PAPER

Growth inhibition of selected microorganisms by an association of dairy starter cultures and probiotics

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Abstract

Several growth curves for selected pathogens and hygiene indicators alone and *vs* selected dairy starter cultures (LAB) and commercial probiotics have been performed. All strains for LAB and commercial probiotics were inoculated as pure cultures into skim milk to get an initial cocci:bacilli:enterocci ratio of 2:1:1 and a concentration of approx 10^7 cfu mL^{-1} until challenge *vs* selected pathogens and hygiene indicators. Selected pathogens came from the collection of the Laboratorio di Ispezione degli Alimenti di O.A. or were reference strains (*Escherichia coli*, CSH26 K12, *Staphylococcus aureus*, 27R, *Salmonella* Derby 27, *Pseudomonas fluorescens* ATCC 13525, *Listeria innocua* ATCC 33090). Each strain was inoculated into skim milk to get an initial concentration of approx 10^6 cfu mL^{-1}. Growth curves in skim milk for the following challenges were studied: i) sterility control; ii) association LAB; iii) association of LAB *vs* each selected pathogen or hygiene indicator; iv) selected pathogen or hygiene indicator alone. The challenges were carried out in BHI broth and in skim milk at 37°C. The highest reduction was observed in milk but in general the association of LAB and the probiotic was able to limit the growth of pathogens and hygiene indicators.

Introduction

During the recent years health-conscious consumers are looking for natural foods without chemical preservatives that will fit in their healthy lifestyle. Bio-preservation refers to extended shelf life and enhanced safety of foods using microorganisms or their metabolites (Cizeikiene *et al.*, 2013). Lactic acid bacteria (LAB) play a key role in food fermentations where they not only contribute to the development of the desired sensory properties in the final product but also to their microbiological safety (Cenci Goga *et al.*, 2008, 2012). The antimicrobial effect of LAB is mainly related to the production of lactic- and acetic-acids, as well as propionic-, sorbic-, benzoic-acids, hydrogen peroxide, diacetyl, ethanol, phenolic- and proteinaceous-compounds. Some strains are able to synthesize antimicrobial substances – bacteriocins (Cizeikiene *et al.*, 2013). The prevention of foods by natural and microbiological compounds is a novel approach aimed at solving economic losses due to microbial spoilage of raw materials and food products and reducing the incidence of food borne illnesses (Cizeikiene *et al.*, 2013). This study evaluated the effect of a formulation of lactic acid bacteria and probiotics on the fate of selected pathogens in an *in vitro* environment.

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Materials and methods

Starter cultures and probiotics

Bacterial strains used as starter cultures were taken from the collection of the Laboratorio di Ispezione degli Alimenti di Origine Animale: *Lactococcus lactis ssp. lactis*, strain 340; *L. lactis* ssp. *lactis*, strain 16; *Lactobacillus casei* ssp. *casei*, strain 208 and *Enterococcus faecium* UBEF-41. Lactococci and lactobacillus were isolated from traditional cheeses manufactured in small-scale dairy plants in Umbria, Italy, wile *Enterococcus faecium* UBEF-41 was provided by Bromatech Srl (Milano, Italy). The morphological, biochemical and physiological characterisation, together with the acidifying activity of the three lactic acid bacteria has been reported by the authors in previous papers (Cenci Goga *et al.*, 1995; Clementi *et al.*, 1998), while characterization of *Enterococcus faecium* UBEF-41 is available at Unique Biotech Ltd. (http://uniquebiotech.com/products/animal_healthcare/enterococcus_faecium). Growth curves for all starter cultures alone and *vs* selected pathogens have been recorded throughout the whole experiment. Before challenge-test, freeze-dried strains of the starter cultures were grown aerobically in Nutrient Broth (NB, Oxoid CM0001, Basingstoke, UK) at 37°C for 24 h. Each strain was then sub-cultured in Skim Milk (BD Difco, Franklin Lakes, NJ, USA, 232100) at 37°C for 24 h. Total viable cells (TVC) count (on Nutrient Agar, NA, Oxoid CM0003, incubated at 37°C on air for 24 h) at 24 h was approximately 1×10^9 cfu mL^{-1}.

Selected microorganisms

Selected pathogens and hygiene indicators came from the collection of the Laboratorio di Ispezione degli Alimenti di Origine Animale or were reference strains: *Escherichia coli*, CSH26 K-12, *Staphylococcus aureus*, 27R, *Salmonella* Derby 27, *Pseudomonas fluorescens* ATCC 13525, *Listeria innocua* ATCC 33090.

Before challenge-test, freeze-dried strains of the starter cultures were grown aerobically in Nutrient Broth (NB, Oxoid CM0001, Basingstoke, UK) at 37°C for 24 hrs. Each strain was then sub-cultured in Skim Milk (BD Difco, Franklin Lakes, NJ, USA, 232100) at 37°C for 24 h. Total viable cells (TVC) count (on Nutrient Agar, NA, Oxoid CM0003, incubated at 37°C on air for 24 h) at 24 h was approximately $1x10^9$ cfu mL^{-1}. For the test each strain was inoculated into skim milk to get an initial concentration of approx. 10^6 cfu/mL.

Characterization of starter cultures with acidification and challenge growth curves

Acidification

All strains were inoculated as pure cultures into skim milk (BD Difco) to get an initial concentration of about 10^7 cfu mL^{-1}. All strains were incubated at 37°C. The association was

inoculated into skim milk (BD Difco) to get an initial cocci:bacilli:enterocci ratio of 2:1:1 and a concentration of approx 10^7 cfu mL^{-1}. The association was incubated at 37°C and pH was measured with a Foodtrode electrode (Hamilton Company, Reno, NV, USA) hooked to an Eutech pH 2700 (Eutech Instrument Europe B.V. Nijkerk, Netherlands) which recorded pH values continuously with CyberComm 6000 (Eutech Instrument) every minute. The following fourth degree polynomial was used as an empirical model to fit the experimental data:

$$y = a + bx + cx^2 + dx^3 + ex^4 \quad \text{(eq. 1)}$$

where y is pH; x is time; a, c, d, e are the generic regression coefficients of the polynomial under study, as determined by the statistical package Stat Graphics, ver. 6.1 (Manugistics Inc. Rockville, MD, USA).

The first derivative of the equation gives the instantaneous acidification rate and its maximum value (V_m) which corresponds to the inflection point of the acidification curve, whereas the second derivative gives the acceleration and its roots give the x values (t_m) at the inflection points.

Challenge test

Several growth curves for selected pathogens and hygiene indicators alone and *vs* the selected dairy starter cultures and commercial probiotics have been performed. All strains for the selected dairy starter cultures and commercial probiotics were inoculated as pure cultures into skim milk (BD Difco) and incubated at 37°C to get an initial concentration of about 10^9 cfu mL^{-1} after 24 h of incubation. The association was then inoculated into skim milk (BD Difco) to get an initial cocci:bacilli:enterocci ratio of 2:1:1 and a concentration of approx 10^7 cfu mL^{-1} until challenge *vs* selected pathogens and hygienic indicators. All strains for the selected pathogens came from the collection of the Laboratorio di Ispezione degli Alimenti di Origine Animale (*Escherichia coli*, strain CSH26 K12, *Staphylococcus aureus*, strain 27R, Salmonella Derby 27, *Pseudomonas fluorescens* ATCC 13525, *Listeria innocua* ATCC 33090). Strains were inoculated as pure cultures into BHI (BD Difco) and incubated at 37°C (30°C for *Pseudomonas fluorescens*) to get an initial concentration of about 10^9 cfu mL^{-1} after 24 h of incubation. Each strain was then inoculated into skim milk (BD Difco) to get an initial concentration of approximately 10^6 cfu mL^{-1}. Growth curves for the following challenges, in triplicates, were studied: i) sterility control, ii) association of starter cultures (LAB), iii) association of LAB *vs* each selected pathogen or hygiene indicator, iv) selected pathogen or hygiene indicator alone. The challenges were carried out in BHI broth (BD Difco) and in skim milk (BD Difco) at 37°C. Bacterial counts were recorded at time 0, 12 h, 24 h, 30 h, 48 h, 72 h, 120 h, 168 h, 240 h. Microbiological analysis were conducted using the methods described in paragraph 2.4.

Microbiological analysis

For the analysis about 25 mL of sample were transferred aseptically to 225 mL of sterile, buffered, peptone water (Oxoid, CM1049), and homogenised in a stomacher (PBI International) for 1 min at low speed and 1 min at high speed at room temperature. Serial decimal dilutions in buffered peptone water were prepared and triplicate 1 mL or 0.1 mL samples of appropriate dilutions were poured or spread on total count and selective agar plates. Total lactic microbiota was determined on de Man, Rogosa, Sharpe (MRS) Agar (Oxoid) acidified to pH 5.5, incubated at 30°C for 72 h under anaerobic conditions (Gas generating kit, Oxoid, BR0038); *Enterococcus faecium* on Slanetz and Bartley (SB) Agar (Oxoid, CM0377), incubated at 37 °C for 48 h; *Pseudomonas fluorescens* on Pseudomonas agar base (Oxoid, CM0559) added with Pseudomonas CFC Supplement (Oxoid, SR0103), incubated at 25°C for 48 h. *S. aureus* were determined on Rabbit Plasma Fibrinogen (RFP) Agar (Oxoid, CM0961, with RFP Supplement (Oxoid, SR0122), incubated at 37 °C for 48 h. *Escherichia coli* CSH26 K12 on Sorbitol MacConkey agar (McS) Agar (Oxoid, CM0813), overlaid with 5 ml of the same medium and incubated at 30 °C for 24 h. The ISO 6579 method was used (ISO, 2007) for *Salmonella* Derby 27. Briefly, after a pre-enrichment step (buffered peptone water, Oxoid), 18 h at 37°C, and the inoculum onto two selective enrichments, Rappaport Vassiliadis Soya peptone broth (RVS, Oxoid CM0866), 24 h at 42°, and Muller-Kaufmann Tetrathionate-Novobiocin Broth (MKTTn, Oxoid, CM1048), 24 h at 37°C, a loopful of broth was spread onto Xylose-Lysine-Desoxycolate Agar (XLD, Oxoid, CM0469) and Salmonella Chromogenic Agar (Oxoid, CM1007 with Salmonella selective supplement, Oxoid, SR0194). The ISO 11290 method (ISO, 1996) was used for *Listeria innocua* ATCC 33090). Briefly, after a primary (Fraser, Oxoid, CM 0895 with half Fraser selective supplement, Oxoid, SR0166), 24 h at 30°C, and a secondary enrichment medium (Fraser with Fraser supplement, Oxoid, SR0156), 48 h at 35°C, a loopful of broth was spread onto Oxford agar (Listeria selective agar, Oxoid, CM0856 with Listeria selective supplement, SR0140) and PALCAM agar (PALCAM agar base, Oxoid, CM0877 with PALCAM selective supplement, Oxoid, SR0150).

Sensitivity for spread plate was 10^2 cfu g^{-1} and for pour plate was 10 cfu g^{-1} and the 95% confidence limit, as given by the classic formula $2s=2\sqrt{x}$ (Adams and Moss, 2000), ranged between ±37% and ±12% (*i.e.* plates with a number of cfu ranging from 30 to 300). Therefore, no plates with less than 30 cfu were used for data analysis, and when this applied to the lowest dilution, the results were recorded as <300 for pour plate and <3000 for spread plate. Samples without typical colonies were recorded as negative and samples with at least one typical colony in the lowest dilution were recorded as positive, regardless the number of colonies (Cenci Goga *et al.*, 2005).

Analysis of the results

For each sampling the arithmetic means of the three subsamples of each of the three sausages was calculated and then all the values (converted to log for microbiological analyses) were analysed using GraphPad InStat, version 3.0b for Mac OS X and graphs were obtained with GraphPad Prism version 6.0d for Mac OS X.

Results and discussion

Characterization of starter cultures with acidification and challenge growth curves

Acidifying activity of selected starter cultures

Three species of lactic acid bacteria previously isolated from among the most representative ones from sheep milk, curd and cheese in cheese making trials carried out previously (Cenci Goga *et al.*, 1995) along with a commercial probiotic were chosen to be used as a starter. These strains had been previously identified by API 50 CHL and some of them had already been used as a starter in the manufacturing of salami (Cenci Goga *et al.*, 2008; 2012). The acidifying activity of the different species had been preliminarily tested (Cenci Goga *et al.*, 1995; Clementi *et al.*, 1998) as pure cultures and as different associations in sterilized cow milk to assess their suitability to be used as a starter. The parameters describing the acidification kinetics are reported in Table 1 together with the regression coefficients of

the fourth degree polynomial (equation 1) which was used as an empirical model. This adequately fitted the experimental data, since the r^2 values varied from 0.997 to 0.9998 and the actual values were almost exactly superimposed on the empirical model curves (Figure 1). The values of the maximum instantaneous acidification rate (V_m) were of the same order for all strains, except for *Lc. lactis ssp. lactis* strain 16 which had the highest V_m (-3.14×10^{-3} ΔpH/min) and the lowest t_m (350 min) values. These values were not very different from those (-5.5×10^{-3} ΔpH/min and 330 min) observed for *Lc. lactis* SL03 in a study evaluating the acidifying activity of several lactic acid bacteria with a similar procedure (Picque *et al.*, 1992). The final pH values of milk inoculated with the different strains as pure cultures (Figure 1) were lower than those previously obtained in cow milk (Cenci Goga *et al.*, 1995; Clementi *et al.*, 1998) except for *Lb. casei spp casei* which reached the same value in both types of milk. After 900 min, the two strains of *Lc. lactis ssp lactis* reached pH values equal to or lower than those obtained in cow and sheep milk inoculated with *Lc. lactis ssp lactis* strains isolated from cheeses (Ledda *et al.*, 1994). *Enterococcus faecium* gave a one fold pH decrease within about 500 min, like some commercial thermophilic lactobacilli (Chamba and Prost, 1989). The association of the four strains performed well with a final pH of 3.74 and showed the fastest pH drop, within the first 24 h along with a steady descent subsequently. The association mimicked the behaviour of the two *Lc. lactis ssp. lactis* strains in the first 16 h and then, when the lactococci acidification activity decreased (steady pH values after 24 h), mirrored the *Lb. casei spp casei* performance for a continuous pH drop up to 70 hrs. This synergism is of the utmost importance in the production of fermented food such as cheeses and dry salami because the LAB activity is desirable throughout the whole fermentation process (Cenci Goga *et al.*, 2012, Cizeikiene *et al.*, 2013).

Challenge growth curve

The results are shown in Figure 2, which displays the different growth curves of pathogens or hygiene indicators *vs* the association of selected dairy starter cultures and commercial probiotics. Figure 2a shows the evolution of *E. coli* CSH26 K12; panel 2b shows the evolution of *Salmonella* Derby strain 27; panel 2c shows *Staphylococcus aureus*, strain 27R; panel 2d shows *Listeria innocua* ATCC 33090; panel 2e shows *Pseudomonas fluorescens* ATCC 12983 and panel 2f shows the evolution of LAB and *E. faecium*. For the challenge *E. coli vs* LAB, level of *E. coli* in milk dropped, after 48 h, below 10^4 cfu mL^{-1}, and was no longer detectable at 72 h, while in BHI the growth curve of *E. coli* was parallel to control, indicating an effect of pH rather than a direct activity of LAB and/or bacteriocins. A similar behaviour was shown by *Salmonella* Derby with an even faster drop ($<10^4$ cfu mL^{-1} after 30 h). *S. aureus* proved to be more resilient and a substantial reduction in total

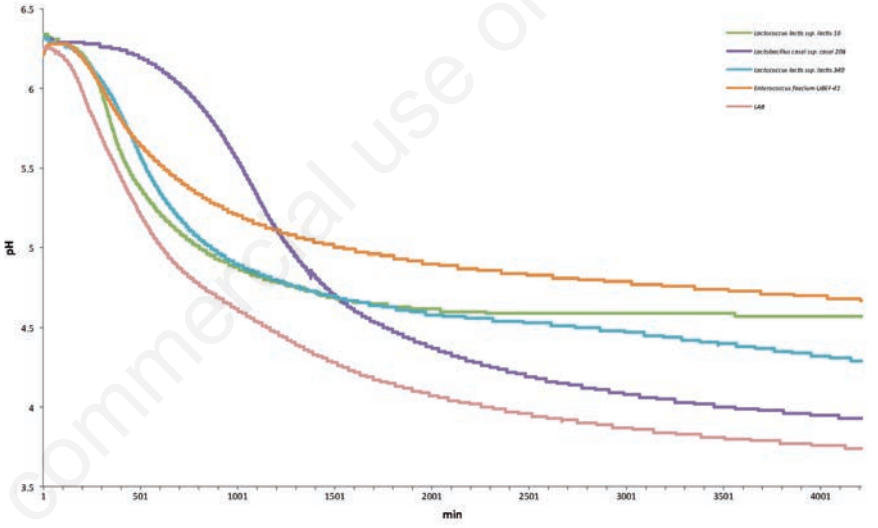

Figure 1. Acidifying curves of selected starter cultures.

Table 1. Acidifying activity of the starter cultures as pure cultures (at 10°C and 37°C) in sterile skim milk. Kinetic parameters and regression coefficients (+/- se) of the acidification curves ad determined by equation 1 ($y = a + bx + cx^2 + dx^3 + ex^4$), see text for explanation.

Strains and associations	*Lc. Lactis lactis* n.16	*Lc.lactis lactis* n.340	*Enterococcus faecium*	*Lb. casei casei* n.208	Association
Kinetic parameters°					
Vm, ΔpH/min	-3.14×10-3	-2.32×10-3	-1.83×10-3	-1.92×10-3	-2.66×10-3
Tm, min	350	436	354	1151	303
pHm	5.74	5.73	5.91	5.25	5.72
Regression coefficients					
a	6.26±0.005	6.26±0.002	6.29±0.002	6.28±0.003	6.27±0.002
b	$2.26\times10^{-3}\pm6.75\times10^{-5}$	$1.26\times10^{-3}\pm2.46\times10^{-5}$	$5.8\times10^{-4}\pm2.82\times10^{-5}$	$1.41\times10^{-4}\pm2.42\times10^{-5}$	$1.3\times10^{-5}\pm2.73\times10^{-5}$
c	$-1.8\times10^{-5}\pm2.75\times10^{-7}$	$-9.5\times10^{-6}\pm7.84\times10^{-8}$	$-7.8\times10^{-6}\pm1.03\times10^{-7}$	$-6.94\times10^{-9}\pm5.48\times10^{-8}$	-1×10-5±1.11×10-7
d	$2.45\times10^{-8}\pm4.13\times10^{-10}$	$1.02\times10^{-8}\pm9.22\times10^{-11}$	$1.03\times10^{-8}\pm1.4\times10^{-10}$	$-1.55\times10^{-9}\pm4.58\times10^{-11}$	$1.49\times10^{-8}\pm1.66\times10^{-10}$
e	$-1.05\times10^{-11}\pm2.05\times10^{-13}$	$-3.37\times10^{-12}\pm3.58\times10^{-14}$	$-4.12\times10^{-12}\pm6.24\times10^{-14}$	6.74× $0^{-13}\pm1.26\times10^{-14}$	$-6.42\times10^{-12}\pm8.20\times10^{-14}$
$r^{2\#}$	0.997	0.999	0.999	0.998	0.999

°V_m is the maximum instantaneous acidification rate, tm and pHm the time and the pH at which Vm occurred. #Significant at P<0.001.

viable cells was observed between 72 and 120 h ($<10^3$ cfu mL^{-1} after 120 h) with a complete elimination after 168 hrs. *L. innocua* was no longer detectable in milk after 120 h, with a steady drop after 24 hrs ($<10^4$ cfu mL^{-1} after 72 h). LAB had an effect on *L. innocua*, also in BHI, with levels below 10^4 cfu mL^{-1} just after 30 h. The direct effect of certain LAB strains on *Listeria* spp. rather than the indirect effect of pH drop has been described (Winkelströter and De Martinis, 2015). *P. fluorescens* had a similar drop both in milk and in BHI and was no longer detectable after only 24 h, proving that the LAB formulation tested in this study is able to limit the growth of this typical spoilage microorganism even in absence of pH drop (Folawe Okorhi, 2014). Figure 2f shows the correct evolution of the LAB formulation and of the probiotic strain both in milk and in BHI. The probiotic strain of *E. faecium* showed a certain susceptibility to pH drop, in fact, after 48 h of incubation in milk as pure culture, a rapid descent was recorded and counts reached values below 10^6 cfu mL^{-1} after 72 h and below 10^4 cfu mL^{-1} after 120 h, when pH had reached levels below 5.5. A similar behaviour has been described in cheese (Reale *et al.*, 2010).

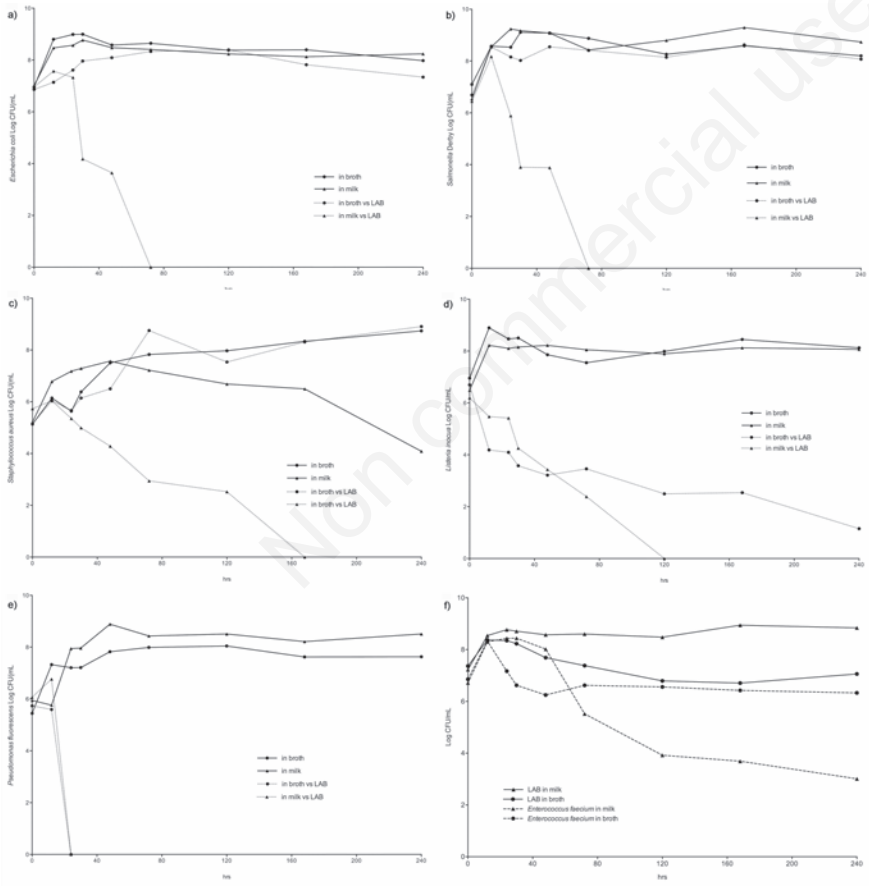

Figure 2. a) Growth curves of *E. coli vs* starter cultures (*Escherichia coli* CSH26 K12). b) Growth curves of *Salmonella* vs starter cultures (*Salmonella* Derby 27). c) Growth curves of *S. aureus vs* starter cultures (*Staphylococcus aureus* 27R). d) Growth curves of *Listeria innocua vs* starter cultures (*Listeria innocua* ATCC 33090). e) Growth curves of *Pseudomonas fluorescens vs* starter cultures (*Pseudomonas fluorescens* ATCC 13525). f) Growth curves of LAB and probiotic (*Lactococcus lactis* ssp. *lactis*, strain 340; *L. lactis* ssp. *lactis*, strain 16; *Lactobacillus casei* ssp. *casei*, strain 208 and *Enterococcus faecium* UBEF-41).

Conclusions

There are certain criteria that lactic acid bacteria starter cultures should meet when being included in food products, such as to compete effectively with indigenous lactic acid bacteria, to produce adequate quantities of lactic acid, to grow in the presence of up to 6% NaCl, to tolerate $NaNO_2$, to grow between 10°C and 40°C, with an optimum between 30°C and 37°C, to be homo-fermentative and catalase-positive, to be non-proteolytic and to produce only very small quantities of H_2O_2, to reduce nitrate, to enhance flavour of the finished sausage, not to produce biogenic amines, not to produce slime, to be antagonistic to pathogenic and other undesirable micro-organisms and tolerant of, or synergistic with, other starter components. Various species have been, and are, used as inoculants in sausage fermentation (Campbell-Platt, 1987). The fermentation of carbohydrates leads to several desirable results: production of organic acids, which lead to the reduced pH value; production of favourable organoleptic compounds; coagulation of meat proteins, thereby decreasing the water holding capacity and thus, facilitating the drying process, which affects the texture and firmness of the end product, and red colour formation due to the reaction of nitrogen monoxide with the heme group in myoglobin at pH 5.4 to 5.5. The association of selected starter cultures and probiotics tested in this research was able to limit the growth of pathogens and hygiene indicators in an in-vitro setting. According to the strong inhibitory activity, our results show that the proposed formulation of LAB and probiotics, could be applied as food preservative and as starter cultures. The LAB application is wide-ranging and most promising if one considers that the indicator microorganisms used in this experiment were suppressed. These paper along with another study on the use of this formulation in salami production (Cenci-Goga *et al.*, 2015) highlights the possibility that food safety and food quality can dramatically be improved by using novel formulations of LAB with antimicrobial activities as starter for food fermentation. It must be considered that in the production of traditional southern European style sausages, the fermentation profile must phase in order to ensure the growth of the added starter culture at the expense of the background flora. Additionally, the acidification profile must not reach values below pH 4.8-5.0 at any time. This will ensure that autochthonous microbiota maintain its activity over a longer period of time; foremost reductase and

flavour-forming activities (Lebert *et al.*, 2007; Marco *et al.*, 2008; Barbut, 2010; Reale *et al.*, 2010). The starter cultures should be specifically selected for traditional fermentation profiles applying temperatures not higher than 24°C (Holley *et al.*, 1988; Scannell *et al.*, 2001; Cenci Goga *et al.*, 2012). On the other hand, in the production of north European and US style sausages the fermentation profile have a very short lag phase in order to rapidly show a fast drop in pH to below 5.3 within 30 hours as a minimum. This ensures an efficient inhibition of background flora and the production time is typically less than 2 weeks at the cost of a general lower consumer acceptability because the faster the acidification the less enzymes enhancing colour and aroma formation are produced (Marco *et al.*, 2008; Barbut, 2010). The proposed formulation is an additional tool in the production of low-acid fermented dry salami that are nowadays the preferred consumer's choice given that the low speed of fermentation does not negatively affect the aroma in the product. Moreover, the incorporation of *E. faecium* is a further asset because researchers postulate that enterococci may contribute to the development of the typical sensory properties of certain fermented meat products, such as salami (Holley *et al.*, 1988). Enterococci are, in fact, capable of modulating the aroma by means of the conversion of amino acids and free fatty acids (González-Fernández *et al.*, 2006; Leroy *et al.*, 2006; Corbiere Morot-Bizot *et al.*, 2007; García Fontán *et al.*, 2007).

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