes evolved similarly over time and the species detected are in accordance with the LAB population described previously evolving in cereal-based fermented foods. *L. plantarum*, *L. brevis*, *E. faecium*, and *P. pentosaceus* are reported as being present in association or not in various cereal-based fermented foods such as *cassava* [37], *hussuwa* [30], *poto poto* [9], and *koko* [28]. In the present study, since the third month of fermentation, a balance was established between lactobacilli species and *Pediococcus pentosaceus* for the entire period of fermentation. Humblot and Guyot [38] have reported a similar evolution in pearl millet slurries, in which only representatives of *Lactobacillaceae* (*Pediococcus* and *Lactobacillus*), *Leuconostocaceae*, and *Enterococcaceae* were detected at the end of fermentation.

The predominant isolates from fermented wheat without vinegar consisted in the association of obligate heterofermentative lactobacilli species, especially *L. brevis* and *P. pentosaceus*. However, in the wheat fermented with vinegar *P. pentosaceus* and *E. faecium* seemed to become dominant over time together with some lactobacilli species (Table 2). The major differences associated with vinegar addition were the dissimilarity of lactobacilli detected in each process and the high amounts of isolates of *E. faecium* detected at all stages of ripening in the presence of vinegar. A large diversity of lactobacilli species was observed in

fermented wheat, with *L. rapi*, *L. buchneri*, *L namurensis*, *L. coryniformis*, *L curvatus*, and *L. pentosus,* which are not frequently reported in wheat or wheat sourdoughs. However, some of them characterize the microbiota of some cereal-based fermented products as demonstrated in *Shanxi* aged vinegar for *L.buchneri* [39] and sorghum sourdoughs for both *L.coryniformis* and *L.curvatus* [40, 41]. Enterococci were detected during the first stages of fermentation of *pozol* and sorghum sourdoughs, whereas during further stages they were replaced by other species of LAB mainly lactobacilli [42, 43]. This feature is in agreement with the results observed here for the wheat fermented without vinegar. Moreover, enterococci were also reported as contaminant bacteria in cereal kernels flour and can be present during the early stages of sourdough production [44].

For pediococci and lactobacilli species, the level of clonal diversity seemed to decrease over time, probably for the benefit of the strains the most adapted to the process conditions. Oguntoyinbo and Narbad [14] have reported similar results for fermented West African cereals where some LAB strains, belonging mainly to the genera *Lactobacillus* and *Pediococcus*, more adapted to environment with rapid acidity potential and hydrolysis of starch can then be able to dominate.

TTGE profiles gave a first view of the dynamic of the main species during the ripening period and showed that a dominant species present in all samples and at all stages of sampling could be assigned to *P. pentosaceus*, *L. fermentum,* or *Leu. fallax* species. The results of the identification of isolates suggest that this dominant species is probably *P. pentosaceus*. It appears thus as the main species evidenced by both approaches. However, some discrepancies between TTGE and culture-dependent methods were observed. Strains of *E. faecium* were isolated from fermented wheat with vinegar and strains of *L. brevis* from fermented wheat without vinegar throughout the ripening period, whereas TTGE patterns showed their probable presence only at late fermentation stages. In addition, species present in small numbers such as *L. curvatus*, *P. acidilactici,* among others were not detectable using TTGE. A possible explanation for such discrepancies in LAB species detection between culture-dependent and culture-independent methods could be DNA extraction, since it is very difficult to extract DNA from all the species with the same efficiency in mixed bacteria population [26] and the limited sensitivity of PCR-TTGE [45]. In fact, TTGE is an interesting method, in first approach, to determine in a rather simple and fast way the level of diversity of samples over time although it allows only presumptive identification using the database.

The primary function of LAB is to ensure the initial acidification of the product via the fermentation of carbohydrates into lactic acid. The latter contributes to the basic, pleasant acidic taste of many fermented foods. Besides, the fermentation of wheat generated a high number of aroma compounds, associated with various flavor notes. Aroma compounds derive from the conversion of food constituents via a series of biochemical reactions mainly catalyzed by microbial enzymes. Diacetyl and 2, 3-pentanedione derive from fermentation of some carbon sources such as citrate and are associated with buttery notes [46]. Diacetyl is widely produced by LAB, including species of the *Leuconostoc*, *Lactobacillus*, and *Pediococcus* genera. Ethanol and acetic acid result from sugar fermentation by heterofermentative LAB species such as *L. brevis*, *L. buchneri,* and *Leu. mesenteroides* detected in the present study. The catabolism of branched-chain, aromatic amino acids and methionine by LAB generates various aroma compounds including acids, alcohols, and aldehydes, which yield a variety of aroma notes [47]. For example, branched-chain aldehydes and branched-chain acids, resulting from the degradation of branched-chain amino acids, are associated with malty and cheesy notes, respectively. Methanol and dimethyl disulfide (DMDS) result from sulfur amino acids and are associated with cooked potatoes and sulfurous notes, respectively. The range of acids and alcohols generated provides potential substrates for esterification reactions catalyzed by LAB esterases [48]. Many esters, associated with fruity flavors, were detected in the present study. Their formation in fermented wheat was likely limited by the amount of alcohols, since we observed significant correlations between the presence of some alcohols (propanol and butanol) and the corresponding esters. The variations observed in the volatile profiles are consistent with the variations in LAB populations. For both volatiles and LAB, profiles were similar regardless the initial addition of vinegar. Moreover, some late changes occurred, suggesting that the viable and metabolically active bacterial population was probably present in higher numbers compared to the low culturable population. The presence of viable but non-culturable (VBNC) LAB has been demonstrated in foods, e.g., in cheese [49] and plants [50].

Conclusion

This first study on fermented wheat *lemzeiet* revealed a high level of LAB species diversity, with at least 16 species identified. *P. pentosaceus* constituted the dominant LAB species in this native LAB population, in association with *Lactobacillus* sp., regardless of the process used. LAB fermentation markedly influenced the profile of wheat volatiles and its changes over the whole ripening period, up to 24 months of ripening. This work allows looking ahead to select cultures, which could be used as starters to produce, at a larger scale, *lemzeiet* with high and controlled quality. It would be of great interest to further investigate

the dynamic of the LAB community in a higher number of samples and during the early stages of the fermentation process when the balance occurs between the species.

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