EVALUATION OF THE *IN VITRO* ANTIMICROBIAL ACTIVITY OF MIXTURES OF *LACTOBACILLUS SAKEI* AND *LACTOBACILLUS CURVATUS* ISOLATED FROM ARGENTINE MEAT AND THEIR EFFECT ON VACUUM-PACKAGED BEEF

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ABSTRACT

A *L. sakei* and a *L. curvatus*-based mixtures exerted an antagonistic activity *in vitro* against 12 different spoilage and pathogenic bacteria. No activity was exerted by the cell-free supernatants. The addition of the two mixtures to sliced vacuum-packaged beef showed a better capability of *L. sakei* to adapt to meat substrate. After 60 days, Total Viable Count (5.8 *vs* 7.4 Log CFU/g), Gram-negative bacteria (2.5 *vs* 6.4 Log CFU/g) and *Enterobacteriaceae* (2.3 *vs* 4.4 Log CFU/g) were significantly lower in *L. sakei*-inoculated samples if compared with control ones; TVC and *Enterobacteriaceae* were also significantly lower in *L. curvatus*-inoculated samples than control ones. The addition of mixtures gave no significant effects on meat pH and colour. The use of high dosage of viable cells could be suggestible in order to exert an early conditioning of meat environment.

Keywords: *Lactobacillus sakei, Lactobacillus curvatus*, biopreservation, vacuum-packed beef, antimicrobial activity

1. INTRODUCTION

Meat preservation is a hard race against spoilage and potential pathogenic microorganisms and restriction methods need to be applied in order to reduce their growth and prolong the shelf-life. Recently, alternative technologies for the decontamination of meat products have been developed and implemented such as bioprotective cultures, natural antimicrobials, gamma, electron and x-ray irradiation, ozone, active packaging, high hydrostatic pressure, ohmic heating and steam pasteurization among the others (DEVLIEGHERE *et al.*, 2004; AYMERICH *et al.*, 2008; ZHOU *et al.*, 2010; LORETZ *et al.*, 2011). All the alternative technologies effort to be mild: their combination, as in the hurdle theory proposed by LEISTNER (2000), may improve their efficacy against pathogens and spoilage microorganisms, without modifying the sensorial qualities of the products.

In chilled vacuum-packaged raw meat, the oxygen source is restricted determining a selective effect on the microbial population; the main spoilage microorganisms associated with these type of food result as psychrotrophic, both Gram-positive bacteria, mainly Lactic Acid Bacteria (LAB) (*Lactobacillus* spp., *Leuconostoc* spp., *Carnobacterium* spp.) and *Brochothrix thermosphacta,* and Gram-negative, mainly represented by *Enterobacteriaceae* (SHAW and HARDING, 1984; HOLZAPFEL, 1998; LABADIE, 1999; NYCHAS and DROSINOS, 2000; FONTANA *et al.*, 2006; ERCOLINI *et al.*, 2009; PENNACCHIA *et al.*, 2011). In vacuum packaged meat, the natural LAB population increases during storage, becoming the predominant microflora: in particular, at chilling temperatures, LAB are able to exert antagonistic actions towards the growth of spoilage and pathogenic microorganisms in beef, pork, poultry and fish (KATLA *et al.*, 2002; YAMAZAKI *et al.*, 2003; CASTELLANO *et al.*, 2008).

In the last years, LAB have received great consideration as bioprotective cultures, leading to the discovery and characterization of several antimicrobial peptides (mainly bacteriocins, organic acids, carbon dioxide, ethanol, hydrogen peroxide and diacetyl), whose activity is well known (VIGNOLO *et al.*, 2000; CLEVELAND *et al.*, 2001; CASTELLANO and VIGNOLO, 2006; AYMERICH *et al.*, 2008; DORTU *et al.*, 2008; RAVYTS *et al.*, 2008). Their action is also due to the lowering of food pH and to the competition for nutrients (VANDENBERGH, 1993).

Different studies indicated that, during the storage, a gradual selection of LAB population occurs in the meat ecosystems, leading to the predominance of few *Lactobacillus* species (VIGNOLO *et al.*, 2010; 2012). *L. sakei* and *L. curvatus* have been observed as the most widespread species in vacuum-packaged beef (YOST and NATTRESS, 2002; FONTANA *et al.*, 2006; STELLA *et al.*, 2013).

Previous studies underlined the abilities of these two species as bioprotective cultures for meat, and their application to vacuum-packaged Argentine beef has already been described (CASTELLANO and VIGNOLO, 2006; CASTELLANO *et al.*, 2008). Their mechanism of action is expressed through the ability to produce not only bacteriocins but even organic acids. Moreover the good adaption to meat environment of *L. curvatus* and *L. sakei* was already proved, showing an important competitiveness in this substrate and an efficient use as an extra hurdle to minimize the risk of listeriosis in different muscle foods (SCHILLINGER *et al.*, 1991; HUGAS, 1998; CASTELLANO and VIGNOLO, 2006; FADDA *et al.*, 2008).

In a previous work 73 Lactobacilli were isolated from 8 lots of vacuum-packaged bovine rump hearts imported in Italy from Argentina, submitted to random amplified DNA-polymerase chain reaction and identified, showing a prevalence of *Lactobacillus sakei* (56 strains grouped in 18 different clusters) and *Lactobacillus curvatus* (8 strains grouped in 6 different clusters) (STELLA *et al.*, 2013).

One strain from each of the most representative clusters obtained of *L. sakei* (≥5 strains) and *L. curvatus* (≥2 strains), for a total 6 *L. sakei* and 2 *L. curvatus* strains, were chosen. Two specific mixtures were prepared (one *L. sakei*-based mixture and one *L. curvatus*-based mixture) and evaluated *in vitro* for their antimicrobial activity against spoilage and potential pathogenic microorganisms. Moreover, the effect of the addition of the two mixtures to sliced vacuum-packaged beef was investigated, considering microbiological and physical-chemical parameters

2. MATERIALS AND METHODS

2.1. Preparation of *Lactobacillus* strains and spoilage and potential pathogenic bacteria

All *L. sakei* and *L. curvatus* strains were stored in cryovials (Microbank™, Pro-Lab Diagnostics, Richmond Hill, Canada) at -70°C until the use. For each strain, a loop of the frozen culture was transferred to a test tube containing 10 mL of MRS broth (Oxoid, Basingstoke, UK) and incubated overnight at 30°C in jars (Anaerojar, Oxoid) with anaerobiosis generators (AnaeroGen, Oxoid). All the strains were re-inoculated into cooled MRS broth tubes and the initial absorbance (540 nm) (Shimadzu, UV1601, McCormick Place, Chicago, IL, USA) was measured. All the tubes were incubated at 15°C and the absorbance was measured after 24 and 48 h. Precultures were collected in exponential growth phase, defined as a change of absorbance of 0.05-0.2 at 540 nm. If necessary, the cultures were diluted before preparing the mixture in order to obtain the similar OD (optical density). Two specific mixtures were prepared (*L. sakei*-based mixture of strains n. 3, 42, 55, 77, 106 and 111 and *L. curvatus*-based mixture of strains n. 25 and 65) adding the same aliquot of broth of each strain.
A selection of 12 spoilage or pathogenic microorganisms was used as target strains for the test: *Escherichia coli* ATCC 25922, *Escherichia coli* 0157:H7 DSM 13526, *Proteus vulgaris* ATCC 8427, *Salmonella* Typhimurium ATCC 14028, *Serratia marcescens* ATCC 14756, *Yersinia enterocolitica* ATCC 23715, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas fluorescens* ATCC 13525, *Pseudomonas putida* ATCC 49128, *Listeria monocytogenes* ATCC 7644, *Listeria innocua* ATCC 33090 and *Staphylococcus aureus* ATCC 6538. Each strain, stored in cryovials at -70°C until the use, was subcultured aerobically overnight at 37°C (30°C for *P. fluorescens* and *P. putida*) in 10 mL TSB tubes (Triptyc Soy Broth, Oxoid). All the strains were re-inoculated into cooled TSB tubes and the initial absorbance was detected. All the tubes were incubated at 15°C and the absorbance was measured after 24 and 48 h. Precultures were collected as reported above.

2.2. Antimicrobial activity test

For the evaluation of the antimicrobial activity, each mixture, prepared as reported above, was inoculated into MRS broth tubes and incubated at 30°C for 48 h in anaerobiosis. After incubation, each of the two broths was spotted by a sterile swab (Carlo Erba, Rodano, I) onto the surface of MRS agar plates, subsequently incubated for 48 h at 30°C in an anaerobic jar. For each spoilage or pathogenic microorganism, 0.2 mL of bacterial suspension were added to a 5 mL share of semisolid agar (BHI, Brain Heart Infusion Broth, Oxoid + agar 0.7%), maintained in a water bath (45°C) and then poured over the MRS plates previously spotted with each mixture. To avoid the dispersion of Lactobacilli from the spot into BHI, a little amount (3-4 drops) of the inoculated semisolid medium was firstly distributed by a sterile Pasteur pipette (Carlo Erba) on the surface of the spot; after solidification (about 3 minutes at room temperature), the remaining BHI was poured

on the plates. After aerobic incubation at 37°C (30°C for *P. fluorescens* and *P. putida*) for 24 h, the plates were checked. A clear zone around the *Lactobacillus* spot indicated the inhibition of the target microorganisms. The tests were conducted in triplicate.

2.3. Antimicrobial activity of cell-free supernatants against spoilage and potential pathogenic microorganisms

In order to determine if the inhibition was due to the production of antagonistic compounds, the cell-free supernatants of the cultured mixtures were tested against the same bacteria. The mixtures were subcultured in MRS broth as described above. After 48 h of incubation, an aliquot of each culture was centrifuged at 7700 rpm for 10 min. For each broth, pH was measured by a pH meter (Amel Instrument, 334-B, Milan, I): three independent measurements were performed on each sample. The supernatants obtained were subsequently filtered by 0.2 μm filters (Sacco, Cadorago, I) and maintained at 4°C. Each of the 12 target strains was inoculated into 10 mL TSB tubes and prepared as described in section 2.2; 1 mL of inoculated TSB was then transferred into 20 mL flasks of Tryptic Soy Agar (Oxoid), maintained in a water bath at 45°C, carefully mixed and poured in sterile Petri plates. Once the medium was solidified, blank discs (Oxoid) were dipped with the supernatant of each mixture and placed onto the plates, subsequently incubated at 37°C (30°C for *P. fluorescens* and *P. putida*) for 24 h. Clear zones around the discs were recorded. Finally, in order to evaluate if the eventual inhibition was due to the production of organic acids, the pH of cell-free supernatants were adjusted to 6.5 with NaOH (1 N) (Sigma Aldrich, St. Luis, USA) and the same test was repeated. All the tests were performed in triplicate.

2.4. Preparation and inoculation of vacuum-packaged meat slices

Two bovine rump hearts were sliced in a commercial cutting plant. From each meat cut, a total of 42 slices (1-cm thick, 50 g of weight) were obtained and inserted into individual sterile plastic bags, with a diffusion coefficient of 6/14 cm^3 m^{-2} atm^{-1} 24 h^{-1} to oxygen at 25°C and 75% relative humidity (Cryovac, Elmwood Park, NJ). The 42 slices obtained from each rump heart were divided into two series (each series including 21 discs) inoculated as follows:

- CLS (Control samples *L. sakei*), inoculated with 0.5 mL of sterile saline solution;
- LS (*L. sakei*), inoculated with 0.5 mL of a mixture of the six strains of *L. sakei* (final concentration of 5 Log CFU g^{-1});

and

- CLC (Control samples *L. curvatus*), inoculated with 0.5 mL of sterile saline solution;
- LC (*L. curvatus*), inoculated with 0.5 mL of a mixture of the two strains of *L. curvatus* (final concentration of 5 Log CFU g^{-1}).

A loop of the frozen culture of each strain was transferred to a test tube containing 10 mL of MRS broth (Oxoid) and incubated overnight at 30°C in jars (Anaerojar, Oxoid) with anaerobiosis generators. All the strains were re-inoculated into cooled MRS broth tubes and the initial absorbance (540 nm) was detected. All the tubes were incubated at 15°C and the absorbance was measured after 24 and 48 h. Precultures were collected in exponential growth phase. The bacterial cells were pelleted by centrifugation at 7700 rpm for 10 min at 4°C and washed twice in 10 mL of 0.1 M phosphate buffered saline (PBS) with pH 7.0. Cell density of each strain was determined by microscopy (1000x) (Meiji Techno America,

USA). An average value from 10 randomly picked fields of view was considered. As needed, precultures were diluted in 0.85% NaCl solution to obtain 5 Log CFU mL^{-1} suspensions prior to inoculate the products.
L. sakei-based mixture of strains n. 3, 42, 55, 77, 106 and 111 and *L. curvatus*-based mixture of strains n. 25 and 65 were finally prepared adding the same aliquot of each strain at a final concentration nearly of 5 Log CFU mL^{-1} (each *L. sakei* strains has a final concentration of 4.22 Log CFU mL^{-1}, each *L. curvatus* strains has a final concentration of 4.70 Log CFU mL^{-1}).
After inoculation, the plastic bags were submitted to a vacuum pump (final vacuum of 99%), sealed using a packaging machine (Orved VM 16, Musile di Piave, I) and immediately stored at 4°C. Samples were submitted in triplicate to analyses after inoculation (T0) and after 10 (T10), 20 (T20), 30 (T30), 40 (T40), 50 (T50) and 60 (T60) days of storage.

2.5. Microbiological analyses

10 g of each sample were diluted in physiological saline (0.85% NaCl) with 0.1% peptone and homogenized in a Stomacher for 60 s (Seward Stomacher 400 Blender Mixer Homogenizer, International PBI, Milano, IT). Serial 10-fold dilutions were prepared and the following parameters were evaluated: Total Viable Count (TVC) was performed on Plate Count Agar (PCA, Biogenetics, Ponte San Nicolò, I) (ISO 4833:2003) and incubated at 30°C for 48h; Lactobacilli were enumerated on MRS agar (Oxoid) (ISO 15214:1998) incubated at 30°C for 48h in anaerobiosis, Gram-negative bacteria were enumerated on Tryptone Soy Agar (Oxoid) supplemented with 10 UI mL^{-1} of penicillin G (Oxoid) (TSAP) and incubated at 30°C for 48h; the number of *Enterobacteriaceae* was determined on Violet Red Bile Glucose Agar (VRBGA, Biogenetics) according to the ISO 21528-2:2004 method.

2.6. Determination of pH and colour parameters evaluation

At each sampling time, pH was measured by a pH meter (mod. XS pH6, Ghiaroni &C., Buccinasco, Italy): three independent measurements were performed on each trimmed sample diluted 1:5 with distilled water; means were then calculated. The surface colour of the meat was assessed 45 min after opening the packages, in order to allow blooming (deoxymyoglobin oxygenation) on six randomly chosen spots of each sample surface using a Minolta CR-200 Chromameter (Minolta, Osaka, J). *L** (lightness), *a** ("red" index) and *b** ("yellow" index) parameters were determined. Chroma was calculated as a^2+b^2, the hue angle (h) was calculated as h = arctan (b*⁄a*), where h = 0 for red hue and h = 90 for yellowish hue. Total colour differences (ΔE*) between treated and control samples were calculated as: $\sqrt{(L1^*-L2^*)^2 + (a1^*-a2^*)^2 + (b1^*-b2^*)^2}$.
A ΔE* more than 2.3 means a variation hardly perceptible to the human eye, while ΔE* more than 3.0 a variation well perceptible to the human eye.

2.7. Statistical analysis

The experimental data from inhibition halos were analyzed by a two-way univariate analysis of variance, performed with MIXED procedure of SAS software (SAS Inst. Inc., Cary, NC, 2006) in order to in order to test mean inhibition halos size differences for the comparisons of interest at *Lactobacillus* mixtures by target strains levels.
Data from meat inoculation tests were also analyzed by a two-way univariate analysis of variance using the same SAS procedure to test response variable mean differences at the levels of interest of *Lactobacillus* mixtures by time. For all statistical evaluations, threshold

levels of $P \leq 0.05$ and $P \leq 0.01$ were considered for significance. A two-way multivariate analysis of variance was also performed on color parameters, considering the vector of values L*, a*, b* as the response variable; GLM Procedure of SAS software was used.

3. RESULTS AND DISCUSSIONS

3.1. Antimicrobial activity against spoilage and potential pathogenic microorganisms

The mean rays of the inhibition halos obtained from antimicrobial evaluation of *L. sakei* mixture and *L. curvatus* mixture are reported in Table 1. The two mixtures exerted an antagonistic activity, producing evident halos against all the 12 target strains tested (66.7% of the halos induced by *L. curvatus* mixture and 52.8% of halos produced by *L. sakei* mixture were >10 mm). Generally, *L. curvatus* mixture resulted significantly more effective if compared to *L. sakei* mixture ($P = 0.0383$), showing also a higher prevalence of halos > 20 mm (19.4% of the plates inoculated with the *L. curvatus* mixture *vs* 5.5% of those inoculated with *L. sakei* mixture). Considering the different target strains, *L. curvatus* mixture produced significantly wider halos against *Y. enterocolitica* ($P = 0.0383$) and *P. aeruginosa* ($P = 0.0325$).
If we consider the results of the target strains clustered in homogenous categories, it is evident that the most sensitive belonged to the genus *Pseudomonas*, whose components produced significantly higher halos if compared with *Enterobacteriaceae* ($P < 0.0001$), *Listeria* spp. ($P = 0.0004$) and *Staphylococcus aureus* ($P = 0.0117$), according to the results obtained by Moore et al. (2006) and Tirloni et al. (2014) who underlined that most of the species of *Pseudomonas* fail to grow under acid conditions.
In particular, *P. fluorescens* resulted to be the most susceptible among the 12 target strains tested as significantly wider halos were observed if compared with all the other strains ($P < 0.01$). Secondly, *P. putida* resulted to be significantly more susceptible if compared to *E. coli* O157:H7 ($P = 0.0357$), *E. coli* ($P = 0.0215$), *L. innocua* ($P = 0.0200$), *L. monocytogenes* ($P = 0.0411$), *P. vulgaris* ($P = 0.0084$), *S. marcescens* ($P = 0.0003$) and *S.* Typhimurium ($P = 0.0215$). Finally *P. aeruginosa* produced significantly wider halos if compared to *S. marcescens* ($P = 0.0185$).
Moreover, *Enterobacteriaceae* showed a high variability in susceptibility with differences among the various species due to the many interspecific and intraspecific differences among the bacteria tested (LIU *et al.*, 2013). *Serratia marcescens* was by far the most resistant target strain, and it showed significantly smaller halos if compared to *Y. enterocolitica* ($P = 0.0116$), the most sensitive of *Enterobacteriaceae*.
Many authors highlighted the presence of an evident antagonistic activity of LAB against *Listeria monocytogenes,* microorganism typically related to vacuum-packaged meat products (Jones *et al.*, 2008; Awisheh and Ibrahim, 2009). Even in our study, both *L. monocytogenes* and *L. innocua*, showed the production of modest halos (between 9.7 and 14 mm).
Considering the cell-free supernatants and the pH-adjusted supernatants, no activity was recorded for all the target strains tested, highlighting that the antagonistic effect originates probably from the nutrient competitive exclusion while the involvement of extracellular compounds was not detected in the species considered in this test. The mechanism of the antibacterial activity of *Lactobacillus* strains usually appears to be multifactorial: the well-known production of bacteriocins by *L. sakei* and *L. curvatus* strains, reported in many previous studies (CASTELLANO and VIGNOLO, 2006; CASTELLANO *et al.*, 2008; 2010) was not confirmed in our research.

Table 1: Halos (in mm) expressed as a mean of three replication induced by *Lactobacillus curvatus* mixture and *Lactobacillus sakei* mixture against spoilage or pathogenic target microorganisms.

Target strains	*L. curvatus* mixture	*L. sakei* mixture
Escherichia coli ATCC 25922	8.3±1.5	13.3±3.8
Escherichia coli O157:H7 DSM 13526	14.0±2.6	10.0±2.0
Proteus vulgaris ATCC 8427	10.3±1.2	7.3±1.5
Salmonella Typhimurium ATCC 14028	13.3±4.9	8.3±2.9
Serratia marcescens ATCC 14756	2.3±1.2	2.7±1.2
Yersinia enterocolitica ATCC 23715	19.7±3.1a	13.7±1.5b
Pseudomonas aeruginosa ATCC 27853	21.3±3.5a	10.0±5.0b
Pseudomonas fluorescens ATCC 13525	56.7±23.1	21.7±13.5
Pseudomonas putida ATCC 49128	30.0±34.7	17.3±2.5
Listeria monocytogenes ATCC 7644	14.0±3.6	10.7±1.5
Listeria innocua ATCC 33090	11.7±5.7	9.7±2.3
Staphylococcus aureus ATCC 6538	13.7±1.5	12.7±1.5

Values are expressed as mean ± standard deviation. Different lower-case letters are pointing out significant difference ($P<0.05$) at the levels of interest of experimental mixtures by target strains.

The potential antagonistic activity of LAB towards spoilage microorganisms is strongly favored by the packaging technique: actually, vacuum or modified atmosphere packaging are the main methods for distribution and commercialization of fresh meat. These systems help the extension of shelf-life limiting the replication of *Enterobacteriaceae* and *Pseudomonas* spp., bacterial communities that often dominate aerobic spoilage of fresh meat at temperatures between -1 and 25°C. Dominating microflora composed by LAB contribute to the quality of meat thanks to their carbohydrate and protein catabolism. As underlined also from our results, the inhibitory properties of LAB, are not only related to the production of organic acids (mainly lactic and acetic) or other compounds (organic acids, bacteriocins, hydrogen peroxide e.g.) but bioprotective actions are also due to the competition for nutrients. In fact, apart from the metabolic activity, starter cultures occupy vital niches, thereby discouraging the colonization of undesired microorganisms. In this context, LAB interact with other microorganisms and with the environment, increasing their biomass at a rate that depends on the physical and chemical characteristics of the substrates: in chilled fresh meat, competition for a growth-limiting substrate such as glucose and oxygen interaction among species occurs (GILL, 1976). In this context, the prevalence of certain species will be determined by their relative initial level, affinity for the substrates, substrate availability as well as the relative growth rate of the competing species at different temperatures (CASTELLANO et al., 2008).
As a matter of fact, in order to obtain an important growth inhibition of the target strains, the presence of high loads of live and metabolically active cells, is fundamental.

3.2. Inoculation of vacuum-packaged meat slices

LAB cultures, and in particular *L. sakei* and *L. curvatus*, have been often studied for the application to food with good results thanks to the inhibition of pathogens and spoilage microorganisms and with the aim to extend the shelf-life of raw meat without important changes in its sensorial properties (CASTELLANO and VIGNOLO, 2006).
In this study, the antagonistic activity observed *in vitro* was also revealed on meat (Fig. 1).
Considering the global effect of the application of *L. sakei* mixture to meat during the whole trial, TVC resulted significantly lower in treated samples (LS) if compared with the control ones (CLS) (P=0.0089), reaching at the end of the trial the loads of 5.8±0.4 and 7.4±0.5 Log CFU g^{-1}, respectively.
The addition of *L. sakei* mixture resulted, since the beginning of the trial, in a constantly higher level of LAB in LS samples if compared with CLS ($P < 0.0001$). In particular, in LS samples, LAB reached the plateau level between 8 and 8.5 Log CFU g^{-1} after only 20 days from the beginning of the experiment. In CLS samples, the LAB naturally present on the slices showed a rapid increase from the beginning until T20; afterwards, they reached a plateau level between 6.6 and 7.4 Log CFU g^{-1}. Considering Gram-negative bacteria for the whole period, LS samples values resulted to be significantly lower than CLS ones ($P = 0.0029$). Moreover, *Enterobacteriaceae* resulted to be significantly lower in LS samples considering the whole trial ($P < 0.0001$). In particular LS samples showed a very stable trend (LS T0=2.3±0.5 *vs* T60=2.3±0.5 Log CFU g^{-1}), while in CLS samples, *Enterobacteriaceae* reached a value of 4.4±1.9 Log CFU g^{-1} at T60; anyway such level of contamination is not generally associated to evident sensorial spoilage of raw meats.
Considering the effect obtained from the application of *L. curvatus* mixture to meat in the whole experimental period, TVC resulted significantly lower in treated samples (LC) than in control (CLC) ones ($P = 0.0013$). The addition of *L. curvatus* mixture resulted, since the beginning of the trial, in a constantly higher level of LAB in LC samples if compared with CLC ($P < 0.0001$). In particular, in LC samples, LAB reached the plateau level between 7.9 and 8.4 Log CFU g^{-1} after only 20 days from the beginning of the experiment, according with LS results. In CLC samples, the LAB naturally present in the product, characterized in this case by a higher load if compared with CLS (3.5±0.6 Log CFU g^{-1} at T0), showed a rapid increase from T20; afterwards, they reached a plateau level between 6.1 and 7.5 Log CFU g^{-1}.
Considering Gram-negative bacteria, the loads resulted to be quite comparable between LC and CLC samples until T20 and then very highly fluctuant data were obtained; considering the whole period no significant differences were recorded ($P = 0.3325$).
Enterobacteriaceae resulted to be constantly lower in LC samples until T60, showing a general significant difference ($P = 0.0225$): in particular they showed a very stable trend for the whole study (LC T0=2.3±0.6 *vs* T60=3.0±1.5 Log CFU g^{-1}). In CLC samples, *Enterobacteriaceae* showed an increasing trend since the beginning of the trial, even if not reaching the threshold level of 5 Log CFU g^{-1} (CLS T0=2.5±0.8 *vs* T60=4.0±1.8 Log CFU g^{-1}).
The effect of the inoculation with *L. sakei* mixture resulted generally more evident than the treatment with *L. curvatus* mixture, suggesting a better capability to adapt to vacuum packaged meat substrate. The better adaptation of *L. sakei* mixture (LS) confirmed the preponderance of *L. sakei* in long shelf-life vacuum packaged meat LAB population, as highlighted in the previous study (STELLA *et al.*, 2013).

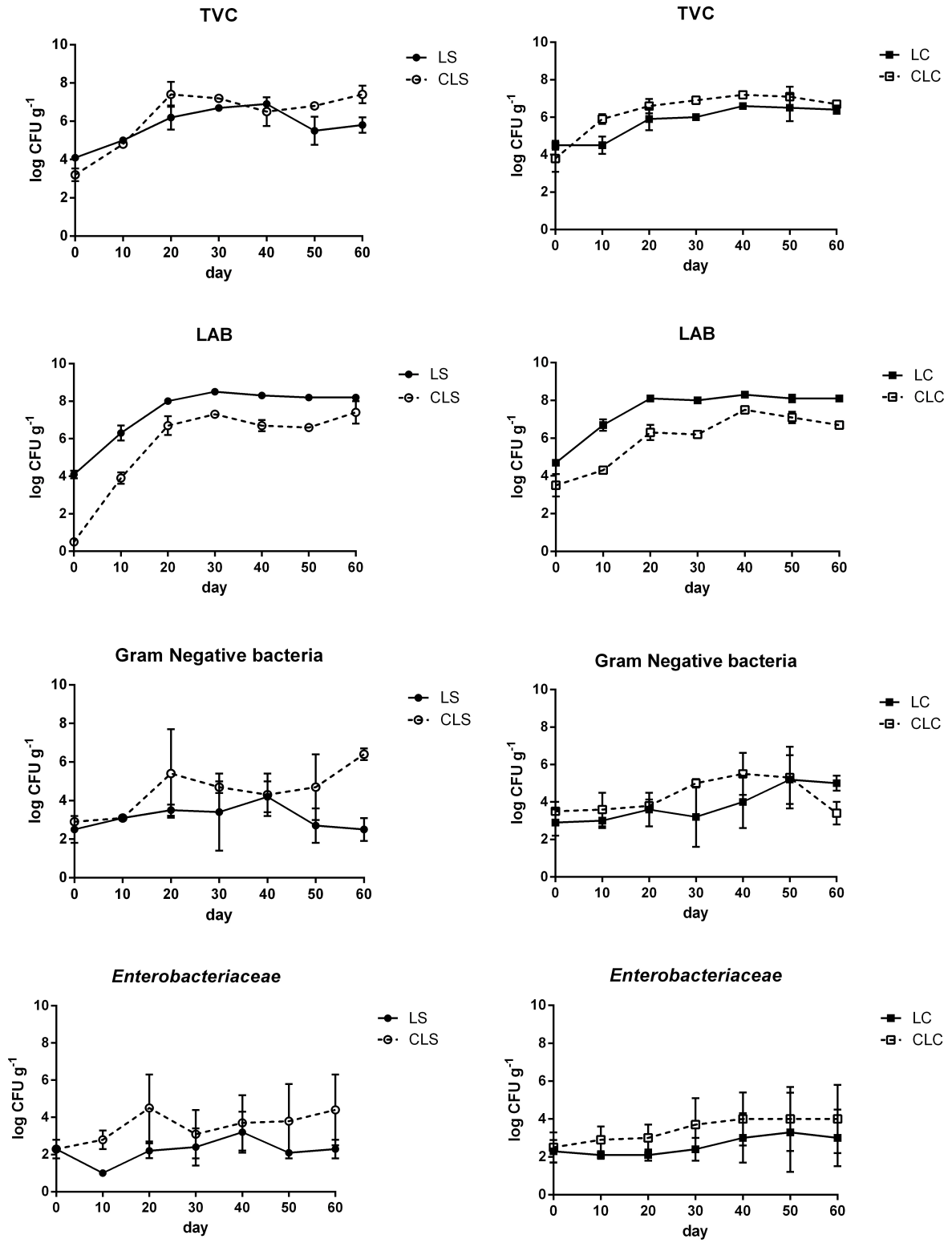

Figure 1: Results of total viable count (TVC), Lactic Acid Bacteria (LAB), Gram-negative bacteria and *Enterobacteriaceae*.
CLS= Control samples for *Lactobacillus sakei*; LS= samples inoculated a mixture of the six strains of *Lactobacillus sakei*; CLC= Control samples for *Lactobacillus curvatus*; LC= samples inoculated with a mixture of the two strains of *Lactobacillus curvatus*.

In any case, the capability of both of the two LAB mixtures to inhibit the growth of spoilage bacteria, clearly demonstrated *in vitro*, was also confirmed on meat substrate, also if it resulted more limited. This could be explained by the different growth rates and competitiveness of the cultures if applied to a complex matrix like meat: the adaptation to a substrate depends especially on the metabolic activity of cultures, which occupy vital niches, thus discouraging colonisation of undesired microorganisms. Generally, an antagonistic effect was detected both for *L. sakei* and *L. curvatus* treatments: despite the promising current knowledge and laboratory studies, LAB strains often suffer from a limited effectiveness in foods; among the others, the main factors involved are the poor adaptation to food environment, the inactivation of antimicrobial compounds through proteolytic enzymes or the binding to food ingredients and the pH buffering action (HOLZAPFEL *et al.*, 1995). In our case, the production of organic acids by the cultures inoculated on meat is supposable, even if their activity could be limited by the buffering capacity of meat. The metabolic activity of LAB population of vacuum packaged meat could be deduced by the slight reduction of pH of meat during the trial, with a 0.25 and 0.39 decrease in meat treated with the two mixtures (LS: T0 = 5.48 *vs* T60 = 5.23; LC: T0 = 5.80 *vs* T60 = 5.51), very close to the decrease observed (0.30-0.44) in control samples (CLS: T0 = 5.71 *vs* T60 = 5.27; CLC: T0 = 5.75 *vs* T60 = 5.45).

3.3. Determination of colour parameters

The addition of LAB mixtures did not result in an evident modification of meat colour. The multivariate analysis of the data evidenced some significant differences in whole meat colour between inoculated and control samples both for *L. curvatus* (T10 and T40) and *L. sakei* (T10, T30, T40 and T60). In any case, the analysis of the single colour parameters (Table 2) did not give univocal results, with few significant differences but without any clear trend.

However, the application of LAB cultures did not negatively affect meat colour for the whole period considered.

During the storage the hue value, a form of data reduction involving both a* and b*, and plottable in cylindrical coordinates when chroma and L* are known, is the main parameter used to attest the colour display life: in this study an increase of hue values between T0 and T10 was detected, afterwards they reached a stability level, without pointing out any significant difference among the series.

Chroma, also termed saturation index, used as an indicator of the loss of colour saturation, was characterized by a slight reduction during the whole period and did not show important differences between treated and control samples.

In three sampling times (T10, T20 and T50) a perceptible total colour difference (expressed as ΔE) between LS samples and CLS samples was not detected ($\Delta E<2.3$), in two sampling times (T0 and T30) this difference was acceptable ($2.3<\Delta E<3$), while in 2 sampling times (T40 and T60) the samples got $\Delta E >3$, highlighting very strong, perceivable differences in meat colour (Table 2). Considering LC and CLC, in four sampling times (T0, T20, T30 and T50) a perceptible total colour differences between LS samples and CLS samples (expressed as ΔE) was not detected, in four sampling times (T0, T20, T30 and T50) this difference was acceptable ($2.3<\Delta E<3$), while just only in one sampling time (T40) the samples got $\Delta E >3$.

Table 2: Values of L*, a*, b* and Hue angle values measured on LS, CLS, LC and CLC samples.

Samples	T0	T10	T20	T30	T40	T50	T60
LS							
L*	44.5±1.7	44.2±1.5	43.1±2.1	42.5±5.5	43.7±2.8B	44.8±2.0	44.8±3.0a
a*	23.1±2.2	20.8±3.6	20.1±1.2	19.7±1.6B	20.8±1.1A	16.9±2.1	16.9±1.1B
b*	15.8±1.9	12.8±4.1b	13.3±1.0	13.0±1.0b	14.5±0.3	13.2±0.9	13.4±1.2
Hue-Angle	34.41	40.82	42.06	41.40	44.56	41.69	42.25
Chroma	27.95	19.50	19.88	19.67	20.71	19.76	19.94
CLS							
L *	44.6±3.5	43.5±1.9	41.7±4.3	44.4±1.9	51.0±1.3A	45.7±1.9	40.9±5.4b
a*	20.9±1.6	21.7±0.8	19.8±1.1	23.1±3.8A	17.6±3.2B	17.4±1.5	20.0±1.2A
b*	14.0±1.5	14.9±0.8a	12.4±2.1	15.0±2.2a	14.9±1.2	13.7±1.0	13.7±1.3
Hue-Angle	33.88	45.17	39.96	45.51	45.25	42.84	42.95
Chroma	25.14	20.93	19.25	21.06	20.96	20.13	20.16
ΔE (LS-CLS)	2.83	2.23	1.68	2.73	7.38	1.03	3.89
LC							
L*	39.7±3.5	42.0±1.6	44.8±3.8	47.2±3.1	46.3±1.8	42.5±1.4	45.4±6.0
a*	22.3±2.4	22.8±2.4	20.6±1.4	19.6±1.7	19.0±2.0B	19.3±1.9	21.1±1.8
b*	13.5±1.1	15.2±2.3A	14.8±1.1	14.5±1.3	13.1±2.2b	14.3±0.6	15.4±1.1
Hue-Angle	31.12	45.78	45.02	44.55	41.55	44.12	46.15
Chroma	26.09	21.16	20.88	20.71	19.72	20.56	21.30
CLC							
L *	40.7±3.2	42.0±4.9	44.5±1.8	48.2±3.5	43.8±3.1	42.8±3.6	48.2±4.4
a*	23.0±2.7	20.9±1.3	19.8±1.5	20.2±0.9	22.5±1.6A	21.0±4.0	20.1±1.2
b*	14.8±2.7	12.4±3.2B	13.4±1.4	14.5±1.2	15.1±1.6a	15.0±1.7	15.2±1.1
Hue-Angle	32.66	39.96	42.20	44.45	45.61	45.38	45.77
Chroma	27.34	19.25	19.92	20.67	21.10	21.01	21.16
ΔE (LC-CLC)	1.73	2.80	1.44	1.06	3.27	0.70	2.77

Values are expressed as mean ± standard deviation. CLS= Control samples for *Lactobacillus sakei*; LS= samples inoculated a mixture of the six strains of *Lactobacillus sakei*; CLC= Control samples for *Lactobacillus curvatus*; LC= samples inoculated with a mixture of the two strains of *Lactobacillus curvatus*.
Different lower-case or upper-case letters are pointing out significant difference, respectively at $P<0.05$ or $P<0.01$, at the levels of interest of *Lactobacillus* mixtures by time.

4. CONCLUSIONS

Historically *L. sakei* and *L. curvatus* have been recognized for their useful role in food biopreservation by contrasting the growth of spoilage and pathogenic microorganisms without the production of sensorial changes. *L. sakei* mixture and especially *L. curvatus* mixture tested in this work showed promising antimicrobial activity *in vitro* against a wide number of spoilage and pathogenic bacteria. No activity was recorded in the supernatants and in the pH adjusted supernatant, for all the target strains tested, highlighting that the antagonistic effect originates probably from the nutrient competitive exclusion. Moreover, the effect of the addition of the two mixtures to sliced vacuum-packaged beef was investigated: the high loads detected on meat, that is a lesser inhibiting effect of the two bacteria on meat than that demonstrated *in vitro*, could be related to the slighter competitiveness of the cultures if applied to a complex substrate and to the buffering

capacity of meat, which decreased the potential action of organic acids. The use of higher dosage of LAB cultures could be suggested as an effective mean to determine an early conditioning of meat environment, in order to prevent the growth of spoilage bacteria and prolong vacuum packaged raw meat shelf-life.

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