Lactococcus lactis and Lactobacillus sakei as bio-protective culture to eliminate Leuconostoc mesenteroides spoilage and improve the shelf life and sensorial characteristics of commercial cooked bacon

Giuseppe Comi, Debbie Andyanto, Marisa Manzano and Lucilla Iacumin*

Department of Food Science, University of Udine, Via Sondrio 2/A, 33100 Udine, Italy.

Running headline: Quality improvement of cooked bacon.

*Corresponding author:
Lucilla Iacumin, PhD
Dipartimento di Scienze degli Alimenti, Università degli Studi di Udine
Via Sondrio 2/A, 33100 Udine, Italy
e-mail: lucilla.iacumin@uniud.it;
Phone: +39 0432 558126;
Fax. +39 0432 558130.
Abstract

Cooked bacon is a typical Italian meat product. After production, cooked bacon is stored at 4 ± 2 °C. During storage, the microorganisms that survived pasteurisation can grow and produce spoilage. For the first time, we studied the cause of the deterioration in spoiled cooked bacon compared to unspoiled samples. Moreover, the use of bio-protective cultures to improve the quality of the product and eliminate the risk of spoilage was tested. The results show that *Leuconostoc mesenteroides* is responsible for spoilage and produces a greening colour of the meat, slime and various compounds that result from the fermentation of sugars and the degradation of nitrogen compounds. Finally, *Lactococcus lactis* *spp. lactis* and *Lactobacillus sakei* were able to reduce the risk of *Leuconostoc mesenteroides* spoilage.

**Keywords:** Cooked bacon, spoilage, bio-protective cultures.
1. Introduction

The tradition of Italian charcuterie has ancient origins and is commonly traced back to the Roman period. Forty-three of the 315 typical traditional Italian meat products consist of different types of bacon. The most popular type is cooked bacon. Bacon is derived from the central part of the cover fat from the half of the carcass that goes from the sternal region to the inguinal. The meat is worked in different ways (natural, cured, smoked, or cooked) and then stretched or rolled in shape. The production process is reported in Table 1. The brine is composed of water and various ingredients, such as salt, fructose, dextrose, spices, ascorbic acid (E 316) and nitrate (E 252), depending on the recipe and the concentration of the ingredients. Sucrose may be used, too. The shelf life is approximately 90 days.

After pasteurisation, the microorganisms that survived pasteurisation can grow and spoil the product during storage. The species involved in the spoilage of cooked meats are heterogeneous. *Brochothrix thermosphacta*, *Enterococcus faecalis*, *Lactobacillus sakei*, *Leuconostoc mesenteroides* subsp. *mesenteroides*, *Leuconostoc carnosum* and *Carnobacterium divergens* are widely known to negatively affect the flavour, texture, and colour of sliced and vacuum packed meat products (Bjorkroth et al. 1998; Metaxopoulos et al., 2002; Cantoni et al., 2008; Audenaert et al., 2010; Vasilopoulos et al., 2008, 2010a, 2010b; Comi et al., 2012). Moreover, the growth of heterofermentative bacteria can cause the packaging to swell or rupture due to the presence of CO₂. Bacterial activity is induced by the presence of the sugars that are added with the brine during tumbling, resting, or cooling for a long time. Lactic acid bacteria (LAB) and *B. thermosphacta*, which can come from the fresh meat, from the handling-related operations during processing or from the environment, are able to produce discoloration, greening and slimes (Bjorkroth et al., 1998; Audenaert et al., 2010, Vasilopoulos et al., 2010a,b; Comi et al., 2012). Improper cooking and sanitisation practices are frequent in craft cooked meat plants; consequently, the precautions are not effective in eliminating the handling-related post-contamination or the presence of...
thermotolerant bacteria (Franz and von Holy, 1996; Comi et al., 2012). Therefore, the use of various post-cooking or post-slicing technologies, such as high pressure, antimicrobial treatments, organic acids and protective cultures, for the bio-preservation of cooked meat products has been suggested (Metaxopoulos et al., 2002; Vermeiren et al., 2004).

The purpose of this study was to identify and characterise the spoilage-associated microorganisms in artisanal cooked bacon and to examine the use of bio-protective cultures to reduce the risk of Leuconostoc mesenteroides spoilage.

2. Materials and methods

2.1 Identification of microorganisms responsible for the spoilage

2.1.1. Samples and sampling procedures.
Two hundred cooked bacon of an artisan meat cooking plant were prepared following the traditional procedures of the plant (the ingredients and concentrations are subject to confidentiality). After cooking, the products were cut into 200 pieces (100 g each), packaged under vacuum, pasteurised at 85 °C for 15 minutes and stored at 4 ± 2 °C. The packaging film was Combiflex PE/PA 2010 (60/150) (Niederwieser, S.p.a., Italy). The shelf-life of the products is 90 days, but just at 30 days, 40 (20 % of total) cooked bacon were spoiled, showing greening parts, slime and slightly inflated packages. At 30th day, 10 out of 40 spoiled and 10 out of 160 unspoiled cooked bacon samples were randomly collected and analysed. At 90 days 10 spoiled and 10 unspoiled were also analysed. The collected samples were subjected to visual and olfactory inspection and to microbiological and physico-chemical analyses. The volatile compounds of the slightly inflated packages were also studied. The rest of the products (150 samples) remained unspoiled till the end of the shelf-life.

2.1.2. Microbiological analysis
Ten grams of each sample was tenfold diluted in bacteriological peptone water (0.1 g/L peptone; 7
g/L NaCl) and homogenised in a Stomacher (P.B.I: International, Italy). Further decimal dilutions were made in the same solution, and the following microbiological analyses were performed in triplicate on agar plates: the total viable count (TVC) on Plate Count Agar (PCA, Oxoid, Italy) incubated at 30 °C for 48 hours (ISO 6887), LAB on de Man-Rogosa-Sharpe (MRS) agar (pH 6.2; Oxoid, Italy) at 30 °C for 48-72 h (ISO 15214) in a microaerophilic environment (gas pack anaerobic system, BBL, Becton Dickinson, USA), Clostridia on Differential Reinforced Clostridia Medium (DRCM) (VWR, USA) that was incubated at 37°C for 24-48 h in an anaerobic jar with an anaerobic kit (gas pack anaerobic system, BBL, Becton Dickinson, USA), Salmonella spp. according to the ISO 6579 method, briefly: (1) a pre-enrichment in buffered peptone water for 18 h at 37 °C; (2) a selective enrichment of 0.1 mL of pre-enriched culture in 10 mL on Rappaport- Vassiliadis Soy (RVS) broth for 24 h at 42 °C and of 1 mL of pre-enriched culture in 10 mL of Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn) broth for 24 h at 37 °C; (3) selective isolation by streaking 10 μL of RVS and 10 μL of MKTTn on plates of XLD agar and brilliant green agar for 18-24 h at 37 °C; Confirmation of any presumptive colony was performed by Salmonella Latex agglutination test (Oxoid, Italy); and Listeria monocytogenes according to the ISO 11290 method, briefly: (1) a primary selective enrichment in 225 mL of Half Fraser Broth for 24 h at 30 °C; (2) a secondary selective enrichment of 0.1 mL of primary enriched culture in 10 mL of Fraser Broth for 48 h at 37 °C; (3) a selective isolation obtained by streaking 10 μL of the secondary enriched culture on Listeria Selective Agar according to Ottaviani and Agosti (ALOA) for 48 h at 37 °C.

Sixty (60) colonies (30 from the spoiled and 30 from the unspoiled samples) grown on MRS agar were randomly collected. Briefly, from one agar plate containing between 30 and 300 colonies of each samples, 3 colonies were isolated. The colonies were selected independently of morphology, colour or size. The isolates were streaked on MRS agar and then stored at -80 °C in MRS broth supplemented with glycerol (30% final concentration, Sigma-Aldrich, Germany). The isolates were
subjected to Gram staining and a catalase test and were then identified according to the molecular
method (PCR-DGGE and sequencing) reported by Iacumin et al., (2009). In particular, the DNA
was amplified with primers P1V1GC (GC-GCGGCGTGCCTAATACATGC) and P2V1
(TTCCCCACGCGTTACTCACC) (Cocolin et al., 2001; Rantsiou et al., 2005), the PCR products
were run in DGGE, and the isolates were grouped according to the migration profile. Where
possible, at least three isolates for each group were subjected to sequencing for identification
purposes. A culture-independent approach was also used to identify the strains found in both the
spoiled and unspoiled cooked bacon: 10 mL of the homogenised sample in bacteriological peptone
water was centrifuged at 10000xg, and the pellet was then subjected to total DNA extraction, PCR-
DGGE, cloning of the DGGE bands and sequencing, following the protocol used by Iacumin et al.,
(2009). The 60 colonies were then evaluated at strain level in order to choose the three strains to use
as cultures for the challenge test against starter cultures. The test divided them in three main
genotipic groups, also based on high, medium and low fermentation speed (data not shown). The
isolates of unspoiled bacons had low fermentation speed and growth and this can explain the
reduced concentration at the end of shelf-life of the products and consequently they can not spoil
them.

2.1.3. Physico-chemical analysis and Total Volatile compounds determination

The potentiometric measurement of pH was made using a pH meter (Radiometer, Copenhagen,
Denmark). The volatile compounds in 10 spoiled and 10 unspoiled cooked bacon samples, all
packaged under vacuum, were analysed at the end of their shelf-life (90 days). The volatile
compounds were determined by SPME-GC-MS on Finnigan Trace DSQ (Thermo Scientific
Corporation, USA) with a Rtx-Wax capillary column (length 30 m x 0.25 mm id.; film thickness
0.25 µm; Restek Corporation, USA) according to the method reported in Chiesa et al., (2006). The
volatiles compounds were then identified by comparison of their mass spectra and the retention
times with those of standard compounds, or by comparison of the mass spectrum with those
of the mass spectrum Wiley library (Wiley library 10 vers.) and the self-made library. The results represent the average of all 10 samples analysed in triplicate.

2.1.4. Statistical analysis

The microbial concentrations of the spoiled and the unspoiled cooked bacon were compared using one-way analysis of variance. The means were separated by Tukey’s honest significant difference test using the StatGraphics software package from Statistical Graphics (Rockville, Maryland).

2.2. Evaluation of starter cultures to eliminate microorganisms responsible for spoilage

2.2.1 Sample preparation, storage conditions and sampling methods for the trials using bio-protective starter culture

New pieces of cooked bacon were prepared and after chilling were inoculated with the starter cultures. The pasteurized phase was eliminated. In particular the pieces were divided into 3 lots of 10 pieces (50 g each). Lot 1 was directly inoculated with a suspension of *Leuc. mesenteroides* at a final concentration of 3 log CFU/g and used as a control. Lot 2 was inoculated with a mixture of *Leuconostoc mesenteroides/Lactococcus lactis* subsp. *lactis* (ratio 1/1) at a final concentration of 3 log CFU/g. Lot 3 was inoculated with *Leuc. mesenteroides/L. sakei* at a ratio of 1/1 and a final concentration of 3 log CFU/g. Each inocula were carried out spreading 0.1 mL of the bacterial suspension (about 6.3 log CFU/ml) on the bacons surface. After inoculation, the bacon pieces were packed under vacuum using the packaging film Combiflex PE/PA 2010 (60/150) (Niederwieser S.p.a., Italy) and stored at 4 ± 2 ºC for 90 days, at the end of their commercial shelf-life. At the end of storage, the presence of greening and of slime was evaluated, and the pH was measured.

*L. sakei* (B-2 Safe Pro® – CHR HANSEN) and *Lc. lactis* subsp. *lactis* (Rubis – CHR HANSEN) were obtained from the Italy branch supplier of Chr. Hansen, Denmark. The lyophilised cultures were resuspended in peptone water [0.1% sodium chloride and 0.7% peptone (Oxoid, England)] and left for 1 h at room temperature for complete rehydration. Appropriate dilutions were made, and 0.1
mL of each dilution was plated on MRS agar (de Man-Rogosa-Sharpe agar, pH 6.2, Oxoid, Italy) and incubated at 30 °C for 48-72 h in a microaerophilic environment (gas pack anaerobic system, BBL, Becton Dickinson, USA) to measure the concentration. Appropriate suspensions of the strains were mixed with *Leuc. mesenteroides* and directly inoculated on pieces of bacon, and the final bacterial cell concentration was approximately 3 log CFU/g.

The inoculum of *Leuc. mesenteroides* was represented by the a mix of three different strains isolated from spoiled cooked bacon and responsible for the greening color and spoilage. The three strains were chosen among the three genotypic groups. One of them had fast, one medium and one low fermentation speed. Despite different times of growth, the strains selected were able to produce the spoilage.

These microorganisms were grown on MRS agar, and a suspension was made for inoculation as described above. The inoculated samples (Lot 1,2,3) were analysed according to the microbial analysis above reported.

The bacterial population, using all the MRS plates from –3 to –7 dilutions was suspended in Phosphate Buffered Saline (one tablet Phosphate Buffered Saline, Sigma-Aldrich P4417-100TAB, St. Louis, USA, dissolved in 200 mL H₂O) and used for Bulk formation as previously described by Iacumin et al., (2011). One hundred μL of the bulk was used for DNA extraction and subjected to PCR-DGGE as above reported.

### 2.2.2 Sensory analysis

To evaluate the influence of bioprotective culture on the organoleptic characteristic of the product, a sensory analysis was performed using the triangle test methodology (ISO 4120:2004).

New pieces of cooked bacon, prepared following the traditional procedure of the plant, were divided into 3 lots. The lot A (10 pieces of 50 g each) was inoculated with a suspension of *L. sakei* and Lot B (10 pieces of 50 g each) by *Lc. lactis* subsp. *lactis*, both the strains were obtained from
the Italy branch supplier of Chr. Hansen, Denmark. The final concentration was for both 3 log

CFU/g. Each inocula were carried out spreading 0.1 mL of the bacterial suspension (about 6.3 log

CFU/ml) on the bacons surface. Lot C (10 pieces of 50 g each) was the control obtained with post-
pasterurization and without adding bioprotective starter cultures. All the bacon pieces were packed

under vacuum using the packaging film Combiflex PE/PA 2010 (60/150) (Niederwieser, S.p.a.,

Italy), and stored at 4 ± 2 ºC.

All the bacon pieces were packed under vacuum using the packaging film Combiflex PE/PA 2010

(60/150) (Niederwieser, S.p.a., Italy), and stored at 4 ± 2 ºC. Ten days before the end of their

commercial shelf-life, all the lots were used for the triangle test.

The triangle test was used to compare the lot A and B to Lot C. Twenty non-professional assessors

were presented with three products, two of which were identical. The assessors were asked to state

which product they believed was the odd one out. The assessors who indicated there were two

distinct samples were asked to identify the best one.

3.0 Results and discussion

3.1 Microbial analysis of spoiled and unspoiled cooked bacon

The concentration of LAB in the spoiled product was significantly different from the concentration

in the unspoiled product (p < 0.05). In particular it was 8.7 ± 0.1 CFU/g in spoiled and 4.0 ± 0.2

CFU/g in unspoiled. Similarly, a significant difference (p < 0.05) was observed in the TVC

concentration between the spoiled and unspoiled product. The spoiled samples presented a TVC

concentration of 6.3 ± 0.3 CFU/g, whereas the unspoiled product presented a TVC concentration of

3.0 ± 0.5 CFU/g. The colonies growth on TVC were presumptively identified as LAB, by GRAM

stain and catalase tests. The difference between the TVC and LAB concentration depends on the

media used. Plate Count Agar used for TVC count tends to limit the growth of LAB and for this

reason LAB concentration valued in MRS was higher than the one valued on PCA.
The data of the spoiled bacon show that LAB can develop in vacuum-packed meat products that are stored at 4-8 °C and cause spoilage (Comi and Iacumin, 2012). The differences observed in the levels of TVC and LAB are reflected by significant differences in the pH (p < 0.05). The pH values of the unspoiled products were approximately 5.6 ± 0.1, and the spoiled products showed pH values of approximately 5.3 ± 0.1. Moreover, a green colour, a slime and a slight inflation of the packaging was observed in spoiled products. Diez et al., (2013) in a previous study demonstrated that L. mesenteroides grew rapidly and influenced the drop in pH and produced milky exudates (slime) in vacuum-packaged morcilla de Burgos a traditional blood sausages from Spain, during cold storage. In leuconostocs the pathways/genes associated with many types of spoilage reactions, such as meat greening, gas and slime production, and pH changing are well studied and known (Jääskeläinen et al., 2013).

At 90 days (end of the shelf-life) the LAB concentration of the unspoiled bacons did not change. It was at level of 4.1 ± 0.5 CFU/g and quite similar than the one at 30 days (p < 0.05). At 90 days also the LAB concentration of the spoiled bacons did not deeply change, being at level of 8.5 ± 0.5 CFU/g (p < 0.05). Similar results were obtained by the TVC counts. At this time the pH of the unspoiled bacons remained quite unchanged, conversely in the spoiled was reduced at level of 5.1 ± 0.2. In these samples the greening colour changed to dark green and the slime persisted. These data demonstrated that in the unspoiled bacons no spoilage was observed because LAB did not growth.

3.2 Microbial identification

All the 60 LAB isolates were Gram positive and catalase negative. The isolates were also subjected to molecular identification, and only one species was found: Leuc. mesenteroides (Accession Number KC545920.1). The direct identification of the bacteria by a culture independent method (PCR-DGGE and sequencing of the DGGE band) confirmed the traditional identification of the isolates. The DGGE profile of the sample treated using the culture independent method was
composed by one only band on the gel, which corresponded to *Leuc. mesenteroides*. Leuconostocs and *L. sakei* are considered to be the bacteria responsible for the spoilage of the cooked meat product stored at 4 °C (Samelis et al., 2000, Metaxopoulos et al., 2002; Cantoni et al., 2008; Vasilopoulos et al., 2010; Comi et al., 2012; Comi and Iacumin, 2012). In this study, the spoiled bacon was contaminated with up to 8 log CFU/g LAB, almost 4 log CFU/g more than the unspoiled cooked bacon (p < 0.05). The levels of contamination in spoiled and unspoiled cooked bacon were similar to those present in vacuum and Modified Atmosphere Packaged (MAP) cooked meats from other countries (Nielsen et al., 1983; Samelis et al., 2000). Samelis et al., (2000) found a concentration of LAB up to 7 log CFU/g in sliced cooked ham packaged under vacuum after 15-30 days of storage at 4 °C. In our study, *Leuc. mesenteroides* was the only species present in the unspoiled cooked bacon at a concentration level of 4 log CFU/g. Our data are not in agreement with the ones of other authors (Bjorkroth et al., 1998; Metaxopoulos et al., 2002; Cantoni et al., 2008; Audenaert et al., 2010; Vasilopoulos et al., 2008, 2010a, 2010b; Comi et al., 2012), that found different LAB. Probably *Leuc. mesenteroides* was the predominant species that was selected in that plant and consequently was the main responsible of the spoilage. However, *Leuconostoc* were highly concentrated in the spoiled cooked bacon. Our data demonstrate that *Leuconostocs* were more profuse because these bacteria grew faster than other LAB at 4 °C, as demonstrated by Comi and Iacumin (2012). It is well-known that in cooked meat products packaged under vacuum, *Leuconostocs*, as well as some others LAB species, are the most prevalent because of the combined effects of pH, Aw (water activity), salinity of the brine and smoke (Samelis et al., 2000; Comi and Iacumin, 2012). During the spoilage, *Leuconostocs* produce lactic acid, acetic acid, ethanol, CO₂ and various other compounds that can lead to off-flavours (Cantoni et al., 2008). *Leuc. mesenteroides* produced hydrogen peroxide in vitro. This compound is responsible for the greening in meat products because hydrogen peroxide oxidises myoglobin to cholemoglobin (Collins et al., 1993; Comi and Iacumin, 2012; Jääskeläinen et al., 2013). In the spoiled bacon samples considered in the present study, greening areas were observed by visual analysis.
Three main genotypic groups were observed after the identification of the *Leuc. mesenteroides* at strain level. One group isolated from unspoiled bacon had low fermentation speed and growth at 4°C. Conversely one group had high and one medium fermentation and growth speed. The group with higher fermentation and growth speed was isolated from the spoiled bacon. The group with a medium fermentation and growth speed belonged to either unspoiled or spoiled bacon (data not shown). It was assumed that the *Leuconostoc* strain of the spoiled bacon had a greater vitality than the unspoiled one, consequently they were able to produce the spoilage within 90 days of storage. It was assumed that the strains of the unspoiled bacon were less effective and consequently were not able to spoil the bacon within the end of their shelf-life. The lower activity was demonstrated by the concentration reached at 90 days that did not change in respect to 30 days in unspoiled bacon.

*Listeria monocytogenes* spp. and *Salmonella* spp. were not detected in any of the tested samples, Clostridia were not detected (less than 10 CFU/g).

3.3 Analysis of the volatile compounds

The analyses of the volatile fractions from the spoiled and unspoiled cooked bacon packaged under vacuum are presented in Table 2. This table shows the mean retention times, the individual compounds and the concentrations of these compounds expressed in µg/kg product from ten analytical runs. The data suggest that the differences observed between the levels of volatile compounds in the spoiled and unspoiled bacon were due to *Leuconostoc* activity. In particular, we found an abundance of heterolactic fermentation products, such as acetic acid, ethanol other carboxylic acids and ketones; aldehydes were not identified because these compounds were most likely transformed into carboxylic acids. Alcohols were mainly present in the spoiled cooked bacon. The analysis was performed on both spoiled and unspoiled cooked bacon after 30 days of storage, because the greening and the slime presence in spoiled appeared after 30 days of storage. To better interpret the results obtained from the analysis of the headspace, the 23 identified substances were split into 5 classes: ketones (4), carboxylic acids (5), alcohols (9), terpenes (2), and miscellanea (3).
According to our data, *Leuc. mesenteroides* produce various compounds by fermenting sugars and metabolised amino acids. The concentrations of some volatile compounds were higher in the spoiled bacon than in the unspoiled bacons. Several of the compounds were ascribed to the glucose and amino acid metabolism of the LAB, to the oxidation and auto-oxidation of lipids (Montel et al., 1998) and to the endogenous reactions that occur during cooking (Mottram, 1998). The five identified acids are all typical metabolites from LAB, coliforms and *E.coli* fermentation of sugars and the degradation of amino acids (Leroy et al., 2009). The presence of certain organic acids, including acetic, propanoic, 2-Methyl-propanoic, 3-Methyl-butanoic and Hexanoic acid, is due to the degrading activity of leuconostoc (Comi and Iacumin, 2012; Diez et al., 2009). *Leuc. mesenteroides* can increase the concentration of acetic acid and aldehydes in meat product and in blood sausage (Diez et al., 2009). Our data confirm the increasing of the acetic acid concentration but do not show the presence of aldehydes. It could be concluded that the aldehydes were not present because these compounds were most likely transformed into carboxylic acids by *Leuc. mesenteroides*, such as hexanal trasformed in hexanoic acid. It is also possible that the acids with more than three carbon atoms can be derived by the lipolytic enzymes in the leuconostocs and in the meat or by lipid oxidation (Chiesa et al., 2006; Comi et al., 2000). Because the brine injected into the meat before production contained up to 0.5% sugars (w/v), we assumed that the organic acids found in the bacon were produced by LAB fermentation. Ethanol was the main alcohol produced and was mainly present in spoiled product. The total concentration of alcohol in the spoiled product was higher than that in the unspoiled product. Leuconostocs most likely produced fewer ketones and carboxylic acids than alcohols. Alcohols can be derived from sugar fermentation (Kandler, 1983; Diez et al., 2009), from aldheydes, ketones or from amino acid (leucine, valine and phenylalanine) catabolism and all these activities are typical of *W. viridescens* and leuconostocs (Comi et al., 2014a,b,2011; Deetae et al., 2007; Smit et al., 2005; Bedaguê et al., 1993). In particular, the higher alcohol levels found in the spoiled bacon were produced by the *Leuc. mesenteroides* conversion of aldehydes, ketones and amino acids. In addition, the higher alcohol
amounts can be also demonstrated by the higher level of *Leuc. mesenteroides* concentration found in spoiled products, considering that the strains isolated from them had a growth and fermentation speed faster than the strains isolated in unspoiled product. The increase of alcohol in spoiled bacon is associated with the increase of the leuconostoc concentrations (heterofermentative microorganisms). In meat products, the aldehyde levels usually increase with the fermentative activity of a starter consisting of LAB and Coagulase Negative Catalase Positive Cocci or after degradation through the Strecker reaction (Comi et al., 2000). Unlike the results obtained by other authors (Comi et al., 2000; Tjener et al., 2003), no aldehydes were identified in our study. This result can be explained by the degradation and reduction of these compounds by leuconostocs, which converted them in alcohols (Bedarguè et al., 1993; Comi and Iacumin, 2012; Comi et al., 2014a,b).

The identified ketones were primarily derived from the oxidation of fatty acids (2-propanone, 2-butanone, 3-hydroxy-2-butanone, 2-pentanone) and by LAB activities (Berdague et al., 1993; Jääskeläinen et al., 2013). Unlike the ketones identified in other studies (Comi et al., 2000; Tjener et al., 2003, Comi and Iacumin, 2012), diacetyl (2,3-butanedione) was not found in our study. This was probably due on the absence of oxygen because the cooked bacon was under vacuum packaged. Jääskeläinen et al., (2013) found that in *Leuc. gasicomitatum* the diacetyl production is minimal in meat packaged under anaerobic condition and on the contrary the respiration enormously increased the accumulation of acetoin and diacetyl under a high-oxygen modified atmosphere. It is also possible that diacetyl may have been reduced to 3-hydroxy-butanone (acetoin), which was found in our study. The absence of diacetyl did not allow to impart a buttery aroma and flavor to spoiled cooked bacon. However, considering the microbial population identified in spoiled cooked hams, it is also possible that the ketones were derived from the degradation of alkanes (Montel et al., 1998).

Three out of 4 ketones detected were found in higher concentration in unspoiled than in spoiled. This was explained by a greater activity of the leuconostocs in spoiled bacon that consequently produced higher amount of alcohol and less ketones (Bedarguè et al., 1993; Comi et al., 2014a,b).
Among the aromatic hydrocarbons (terpenes), two compounds were identified both in the unspoiled and spoiled cooked bacon. These compounds are typically found in raw materials and most likely originated from various contaminations in the animal feedstuffs and spices (nutmeg, black pepper) because these compounds can be found in plants (Van Straten, 1977; Comi et al., 2000) that are eaten by animals. For these reasons, a large difference was not observed between the terpene concentrations of the spoiled and unspoiled cooked bacon.

3.4 Challenge test

Table 3 shows the results obtained using the bioprotective culture: their use can eliminate the spoilage produced by leuconostocs. In fact, cooked bacon with bio-protective cultures did not present greening, slime or inflated packaging at the end of the shelf life. In contrast, a sticky-white slime and a greening colour were observed in the cooked bacon inoculated only with *Leuc. mesenteroides*. The bio-protective cultures grew during the storage of the products, and at the end of the shelf-life, the ratio *Leuc. mesenteroides*/*Lc. lactis* subsp. *lactis* was about 1/100. In fact the DNA of *Leuc. mesenteroides* was retrieved by PCR-DGGE at a sample decimal dilution of $10^{-3}$, whereas DNA of *Lc. lactis* subsp. *lactis* at a decimal dilution of $10^{-5}$ (Table 4). On the other hand, at the end of the shelf-life, the ratio of *Leuc. mesenteroides*/*L. sakei* was about 1/1000. In fact the DNA of *Leuc. mesenteroides* was retrieved by PCR-DGGE at a decimal dilution of $10^{-3}$, whereas the DNA of *L. sakei* at a dilution level of $10^{-6}$ (Table 4). On the basis of the obtained results, can be assumed that the bioprotective cultures, competing for the substrate with *Leuc. mesenteroides*, inhibited their growth. This assumption is also confirmed by the results obtained for the control sample (Lot 1), where the DNA of *Leuc. mesenteroides* was identified at a dilution level of $10^{-6}$ (Table 4). *Leuc. mesenteroides* concentration in Lot 1 was $6.0 \pm 0.3$ UFC/g. Consequently, the growth of the bio-protective cultures eliminated the spoilage caused by leuconostocs, and the products resulted acceptable at the end of the shelf life (90 days).
The use of LAB as bio-protective cultures to prolong the shelf life of the meat products, including cooked meat product, is a new concept that has been suggested by many authors (Metaxopoulos et al., 2002; Vermeiren et al., 2004; Comi et al., 2011). Kotzekidou and Bloukas (1996) noticed that cooked ham supplemented with protective cultures had lower total aerobic bacteria, micrococci, staphylococci and *B. thermosphacta* counts than control hams, which had higher populations of LAB and lower populations of pseudomonads. Additionally, Metaxopoulos et al., (2002) and Vermeiren et al., (2006) concluded that LAB affected the spoilage microflora growth and did not negatively affect the organoleptic properties of the products. Comi et al., (2012) demonstrated that starter cultures (*L. sakei, L. curvatus* and *Lc. lactis* subsp. *lactis*) extend the shelf life of cooked ham slices packed in MAP. The microorganisms influenced the flavour, odour and stability of the colour of the sliced cooked ham.

### 3.5 Sensorial analysis

The acceptability of the bacon added with starter cultures was confirmed with the triangular test by the panel composed by 20 not-professional assessors. They established that did not exist any difference between Lot A and B (with bio-protective culture): the slice resulted compact and homogeneous; the lean part was a ruby red color and the color of the fat was white, typical of the product; the consistency was compact but not elastic; the bouquet was delicate and distinctive; taste was sweet and delicate and there was no perception of spices or flavors; acidity was not perceived. On the other hand, Lot C was indicated as different by the 100% of the assessors, who stressed as follows: the slice resulted non compact and the presence of irregular holes was observed; the lean part was a ruby red color with the presence of green coloring and slime; the color of the fat was white, typical of the product; the consistency was compact but not uniform; the bouquet was altered and not distinctive of the product.

### 4. Conclusion
Data demonstrated that bio-protective cultures inhibited *Leuc. mesenteroides* growth and eliminated the greening colour of the meat, the slime, the package inflation, the off-flavours and the off-odours. Therefore, the bio-protective cultures evaluated in this study can improve the shelf life and eliminate the growth of spoilage microorganisms. In particular this is the first time that a *Lc. lactis* subsp. *lactis* is used in order to improve the shelf-life of cooked meat as cooked bacon.

**Conflict of interest**

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

**References**


Table 1: Process steps of traditional cooked bacon production

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<tr>
<th>Step</th>
<th>Temperature/time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw material</td>
<td>4 ± 2 °C</td>
</tr>
<tr>
<td>Trimming, Squaring</td>
<td>12 °C</td>
</tr>
<tr>
<td>Brine injection</td>
<td>7 °C</td>
</tr>
<tr>
<td>Churning</td>
<td>7 °C – 8 hours</td>
</tr>
<tr>
<td>Resting</td>
<td>2-4 °C – 7 days</td>
</tr>
<tr>
<td>Drying</td>
<td>44-55 °C – 1 hour</td>
</tr>
<tr>
<td>Smoking</td>
<td>66 °C – 24 hours</td>
</tr>
<tr>
<td>Cooking</td>
<td>72 – 78 °C</td>
</tr>
<tr>
<td>Chilling</td>
<td>2-4 °C</td>
</tr>
<tr>
<td>Packaging</td>
<td>4 °C</td>
</tr>
<tr>
<td>Pasteurization</td>
<td>85 °C – 15 minutes</td>
</tr>
<tr>
<td>Storing</td>
<td>4 ± 2 °C</td>
</tr>
</tbody>
</table>
### Table 2: Volatile compounds in unspoiled and spoiled bacon

<table>
<thead>
<tr>
<th>RT</th>
<th>Compounds</th>
<th>Unspoiled Mean (± SD)</th>
<th>Spoiled Mean (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ketones</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.29</td>
<td>2-Propanone</td>
<td>2.51 (0.02a)</td>
<td>1.65 (0.08b)</td>
</tr>
<tr>
<td>3.17</td>
<td>2-Butanone</td>
<td>6.04 (0.03b)</td>
<td>9.33 (0.01a)</td>
</tr>
<tr>
<td>4.72</td>
<td>2-Bentanone</td>
<td>8.45 (0.07a)</td>
<td>0.28 (0.01b)</td>
</tr>
<tr>
<td>17.63</td>
<td>3-Hydroxy-2-butanone</td>
<td>37.35 (0.64a)</td>
<td>5.80 (0.19b)</td>
</tr>
<tr>
<td></td>
<td>Sum</td>
<td>54.35 (</td>
<td>17.06</td>
</tr>
<tr>
<td></td>
<td>Alcohols</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.87</td>
<td>Ethanol</td>
<td>16.90 (0.08b)</td>
<td>26.11 (0.02a)</td>
</tr>
<tr>
<td>6.64</td>
<td>2-Butanol</td>
<td>1.28 (0.04b)</td>
<td>5.82 (0.26a)</td>
</tr>
<tr>
<td>7.28</td>
<td>1-Propanol</td>
<td>4.54 (0.12b)</td>
<td>28.18 (0.71a)</td>
</tr>
<tr>
<td>10.22</td>
<td>2-Methyl-1-propanol</td>
<td>0.75 (0.30b)</td>
<td>2.73 (0.26a)</td>
</tr>
<tr>
<td>11.1</td>
<td>2-Propen-1-ol</td>
<td>0.04 (0.01b)</td>
<td>0.30 (0.03a)</td>
</tr>
<tr>
<td>11.6</td>
<td>2-Pentanol</td>
<td>0.17 (0.02b)</td>
<td>0.63 (0.11a)</td>
</tr>
<tr>
<td>15.42</td>
<td>3-Methyl-1-butanol</td>
<td>4.83 (0.14b)</td>
<td>8.36 (0.02a)</td>
</tr>
<tr>
<td>16.76</td>
<td>3-Methyl-3-buten-1-ol</td>
<td>3.07 (0.04a)</td>
<td>0.15 (0.02b)</td>
</tr>
<tr>
<td>25.17</td>
<td>2,3-Butanediol</td>
<td>3.18 (0.04a)</td>
<td>0.12 (0.01b)</td>
</tr>
<tr>
<td></td>
<td>Sum</td>
<td>28.76 (</td>
<td>72.39</td>
</tr>
<tr>
<td></td>
<td>Terpenes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.67</td>
<td>α-Pinene</td>
<td>0.24 (0.01a)</td>
<td>0.28 (0.12a)</td>
</tr>
<tr>
<td>12.06</td>
<td>δ-3-Carene</td>
<td>0.04 (0.02a)</td>
<td>0.17 (0.03a)</td>
</tr>
<tr>
<td></td>
<td>Sum</td>
<td>0.28 (</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Carboxylic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22.23</td>
<td>Acetic acid</td>
<td>5.48 (0.11b)</td>
<td>7.51 (0.21a)</td>
</tr>
<tr>
<td>24.28</td>
<td>Propanoic acid</td>
<td>0.49 (0.10b)</td>
<td>0.17 (0.02a)</td>
</tr>
<tr>
<td>24.95</td>
<td>2-Methyl-propanoic acid</td>
<td>0.18 (0.00a)</td>
<td>0.08 (0.00b)</td>
</tr>
<tr>
<td>27.04</td>
<td>3-Methyl-butanolic acid</td>
<td>1.88 (0.07a)</td>
<td>1.04 (0.02b)</td>
</tr>
<tr>
<td>30.34</td>
<td>Hexanoic acid</td>
<td>0.09 (0.04b)</td>
<td>0.16 (0.02a)</td>
</tr>
<tr>
<td></td>
<td>Sum</td>
<td>8.11 (</td>
<td>8.97</td>
</tr>
<tr>
<td></td>
<td>Miscellany</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.05</td>
<td>Acetic acid ethyl-ester</td>
<td>2.26 (0.04a)</td>
<td>0.88 (0.07b)</td>
</tr>
<tr>
<td>25.98</td>
<td>Butirolacetone</td>
<td>0.14 (0.03a)</td>
<td>0.12 (0.01a)</td>
</tr>
<tr>
<td>31.12</td>
<td>Dimethylsulfone</td>
<td>0.02 (0.01a)</td>
<td>0.03 (0.01a)</td>
</tr>
<tr>
<td></td>
<td>Sum</td>
<td>2.42 (</td>
<td>1.02</td>
</tr>
</tbody>
</table>

Legend: Mean (mean of 10 samples analysed in triplicate) expressed in µg/kg; Sum of compounds; RT: Retention time. SD: Standard deviation. Data represent the means ± standard deviations of all the samples; Mean with the same letters within a row (following the values) are not significantly differently (P< 0.05).
**Table 3:** Physico-chemical results of the bio-protective effect of the LAB starter used, versus *Leuconostoc mesenteroides*

<table>
<thead>
<tr>
<th>Lot</th>
<th>Microorganisms</th>
<th>Greening</th>
<th>Slime</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Leuc. mesenteroides</em></td>
<td>+</td>
<td>+</td>
<td>5.3 ± 0.2* a</td>
</tr>
<tr>
<td>2</td>
<td><em>Leuc. mesenteroides vs Lc. lactis</em></td>
<td>-</td>
<td>-</td>
<td>5.4 ± 0.1* a</td>
</tr>
<tr>
<td>3</td>
<td><em>Leuc. mesenteroides vs L. sakei</em></td>
<td>-</td>
<td>-</td>
<td>5.4 ± 0.2* a</td>
</tr>
</tbody>
</table>

Legend: Mean (mean of 10 samples analysed in triplicate) expressed in µg/kg; Sum of compounds; RT: Retention time. SD: Standard deviation. Data represent the means ± standard deviations of all the samples; Mean with the same letters within a row (following the values) are not significantly differently (P< 0.05)
Table 4: Plate dilution at which the identified species were detected

<table>
<thead>
<tr>
<th>Lot</th>
<th>Microorganism inoculated</th>
<th>Serial decimal dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10^-3</td>
</tr>
<tr>
<td>1</td>
<td><em>Leuc. mesenteroides</em></td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td><em>Leuc. mesenteroides</em></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>Lc. lactis</em></td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td><em>Leuc. mesenteroides</em></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td><em>L. sakei</em></td>
<td>+</td>
</tr>
</tbody>
</table>