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Detection of different microenvironments and *Lactobacillus sakei* biotypes in Ventricina, a traditional fermented sausage from central Italy

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ABSTRACT

The present study evaluated the physico-chemical and microbiological features of Ventricina, considering for the first time the presence of different compartments deriving from the technology of production. In fact meat pieces (pork muscle and fat cut into cubes of about 10–20 cm^3), mixed with other ingredients and then stuffed into pig bladder, are still distinguishable at the end of the ripening. They appear delimited on the outside by the casing and inside by thin layers consisting of spices (mainly red pepper powder), salt and meat juices. Our results showed that the exterior (portion of the product in contact with the casing), the interstice (area between the different cubes of meat or fat) and the heart (the inner portion of meat cubes) had distinctive values of pH and a_w, and a typical microbial progression, so that they can be considered as different ecological niches, here called microenvironments. The study of lactic acid bacteria population, performed with PCR-DGGE and sequence analysis targeting the V1–V3 region of the 16S rRNA gene (rDNA), highlighted the presence of a few species, including *Lactobacillus sakei*, *Lb. plantarum*, *Weissella hellenica* and *Leuconostoc mesenteroides*. The RAPD-PCR analysis performed on *Lb. sakei*, recognised as the predominant species, allowed the differentiation into three biotypes, with that characterised by the highest acidifying and proteolytic activities and the highest ability to grow in the presence of sodium chloride prevailing. This leading biotype, detectable in the interstice during the entire ripening period, was isolated in the microenvironments exterior and heart starting from the 30th d of ripening, and it was the sole biotype present at the end of the ripening. The analysis of microenvironments through the scanning electron microscopy (SEM) evidenced the presence of micro-channels, which could favour the microbial flow from the interstice to the exterior and the heart. Moreover, the SEM analysis allowed the detection of biofilms, recognised as responsible for the correct colonisation of the different meat niches.

1. Introduction

Traditional Ventricina is a long-cured fermented sausage produced in the geographical area of central Italy, between Abruzzo and Molise regions. A clear and unambiguous identity is conferred to the product by the great sub-ovoid shape (weight of about 1–2.5 kg and Ø of 90–200 mm), and by the production technology, based on the use of pork meat and fat cut into cubes of about 10–20 cm^3 (Tremonte et al., 2005a). Sodium chloride (3%), bell pepper powder (3%) and other spices (chilli, fennel and pepper) are also added. After mixing and kneading, the mixture rests for 12–24 h, and then it is stuffed into the casing (pig bladder). The ripening includes a first drying step at 15°–20 °C for 10 days and a subsequent curing phase for at least three months, made at room temperature according to the artisanal technology.

To date, Ventricina is prepared without the use of starter cultures, and no quality check during the fermentation process is carried out (Coppola and Tremonte, 2012). In this condition, the fermentation process is brought by the microbial communities deriving from the raw materials and the environment (Aquilanti et al., 2016), and it is also affected by the production process (Chevallier et al., 2006). Consequently, both safety and final quality of Ventricina cannot be ensured unless the microbial ecology and its development are carefully examined and, eventually, corrected. In this context, numerous scientific studies dealt with the description of microbial dynamics responsible for the fermentation of different meat products (Iacumin et al., 2012; Talon et al., 2007; Tremonte et al., 2010). Moreover, the predominance of lactic acid bacteria (LAB) and coagulase-negative cocci (CNC) during the ripening of southern European fermented meats was well documented (Aquilanti et al., 2016; Greppi et al., 2015), along with their roles in the promotion of proteolytic and protective activities (Basso et al., 2004; Casaburi et al., 2016; Comi et al., 2016; Di Luccia et al., 2016; Tremonte et al., 2007). On the other hand, only few studies are available

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on particular types of fermented sausages, which, as in the case of traditional Ventricina, significantly differ by other artisanal or industrial products.

In the light of previous findings, the present study aimed at the description of some physico-chemical and microbiological aspects of traditional Ventricina, whose particular technology of production, based on the use of large meat and fat cubes, causes the formation of different compartments, here called microenvironments.

2. Materials and methods

2.1. Sampling

Ventricina productions were monitored during the wintertime in two different meat factories located in the Molise region. They were called plants A and B in this study. Plants were selected on the basis of their traditional technology of production, which means without the use of starter cultures or mechanically minced meat, and without the control of ripening parameters. Pork meat (70% muscle and 30% fat) was obtained by the same local supplier and then equally divided between the two plants. Hence, the production technology was separately performed in the two plants following the traditional technology already described by Tremonte et al. (2005a). Briefly, the meat was cut by hand into cubes (about 20 cm^3) and mixed with other ingredients (30 g/kg NaCl and 25 g/kg red sweet pepper powder). After a 24 h rest phase at room temperature, the mixture was stuffed into pig bladder previously washed with a saturated NaCl solution added with fresh garlic and orange peel (about 10 g/L and 20 g/L, respectively). The subsequent step of maturation was performed for 90 days at room temperature (first 7 d at 15–17 °C in room with fireplace, followed by 83 d at about 13 °C).

Two samples from each plant were taken at 0, 15, 30 and 90 days of ripening and transported to the laboratory in refrigerated bags. Each Ventricina sample was cut into two halves, and the analyses were carried out on each half withdrawing three sub-samples representative of three different microenvironments: exterior (OUT), portion of the product in contact with the casing; interstice (GAP), area between the different cubes of meat or fat; heart (HRT), the inner portion of meat cubes.

2.2. Physico-chemical analyses

Potentiometric measurement of pH was performed by inserting a pin electrode of a pH-meter (Crison 2001) directly into each sub-sample from each half as described by Tremonte et al. (2005b). Activity water was determined on the same samples utilising a Water Activity Meter AQUALAB CX-2 (Decagon Devices, USA). The results were expressed as the mean of four determinations (two for each half) performed at each sampling time.

2.3. Microbial counts

For each sampling time, microbiological analyses were performed on about 10 g of meat aseptically withdrawn from each sub-sample. Decimal dilutions were made in a sterile solution of 0.1% peptone water to detect bacteria (Tremonte et al., 2014) and *Eumycetes* (Tremonte et al., 2016). After homogenization in a Lab-blender (Stomacher Seward Medical, London, SE1 1PP, UK), subsequent serial dilutions were inoculated in appropriate media as described below.

Total mesophilic bacteria (TMB) were counted on plate count agar (PCA) (Oxoid, Milan, Italy) after incubation at 30 °C for 72 h. Lactic acid bacteria (LAB) were enumerated on de Man, Rogosa, Sharpe (MRS) agar (Oxoid) incubated at 28 °C for 72 h in anaerobic conditions (GENbox anaer, bioMérieux, Marcy-l'Etoile, France). Coagulase negative cocci (CNC) were enumerated on mannitol salt agar (MSA) (Oxoid) after incubation at 28 °C for 48 h. Enterococci were recognised on Slanetz and Bartley medium (SB) (Oxoid) incubated at 37 °C for 48 h. *Enterobacteriaceae* were enumerated on Violet Red Bile Glucose Agar (VRBGA) (Oxoid) after incubation at 37 °C for 48 h. Yeasts and moulds were counted on Rose Bengal Agar (RB) supplemented with chloramphenicol (both from Sigma-Aldrich, Saint Louis, MO, USA) after incubation at 25 °C for 48 h. *Listeria* spp. was enumerated as described by Reale et al. (2008).

Seven colonies from MRS plates with the highest dilution that yielded growth were randomly selected, purified, and stored in Microbanks (Prolab Diagnostics) at −20 °C. Prior to subsequent analyses, isolates were revitalised in MRS broth (Oxoid) following the incubation conditions described above.

2.4. Presumptive identification and PCR-DGGE analysis on lactic acid bacteria

Gram staining, catalase test and microscope observation were used to presumptively identify the isolates belonging to lactic acid bacteria (LAB).

Two milliliters of each overnight culture of presumptive LAB was centrifuged at 14,000 ×*g* for 10 min at 4 °C (Centrifuge 5415 R; Eppendorf, Hamburg, Germany) to pellet the cells and the pellet was subjected to DNA extraction according to Querol et al. (1992), with the addition of lysozyme (25 mg/mL, Sigma) and mutanolysin (10 U/mL, Sigma) for bacterial cell wall digestion. The DNA from each strain was then prepared for DGGE by amplifying the V1 region of 16S rDNA using the following primers: P1V1 (5′-GCG GCG TGC CTA ATA CAT GC-3′) (Cocolin et al., 2001) and P2V1 (5′-TTC CCC ACG CGT TAC TCA CC-3′) (Rantsiou et al., 2005). A GC clamp (5′- CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′) (Sheffield et al., 1989) was attached to the 5′ end of the P1V1 primer. Negative controls without DNA template were included in parallel. PCR and gel processing were performed as described by Testa et al. (2014).

2.5. Sequence analysis

One representative strain from each cluster obtained by DGGE analysis was amplified with primers P1 and P4, as described by Klijn et al. (1991), targeting 700 bp of the V1–V3 region of the 16S rRNA gene (rDNA). After purification (QIAquick PCR purification kit, QIAGEN GmbH, Hilden, Germany), products were sent to a commercial facility for sequencing (Eurofins MWG Biotech Company, Ebersberg, Germany). Sequences were aligned with those in GenBank with the Blast program to determine the closest known relatives, based on the partial 16S rDNA sequence obtained.

2.6. Bio-typing of Lactobacillus sakei by RAPD-PCR

Amplification reactions were performed in a 25 μL reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 200 μM of each dATP, dGTP, dCTP and dTTP, 1.5 mM $MgCl_2$, 1 μM primer, 80 ng DNA and 1.25 U Taq-DNA polymerase (Finnzymes, Finland). Amplifications were performed in a Mastercycler gradient (Eppendorf, Hamburg, Germany) using the primers M13 and D8635, as described by Reale et al. (2005). The amplification products were separated by electrophoresis on 1.5% (w/v) agarose gel (Sigma-Aldrich, Steinheim, Germany) in 0.5× TBE buffer and then subjected to ethidium bromide staining. RAPD-PCR gels were digitally captured and analysed as previously described for DGGE analysis.

2.7. Technological characterisation of Lb. sakei strains

Strains were examined for acid production (pH) in MRS broth. For this purpose, overnight cultures were inoculated (1%, v/v) in the medium and incubated at 28 °C. Measurements of pH were carried out at 0 and 72 h of incubation. Data were reported as acidity score (A_score),

determined with the following formula:

$$A\_score = \frac{\Delta pH\ x_{(0-72)}}{\Delta pH_{max}}$$

where:

$\Delta pH\ x_{(0\ -\ 72)}$ represents the pH difference between 0 and 72 h registered for each strain;

ΔpH_{max} represents the maximum pH difference between 0 and 72 h registered for all the strains.

To assess the growth ability in the presence of NaCl, overnight cultures were inoculated (1% v/v) in MRS broth added with 8% NaCl. The microbial growth was ascertained on cultures incubated at 28 °C and the absorbance at 580 nm was read at 0 and 72 h, using a basic spectrophotometer (Eppendorf, UK). Data were reported as growth score in presence of NaCl (G_NaCl_score), determined with the following formula:

$$G\_NaCl\_score = \frac{\Delta A_{580}\ x_{(72-0)}}{\Delta A_{580\,max}}$$

where:

$\Delta A_{580}\ x_{(72\ -\ 0)}$ represents the A_{580} difference between 72 and 0 h registered for each strain;

ΔA_{580max} represents the maximum A_{580} difference between 72 and 0 h registered for all the strains.

The ability to hydrolyse sarcoplasmic proteins was determined as reported by Mauriello et al. (2002). Data were reported as proteolitic score (P_score), determined with the following formula:

$$P\_score = \frac{Ø\ x_{(72-0)}}{Ø_{max}}$$

where:

$Ø\ x_{(72\ -\ 0)}$ represents the diameter difference of the proteolytic halo between 72 and 0 h registered for each strain;

$Ø_{max}$ represents the maximum diameter difference of the proteolytic halo between 72 and 0 h registered for all the strains.

Data were then analysed with the software IBM SPSS Statistics Base version 21.0.

2.8. Scanning electron microscopy (SEM)

SEM analysis was performed as described by Sorrentino et al. (2013). Briefly, portions of each sub-sample (size 1 to 2 mm^2) were withdrawn and fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer. After 12 h, samples were rinsed 3 times with the same buffer and dehydrated (2 times for each solution) in a graded ethanol series (20%, 40%, 60%, 80%, 95%, 100%) for 10 min each. After drying in a CO_2 critical point dry (Emitech K850), samples were sputter coated with palladium gold in Emitech K550 and observed using a Zeiss DSM 940 A SEM (Milan, Italy).

2.9. Statistical analyses

Statistical analyses were performed following the approach used by Gaglio et al. (2016). Briefly, results from microbiological counts and physico-chemical analyses (pH and a_w) were analysed by a General Linear Model based on ANOVA (IBM SPSS Statistics 21) considering the effect of plants, microenvironments and ripening time. The post-hoc Bonferroni test was used for pairwise comparison. Statistical significance was attributed to P values of <0.05. Statistical data were expressed as mean ± standard error or standard deviation.

3. Results and discussion

3.1. Physico-chemical and microbiological parameters

The pH and the a_w detected in the different microenvironments of Ventricina during the ripening period are reported in Table 1 as the mean values obtained on samples produced in the two artisanal plants A and B. Values resulted in agreement with those commonly found in other fermented sausages from southern Italy (Coppola et al., 1997; Tremonte, 2005a; Urso et al., 2006), even if interesting differences distinguished the various microenvironments at different sampling times. In detail, a significant lowering in the pH value ($P < 0.05$) was detected in GAP at the 15th d, whereas the highest pH drop was observed in OUT and in HRT only at 30 d. Moreover, at 15 and 30 d of ripening pH values registered in GAP were significantly lower than those registered in OUT and HRT. The pH recorded in GAP, showing a rapid decrease up to the 15th d of ripening, seemed to mimic the typical behaviour of salami prepared with starter cultures (Di Luccia et al., 2016; Tabanelli et al., 2012), with the addition of sugar (Casquete et al., 2011), or otherwise characterised by significant levels of LAB (Coppola et al., 1998). On the other hand, pH values detected in OUT and HRT showed the usual behaviour (pH mean values above 5.4) reported for traditional salami obtained without the use of starter cultures (Fonseca et al., 2013; Urso et al., 2006).

In all the microenvironments, the a_w decreased from initial values of about 0.97 to about 0.89 at the end of the ripening, but significant differences emerged among OUT, GAP and HRT starting from the 15th d of ripening (Table 1). In fact, the a_w recorded in GAP at 15 and 30 days of ripening was about 0.03 units lower than that recorded in OUT and in HRT. These data evidenced again different features of the microenvironments, with GAP more similar to fermented salami produced with starter cultures and OUT and HRT comparable with those obtained without starter (Di Luccia et al., 2016; Ranucci et al., 2016).

The behaviour of the main microbial groups colonising the traditional Ventricina is reported in Fig. 1. Statistical analysis highlighted that no significant differences ($P > 0.05$) characterised the samples of the two plants A and B. On the other hand, significant differences in counts ($P < 0.05$) were found depending on the diverse microenvironments and the different sampling time.

Specifically, high microbial loads were recorded already at time 0, and this fact was particularly noticeable for LAB in the microenvironment GAP (5.0 log CFU/g), and for CNC in the microenvironments OUT and GAP (5.3 and 5.0 log CFU/g, respectively). These counts were arguably imputable to the development of autochthonous

Table 1
Values of pH and a_w detected during the ripening of Ventricina (samples A and B). OUT, portion of the product in contact with the casing; GAP, area between the different cubes of meat or fat; HRT, the inner portion of meat cubes.

	Ripening time (days)	OUT	GAP	HRT
pH	0	5.68 (±0.03)^{Aa}	5.73 (±0.03)^{Aab}	5.74 (±0.02)^{Ab}
	15	5.64 (±0.03)^{Aa}	5.18 (±0.02)^{Bb}	5.71 (±0.03)^{Ac}
	30	5.51 (±0.03)^{Ba}	5.24 (±0.02)^{Bb}	5.48 (±0.01)^{Ba}
	90	5.53 (±0.04)^{Ba}	5.56 (±0.01)^{Ca}	5.52 (±0.02)^{Ba}
a_w	0	0.975 (±0.003)^{Aa}	0.972 (±0.004)^{Aa}	0.974 (±0.003)^{Aa}
	15	0.962 (±0.004)^{Ba}	0.944 (±0.004)^{Bb}	0.968 (±0.005)^{ABa}
	30	0.958 (±0.004)^{Ba}	0.933 (±0.002)^{Cb}	0.963 (±0.003)^{Ba}
	90	0.891 (±0.001)^{Ca}	0.892 (±0.003)^{Da}	0.894 (±0.002)^{Ca}

Mean ± standard deviation of four measurements carried out in duplicate for two independent productions (plants A and B). Means in the same row with different superscript small letters are significantly different ($P < 0.05$). Means in the same column with different superscript capital letters are significantly different ($P < 0.05$).

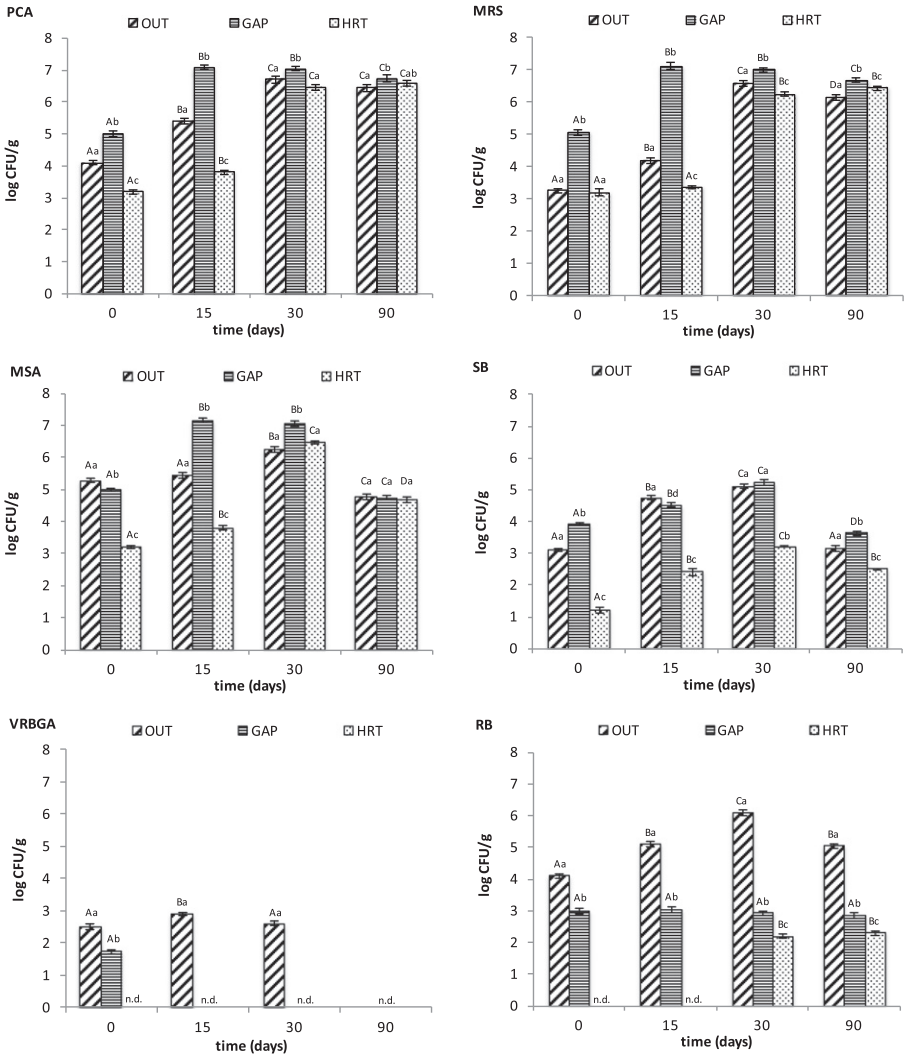

Fig. 1. Microbial loads (expressed in log CFU/g) detected in each microenvironment (OUT, portion of the product in contact with the casing; GAP, area between the different cubes of meat or fat; HRT, the inner portion of meat cubes) during the ripening of Ventricina. Mean ± standard error of four measurements carried out in duplicate for two independent productions (plants A and B). Means with different superscript letters are significantly different ($P < 0.05$).

bacteria in the mixture during the 24 h of resting phase at room temperature, which preceded the stuffing into pig bladder. This datum is in accordance with other studies, which showed a high contamination by LAB of raw meat used for the production of salami (Gaglio et al., 2016). Interestingly, values of pH and a_w registered in the different microenvironments at time zero did not show significant differences (Table 1), and this fact demonstrated the correctness of the mixing and stuffing procedures. On the other hand, microbial counts at time zero generally differed among GAP, OUT and HRT despite the mixing process. The lowest microbial load in HRT was expected, considering that this microenvironment represents the inner portion of meat cubes. However, substantial differences at time zero were also observed among GAP and OUT, especially for LAB. In detail, the low LAB counts registered in OUT (portion of the product in contact with the casing) could be due to a partial inhibition of the growth caused by the high NaCl concentration. On the other hand, CNC, generally recognised as more halotolerant then LAB, showed similar counts at time zero in OUT and GAP, and this datum seems to substantiate our assumption.

TMB, LAB and CNC reached the highest microbial loads between the 15th and the 30th day of ripening, in accordance with other studies on central-southern European salami (Janssens et al., 2012; Ruiz-Moyano et al., 2011). These bacteria, which ordinarily prevailed over the other microbial groups (enterococci, *Enterobacteriaceae*, yeasts and moulds), reached the highest counts in the microenvironment GAP already at the 15th d, whereas 30 d needed in OUT and HRT. Moreover, microbial loads attributable to LAB were significantly different among microenvironments during the entire ripening period, with those counted in GAP always prevailing. A similar observation characterised CNC until the 30th d.

Among the other microbial groups, enterococci showed an increasing trend in the three microenvironments until the 30th d of ripening, but significant differences were generally appreciated among OUT, GAP and HRT. Yeasts and moulds displayed microbial charges significantly different in relation to the ripening time and the microenvironments. *Listeria* spp. was never detectable (data not shown), whereas *Enterobacteriaceae* resulted detectable in GAP at day 0 (below 2 log CFU/g) and in OUT until the 30th d. Other Authors reported the presence of *Enterobacteriaceae* in salami characterised by low acidity levels (Fonseca et al., 2013; Marcos et al., 2007). Remarkably, the microenvironment OUT was characterised by pH values above 5.5 during the entire ripening period (Table 1), and this fact could be the cause of the permanence of undesired microbial groups during the initial ripening period. On the other hand, the microenvironment HRT, characterised by high pH values as well, did not show microbial contamination by *Enterobacteriaceae* and, in general, HRT showed the lowest presence of the other investigated microbial

groups, in confirmation of substantial differences among the three microenvironments.

3.2. Lactic acid bacteria identification

During the ripening period, 168 isolates were collected by MRS plates (84 from each Ventricina sample A and B), and 152 bacteria, presumptively ascribable to LAB on the basis of Gram staining and catalase test, were identified by PCR-DGGE analysis (Fig. S1). Considering a similarity level of 70% as the arbitrary threshold for the identification at species level, the isolates were grouped into 24 clusters (labelled from A to X), plus 5 single isolates (from 1 to 5) which did not convene with the others. According to the migration profiles, one isolate from each cluster and all those grouping alone were subjected to sequencing for identification purposes. The results of sequencing (Table S1) allowed the identification of the 29 isolates. Combining these results with those obtained from the PCR-DGGE cluster analysis, it was possible to identify 102 isolates as *Lb. sakei* (clusters A-L plus single isolate 1), 14 as *Lb. plantarum* (cluster M plus single isolates 2 and 3), 13 as *Weissella hellenica* (clusters N-R plus single isolate 4) and 23 as *Leuconostoc mesenteroides* (clusters S-X plus single isolate 5). Thus, the PCR-DGGE analysis was a useful approach to get information about the LAB species dominating Ventricina. Nevertheless, band profiles of strains belonging to the same species were clustered separately, and this fact was particularly evident in the case of *Lb. sakei*, which was collected in 12 clusters plus one single strain. This result could be due to several factors, such as the operating procedures (Ercolini, 2004) or, also, the appearance of multiple bands with lower intensity within the same species, depending upon PCR conditions (Lorbeg et al., 2009). On the other hand, the distribution of *Lb. sakei* in separate DGGE clusters was already observed by Cocolin et al. (2004), who concluded that *Lb. sakei* strains belonged to different populations.

The results reported above highlighted the predominance of *Lb. sakei*, in agreement with other studies on fermented meat products (Champomier-Vergès and Zagorec, 2015). The community of LAB in Ventricina also included *Lb. plantarum*, *Ln. mesenteroides* and *W. hellenica*. *Lactobacillus plantarum*, along with *Lb. sakei*, represents the most prevalent species among all LAB genera constituting the common microbiota in traditional sausage (Kumar et al., 2015), while different species belonging to the genera *Weissella* and *Leuconostoc* were generally isolated from some common and uncommon fermented meat products (Tamang et al., 2016).

3.3. Distribution of LAB species

The results of the identification of LAB isolated during the ripening of Ventricina allowed the detection of a progressive increase of *Lb. sakei* during time (Fig. 2). This fact is in accordance with studies on other salami (Cocolin et al., 2009; Garcia-Fontan et al., 2007), but in the case of Ventricina some peculiarities emerged. In fact, the three investigated microenvironments showed different distributions of LAB during the ripening. In GAP, all the species were present together only at time 0, whereas in the subsequent sampling times only *Lb. sakei* was detected. A different behaviour was appreciated in the other microenvironments. In fact, in OUT and in HRT different LAB species where detected until the 30th day of ripening, whereas only *Lb. sakei* was valued at 90 days of ripening. Data reported above evidenced, as in the case of the results obtained for pH and a_w (Table 1), substantial differences among subsamples GAP, OUT and HRT, with GAP dissimilar to OUT and HRT. Other studies considered some physico-chemical and microbiological characteristics of Italian fermented sausages in relation to their size (Tabanelli et al., 2016), or to the biodiversity of *Lb. sakei* strains involved in the maturing processes of fermented meats (Pisacane et al., 2015). However, the present study evidenced for the first time the presence of different microenvironments, and consequently different maturation processes, inside one fermented sausage.

3.4. Diversity in Lb. sakei RAPD-PCR profiles

Based on the previous evidences, we tried to obtain a more in depth knowledge of the LAB evolution in the three microenvironments during the ripening of Ventricina. The target of the investigation was represented by *Lb. sakei*, the main species isolated during the entire observation period. Other authors (Amadoro et al., 2015) already reported the predominance of this species in Ventricina. However, no information was given regarding the presence of specific biotypes responsible for the fermentation process, nor their distribution within the Ventricina. In our study, the RAPD-PCR analysis was used to detect the distribution of *Lb. sakei* types in relation to the ripening time and to the isolation from different microenvironments. Considering the migration profiles, a

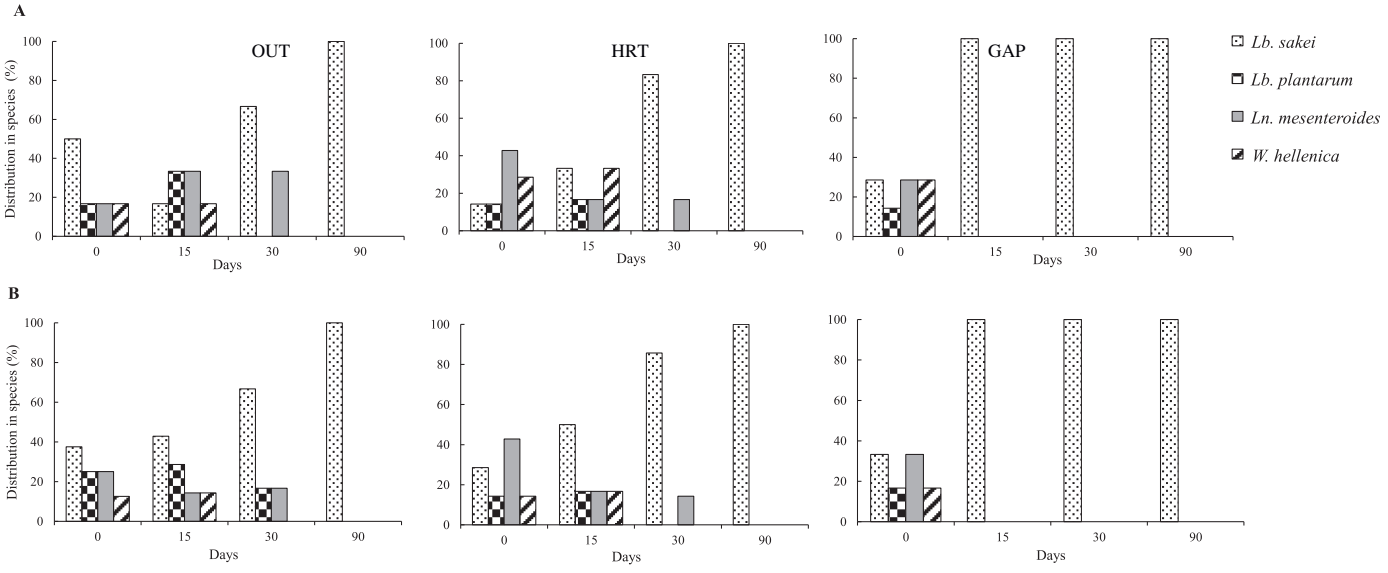

Fig. 2. Distribution in species (%) of lactic acid bacteria detected in each microenvironment (OUT, portion of the product in contact with the casing; GAP, area between the different cubes of meat or fat; HRT, the inner portion of meat cubes) during the ripening of Ventricina. A, sample A; B, sample B.

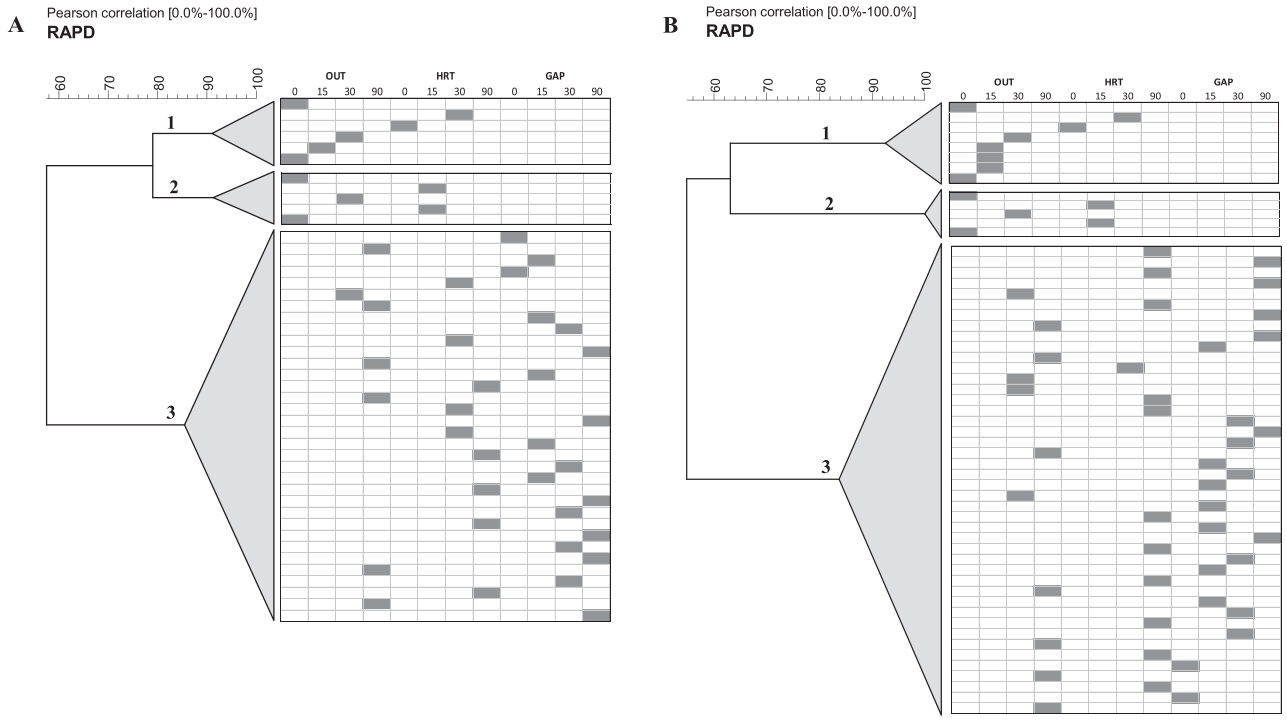

Fig. 3. Cluster analysis of RAPD-PCR profiles of *Lactobacillus sakei* isolated from OUT, HRT and GAP during the ripening of Ventricina. A, sample A; B, sample B.

similarity level of 84% was chosen to distinguish the different biotypes (Fig. 3A and B), and for each Ventricina sample, three clusters were obtained. Clusters 3A and 3B included the majority of profiles belonging to 34 and 44 strains from samples A and B, respectively. These strains were isolated at each sampling time from GAP, and only at 30 and 90 days from OUT and HRT. Vice versa, clusters 1 and 2 (from both Ventricina samples A and B) comprised only those *Lb. sakei* strains isolated in the early stages of ripening from OUT and HRT, whereas no

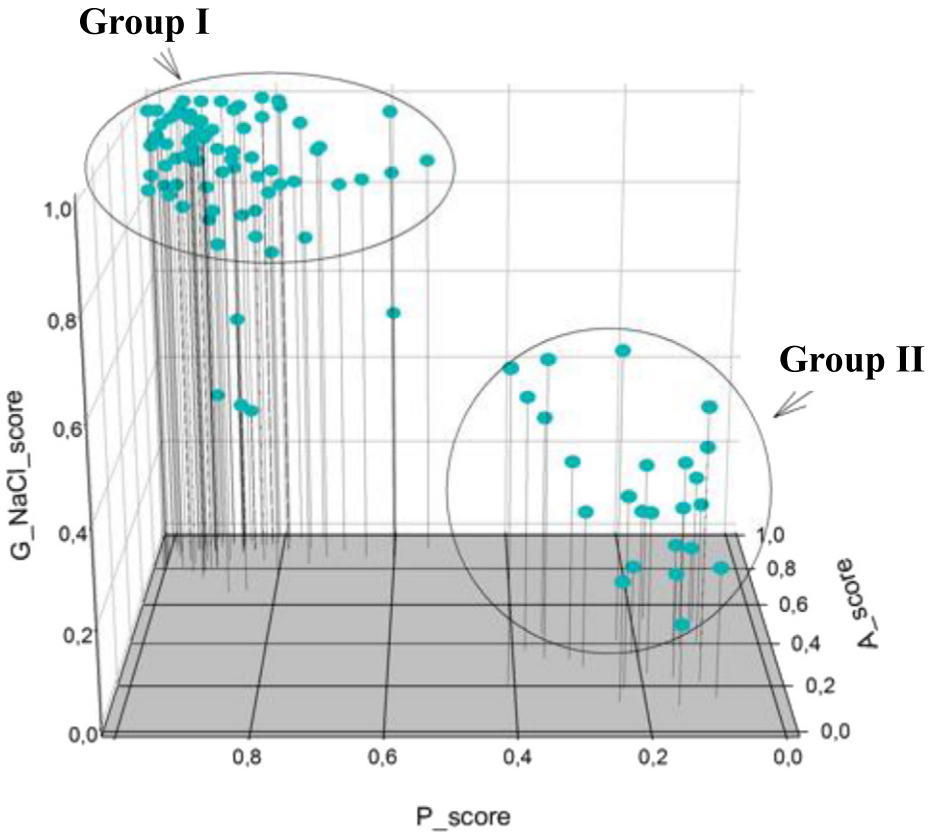

Fig. 4. Scatter Plot 3D of three components analysed on 102 *Lb. sakei* strains from Ventricina (samples A and B).

strain from GAP was included. These results highlighted that different *Lb. sakei* biotypes populated the microenvironments of Ventricina in different ripening periods, with those included in clusters 3A and 3B as the main RAPD type that conducted the fermentation. These results are in agreement with those obtained by Urso et al. (2006) with regard to the distribution of biotypes during the fermentation of traditional Italian fermented sausages, but in Ventricina we also found a compartmentation of the different biotypes.

3.5. Diversity among Lb. sakei isolates

The assessment of technological features attributable to *Lb. sakei* strains allowed the individuation of important differences related to the different biotypes (Fig. 4). In detail, two main groups, called I and II, were individuated and 5 strains were unique. The group I collected 71 strains from the RAPD clusters 3A and 3B (Fig. 3A and B), that is, those biotypes isolated at each sampling time from GAP and only at 30 and 90 days from OUT and HRT. The 5 single strains were from clusters 3A and 3B as well. The group II was composed by all the 24 strains belonging to the RAPD clusters 1 and 2 (Fig. 3A and B), that is, the biotypes isolated by OUT and HRT in the first ripening period. In addition, the analysis of data allowed the ascertainment of different features of *Lb. sakei* strains from the groups I and II. In fact, the former showed high acidifying activity (A_score), high ability to grow in the presence of sodium chloride (G_NaCl_score) and high proteolytic activity (P_score), while the latter showed the lowest scores.

A recent study (Greppi et al., 2015) evidenced that in fermented sausages only a few species, including *Lb. sakei*, may be metabolically active and can really contribute to determine the final characteristics of the products. In the light of the results obtained in our study, there is an evolution of *Lb. sakei* biotypes during time, with those characterised by the highest adaptability to environmental conditions prevailing. Interestingly, in Ventricina all the prevailing *Lb. sakei* biotypes were isolated from the interstice (GAP) that is, the microenvironment characterised by the lowest pH and a_w values already after the 30th day of ripening (Table 1).

3.6. Analysis of scanning electron microscopy

The results of scanning electron microscopy (SEM) corroborated the evidences emerged above. Specifically, Fig. 5 accounts for the SEM analysis of GAP, HRT and OUT after 15 days of ripening, when substantial differences in LAB counts where evidenced in the various microenvironments, as reported previously in Fig. 1. In fact, it was possible to observe a high growth of rod-shaped bacteria in the sub-sample GAP, whereas OUT and HRT were only sparsely populated by bacilli. Moreover, in the Fig. 6A, obtained by the SEM analysis of the sub-sample HRT after 15 days of ripening, the presence of microchannels into one meat cube (HRT) was highlighted. This datum confirms the evidences of Farouk et al. (2012) regarding the structure of meat muscle, and suggests the hypothesis that certain microorganisms are able to reach the core of meat cubes through these microchannels, which are also responsible for the gradual expulsion of liquids. Furthermore, Fig. 6B showed the presence of biofilm formation by rod-shaped bacteria. Analogous findings were evidenced by Chaillou et al. (2005) with regard to the ability of *Lb. sakei* to form functional biofilms to overcome environmental stresses.

4. Conclusions

To our knowledge, only few researches focused on the characteristics of Ventricina, and no study evidenced the presence of different ecological niches in this kind of product, nor in other fermented salami. Our study provided, for the first time, a punctual description of physico-chemical, microbiological and structural features of Ventricina, highlighting a differentiation into three microenvironments having peculiar features. In fact, we observed that the microenvironment GAP had the typical behaviour of salami prepared with starter cultures, in terms of LAB load and pH values, whereas OUT and HRT showed the usual behaviour of traditional salami obtained without the use of starter cultures. In our opinion, the main important scientific result obtained in our study was to ascertain that the formation of the different microenvironments is responsible for the correct maturation of Ventricina. In detail, a specific biotype of *Lb. sakei* coming from the interstice (GAP), was selected during the ripening period, and it progressively prevailed and populated the exterior (OUT) and the inner portion of meat cubes (HRT). We also showed that the microbial flow is favoured by the presence of micro-channels, while the ability of *Lb. sakei* to form biofilm may guarantee the correct colonisation of the different meat niches, related to the harmonisation of the fermentation process.

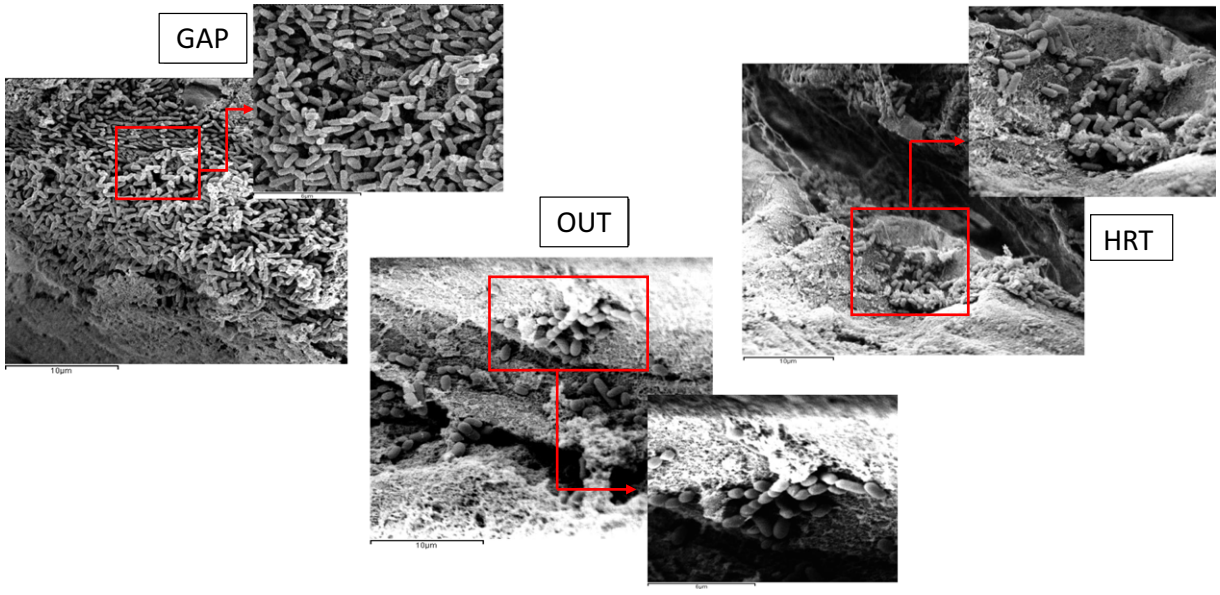

Fig. 5. Scanning electron microscopy images of the three different microenvironments (OUT, HRT and GAP) characterising Ventricina. The red square indicates the area subjected to magnification.

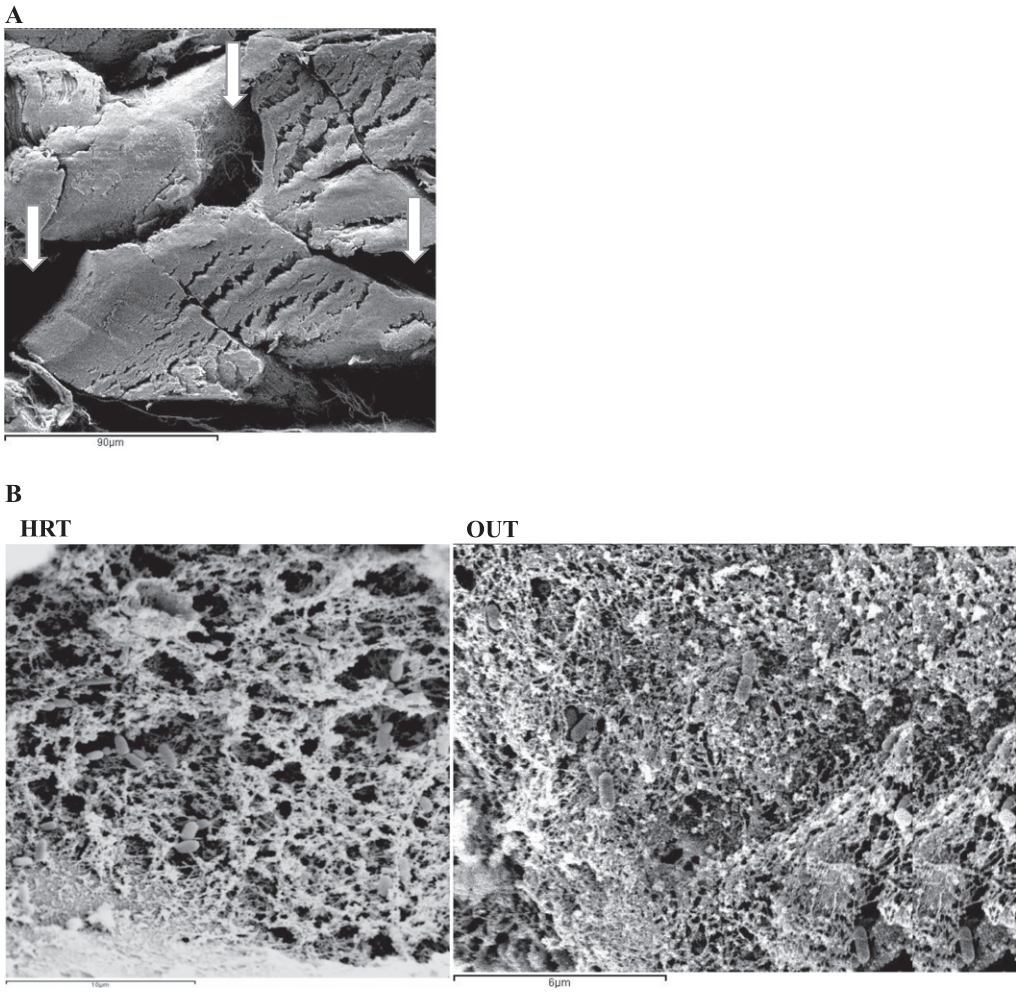

Fig. 6. Scanning electron microscopy images of different microenvironments characterising Ventricina. A, meat cubes from HRT where micro-channels are indicated by white arrows; B, microenvironments HRT and OUT with biofilms formed by rod-shaped bacteria.

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