



UNIVERSITÀ  
DEGLI STUDI  
DI UDINE

## Università degli studi di Udine

Lactococcus lactis and Lactobacillus sakei as bio-protective culture to eliminate Leuconostoc mesenteroides spoilage and improve the shelf life and

*Original*

*Availability:*

This version is available <http://hdl.handle.net/11390/1085982> since 2020-02-27T20:40:13Z

*Publisher:*

*Published*

DOI:10.1016/j.fm.2016.03.001

*Terms of use:*

The institutional repository of the University of Udine (<http://air.uniud.it>) is provided by ARIC services. The aim is to enable open access to all the world.

*Publisher copyright*

(Article begins on next page)

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

4  
5 Giuseppe Comi, Debbie Andyanto, Marisa Manzano and Lucilla Iacumin\*

6  
7 Department of Food Science, University of Udine, Via Sondrio 2/A, 33100 Udine, Italy.

8  
9 **Running headline: Quality improvement of cooked bacon.**

10  
11 \*Correponding author:

12 Lucilla Iacumin, PhD

13 Dipartimento di Scienze degli Alimenti, Università degli Studi di Udine

14 Via Sondrio 2/A, 33100 Udine, Italy

15 e-mail: [lucilla.iacumin@uniud.it](mailto:lucilla.iacumin@uniud.it);

16 Phone: +39 0432 558126;

17 Fax. +39 0432 558130.

18

19

20

21

22

23

24

25

26

27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52

**Abstract**

Cooked bacon is a typical Italian meat product. After production, cooked bacon is stored at  $4 \pm 2$  °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.

## 53 1. Introduction

54

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

64 After pasteurisation, the microorganisms that survived pasteurisation can grow and spoil the  
65 product during storage. The species involved in the spoilage of cooked meats are heterogeneous.  
66 *Brochothrix thermosphacta*, *Enterococcus faecalis*, *Lactobacillus sakei*, *Leuconostoc mesenteroides*  
67 subsp. *mesenteroides*, *Leuconostoc carnosum* and *Carnobacterium divergens* are widely known to  
68 negatively affect the flavour, texture, and colour of sliced and vacuum packed meat products  
69 (Bjorkroth et al. 1998; Metaxopoulos et al., 2002; Cantoni et al., 2008; Audenaert et al., 2010;  
70 Vasilopoulos et al., 2008, 2010a, 2010b; Comi et al., 2012). Moreover, the growth of  
71 heterofermentative bacteria can cause the packaging to swell or rupture due to the presence of CO<sub>2</sub>.  
72 Bacterial activity is induced by the presence of the sugars that are added with the brine during  
73 tumbling, resting, or cooling for a long time. Lactic acid bacteria (LAB) and *B. thermosphacta*,  
74 which can come from the fresh meat, from the handling-related operations during processing or  
75 from the environment, are able to produce discoloration, greening and slimes (Bjorkroth et al.,  
76 1998; Audenaert et al., 2010, Vasilopoulos et al., 2010a,b; Comi et al., 2012). Improper cooking  
77 and sanitisation practices are frequent in craft cooked meat plants; consequently, the precautions are  
78 not effective in eliminating the handling-related post-contamination or the presence of

79 thermotolerant bacteria (Franz and von Holy,1996; Comi et al., 2012). Therefore, the use of various  
80 post-cooking or post-slicing technologies, such as high pressure, antimicrobial treatments, organic  
81 acids and protective cultures, for the bio-preservation of cooked meat products has been suggested  
82 (Metaxopoulos et al., 2002; Vermeiren et al., 2004).

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

86

87

## 88 **2. Materials and methods**

### 89 *2.1 Identification of microorganisms responsible for the spoilage*

#### 90 *2.1.1. Samples and sampling procedures.*

91 Two hundred cooked bacon of an artisan meat cooking plant were prepared following the traditional  
92 procedures of the plant (the ingredients and concentrations are subject to confidentiality). After  
93 cooking, the products were cut into 200 pieces (100 g each), packaged under vacuum, pasteurised at  
94 85 °C for 15 minutes and stored at  $4 \pm 2$  °C. The packaging film was Combiflex PE/PA 2010  
95 (60/150) (Niederwieser, S.p.a., Italy). The shelf-life of the products is 90 days, but just at 30 days,  
96 40 (20 % of total) cooked bacon were spoiled, showing greening parts, slime and slightly inflated  
97 packages. At 30<sup>th</sup> day, 10 out of 40 spoiled and 10 out of 160 unspoiled cooked bacon samples were  
98 randomly collected and analysed. At 90 days 10 spoiled and 10 unspoiled were also analysed. The  
99 collected samples were subjected to visual and olfactory inspection and to microbiological and  
100 physico-chemical analyses. The volatile compounds of the slightly inflated packages were also  
101 studied. The rest of the products (150 samples) remained unspoiled till the end of the shelf-life.

102

#### 103 *2.1.2. Microbiological analysis*

104 Ten grams of each sample was tenfold diluted in bacteriological peptone water (0.1 g/L peptone; 7

105 g/L NaCl) and homogenised in a Stomacher (P.B.I: International, Italy). Further decimal dilutions  
106 were made in the same solution, and the following microbiological analyses were performed in  
107 triplicate on agar plates: the total viable count (TVC) on Plate Count Agar (PCA, Oxoid, Italy)  
108 incubated at 30 °C for 48 hours (ISO 6887), LAB on de Man-Rogosa-Sharpe (MRS) agar (pH 6.2;  
109 Oxoid, Italy) at 30 °C for 48-72 h (ISO 15214) in a microaerophilic environment (gas pack  
110 anaerobic system, BBL, Becton Dickinson, USA), Clostridia on Differential  
111 Reinforced Clostridia Medium (DRCM) (VWR, USA) that was incubated at 37°C for 24-48 h in an  
112 anaerobic jar with an anaerobic kit (gas pack anaerobic system, BBL, Becton Dickinson, USA),  
113 *Salmonella* spp. according to the ISO 6579 method, briefly: (1) a pre-enrichment in buffered  
114 peptone water for 18 h at 37 °C; (2) a selective enrichment of 0.1 mL of pre-enriched culture in 10  
115 mL on Rappaport- Vassiliadis Soy (RVS) broth for 24 h at 42 °C and of 1 mL of pre-enriched  
116 culture in 10 mL of Muller-Kauffmann Tetrathionate-Novobiocin (MKTTn) broth for 24 h at 37  
117 °C; (3) selective isolation by streaking 10  $\mu$  L of RVS and 10  $\mu$  L of MKTTn on plates of XLD  
118 agar and brilliant green agar for 18-24 h at 37 °C; Confirmation of any presumptive colony was  
119 performed by Salmonella Latex agglutination test (Oxoid, Italy); and *Listeria monocytogenes*  
120 according to the ISO 11290 method, briefly: (1) a primary selective enrichment in 225 mL of Half  
121 Fraser Broth for 24 h at 30 °C; (2) a secondary selective enrichment of 0.1 mL of primary enriched  
122 culture in 10 mL of Fraser Broth for 48 h at 37 °C; (3) a selective isolation obtained by streaking 10  
123  $\mu$  L of the secondary enriched culture on Listeria Selective Agar according to Ottaviani and Agosti  
124 (ALOA) for 48 h at 37 °C.

125 Sixty (60) colonies (30 from the spoiled and 30 from the unspoiled samples) grown on MRS agar  
126 were randomly collected. Briefly, from one agar plate containing between 30 and 300 colonies of  
127 each samples, 3 colonies were isolated. The colonies were selected independently of morphology,  
128 colour or size. The isolates were streaked on MRS agar and then stored at -80 °C in MRS broth  
129 supplemented with glycerol (30% final concentration, Sigma-Aldrich, Germany). The isolates were

130 subjected to Gram staining and a catalase test and were then identified according to the molecular  
131 method (PCR-DGGE and sequencing) reported by Iacumin et al., (2009). In particular, the DNA  
132 was amplified with primers P1V1GC (GC-GCGGCGTGCCTAATACATGC) and P2V1  
133 (TTCCCCACGCGTTACTCACC) (Cocolin et al., 2001; Rantsiou et al., 2005), the PCR products  
134 were run in DGGE, and the isolates were grouped according to the migration profile. Where  
135 possible, at least three isolates for each group were subjected to sequencing for identification  
136 purposes. A culture-independent approach was also used to identify the strains found in both the  
137 spoiled and unspoiled cooked bacon: 10 mL of the homogenised sample in bacteriological peptone  
138 water was centrifuged at 10000xg, and the pellet was then subjected to total DNA extraction, PCR-  
139 DGGE, cloning of the DGGE bands and sequencing, following the protocol used by Iacumin et al.,  
140 (2009). The 60 colonies were then evaluated at strain level in order to choose the three strains to use  
141 as cultures for the challenge test against starter cultures. The test divided them in three main  
142 genotypic groups, also based on high, medium and low fermentation speed (data not shown). The  
143 isolates of unspoiled bacons had low fermentation speed and growth and this can explain the  
144 reduced concentration at the end of shelf-life of the products and consequently they can not spoil  
145 them.

#### 146 *2.1.3. Physico-chemical analysis and Total Volatile compounds determination*

147 The potentiometric measurement of pH was made using a pH meter (Radiometer, Copenhagen,  
148 Denmark). The volatile compounds in 10 spoiled and 10 unspoiled cooked bacon samples, all  
149 packaged under vacuum, were analysed at the end of their shelf-life (90 days). The volatile  
150 compounds were determined by SPME-GC-MS on Finnigan Trace DSQ (Thermo Scientific  
151 Corporation, USA) with a Rtx-Wax capillary column (length 30 m x 0.25 mm id.; film thickness  
152 0.25 µm; Restek Corporation, USA) according to the method reported in Chiesa et al., (2006). The  
153 volatiles compounds were then identified by comparison of their mass spectra and the retention  
154 times with those of standard compounds, or by comparison of the mass spectrum with those

155 of the mass spectrum Wiley library (Wiley library 10 vers.) and the self-made library. The results  
156 represent the average of all 10 samples analysed in triplicate.

157

#### 158 2.1.4. Statistical analysis

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

162

#### 163 2.2. Evaluation of starter cultures to eliminate microorganisms responsible for spoilage

##### 164 2.2.1 Sample preparation, storage conditions and sampling methods for the trials using bio- 165 protective starter culture

166 New pieces of cooked bacon were prepared and after chilling were inoculated with the starter  
167 cultures. The pasteurized phase was eliminated. In particular the pieces were divided into 3 lots of  
168 10 pieces (50 g each). Lot 1 was directly inoculated with a suspension of *Leuc. mesenteroides* at a  
169 final concentration of 3 log CFU/g and used as a control. Lot 2 was inoculated with a mixture of  
170 *Leuconostoc mesenteroides/Lactococcus lactis* subsp. *lactis* (ratio 1/1) at a final concentration of 3  
171 log CFU/g. Lot 3 was inoculated with *Leuc. mesenteroides/L. sakei* at a ratio of 1/1 and a final  
172 concentration of 3 log CFU/g. Each inocula were carried out spreading 0.1 mL of the bacterial  
173 suspension (about 6.3 log CFU/ml) on the bacons surface. After inoculation, the bacon pieces were  
174 packed under vacuum using the packaging film Combiflex PE/PA 2010 (60/150) (Niederwieser  
175 S.p.a., Italy) and stored at  $4 \pm 2$  °C for 90 days, at the end of their commercial shelf-life. At the end  
176 of storage, the presence of greening and of slime was evaluated, and the pH was measured.

177 *L. sakei* (B-2 Safe Pro® – CHR HANSEN) and *Lc. lactis* subsp. *lactis* (Rubis – CHR HANSEN)  
178 were obtained from the Italy branch supplier of Chr. Hansen, Denmark. The lyophilised cultures  
179 were resuspended in peptone water [0.1% sodium chloride and 0.7% peptone (Oxoid, England)] and  
180 left for 1 h at room temperature for complete rehydration. Appropriate dilutions were made, and 0.1



181 mL of each dilution was plated on MRS agar (de Man-Rogosa-Sharpe agar, pH 6.2, Oxoid, Italy)  
182 and incubated at 30 °C for 48-72 h in a microaerophilic environment (gas pack anaerobic system,  
183 BBL, Becton Dickinson, USA) to measure the concentration. Appropriate suspensions of the strains  
184 were mixed with *Leuc. mesenteroides* and directly inoculated on pieces of bacon, and the final  
185 bacterial cell concentration was approximately 3 log CFU/g.

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

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

194 The bacterial population, using all the MRS plates from – 3 to – 7 dilutions was suspended in  
195 Phosphate Buffered Saline (one tablet Phosphate Buffered Saline, Sigma-Aldrich P4417-100TAB,  
196 St. Louis, USA, dissolved in 200 mL H<sub>2</sub>O) and used for Bulk formation as previously described by  
197 Iacumin et al., (2011). One hundred µL of the bulk was used for DNA extraction and subjected to  
198 PCR-DGGE as above reported.

199

### 200 2.2.2 Sensory analysis

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

203 New pieces of cooked bacon, prepared following the traditional procedure of the plant, were  
204 divided into 3 lots. The lot A (10 pieces of 50 g each) was inoculated with a suspension of *L. sakei*  
205 and Lot B (10 pieces of 50 g each) by *Lc. lactis* subsp. *lactis*, both the strains were obtained from

206 the Italy branch supplier of Chr. Hansen, Denmark. The final concentration was for both 3 log  
207 CFU/g. Each inocula were carried out spreading 0.1 mL of the bacterial suspension (about 6.3 log  
208 CFU/ml) on the bacons surface. Lot C (10 pieces of 50 g each) was the control obtained with post-  
209 pasterurization and without adding bioprotective starter cultures. All the bacon pieces were packed  
210 under vacuum using the packaging film Combiflex PE/PA 2010 (60/150) (Niederwieser, S.p.a.,  
211 Italy), and stored at  $4 \pm 2$  °C.

212 All the bacon pieces were packed under vacuum using the packaging film Combiflex PE/PA 2010  
213 (60/150) (Niederwieser, S.p.a., Italy), and stored at  $4 \pm 2$  °C. Ten days before the end of their  
214 commercial shelf-life, all the lots were used for the triangle test.

215 The triangle test was used to compare the lot A and B to Lot C. Twenty non-professional assessors  
216 were presented with three products, two of which were identical. The assessors were asked to state  
217 which product they believed was the odd one out. The assessors who indicated there were two  
218 distinct samples were asked to identify the best one.

219

## 220 **3.0 Results and discussion**

### 221 3.1 Microbial analysis of spoiled and unspoiled cooked bacon

222 The concentration of LAB in the spoiled product was significantly different from the concentration  
223 in the unspoiled product ( $p < 0.05$ ). In particular it was  $8.7 \pm 0.1$  CFU/g in spoiled and  $4.0 \pm 0.2$   
224 CFU/g in unspoiled. Similarly, a significant difference ( $p < 0.05$ ) was observed in the TVC  
225 concentration between the spoiled and unspoiled product. The spoiled samples presented a TVC  
226 concentration of  $6.3 \pm 0.3$  CFU/g, whereas the unspoiled product presented a TVC concentration of  
227  $3.0 \pm 0.5$  CFU/g. The colonies growth on TVC were presuntively identified as LAB, by GRAM  
228 stain and catalase tests. The difference between the TVC and LAB concentration depends on the  
229 media used. Plate Count Agar used for TVC count tends to limit the growth of LAB and for this  
230 reason LAB concentration valued in MRS was higher than the one valued on PCA.

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

242 At 90 days (end of the shelf-life) the LAB concentration of the unspoiled bacons did not change. It  
243 was at level of  $4.1 \pm 0.5$  CFU/g and quite similar than the one at 30 days ( $p < 0.05$ ). At 90 days  
244 also the LAB concentration of the spoiled bacons did not deeply change, being at level of  $8.5 \pm 0.5$   
245 CFU/g ( $p < 0.05$ ). Similar results were obtained by the TVC counts. At this time the pH of the  
246 unspoiled bacons remained quite unchanged, conversely in the spoiled was reduced at level of  $5.1$   
247  $\pm 0.2$ . In these samples the greening colour changed to dark green and the slime persisted.

248 These data demonstrated that in the unspoiled bacons no spoilage was observed because LAB did  
249 not growth.

250

### 251 3.2 Microbial identification

252 All the 60 LAB isolates were Gram positive and catalase negative. The isolates were also subjected  
253 to molecular identification, and only one species was found: *Leuc. mesenteroides* (Accession  
254 Number KC545920.1). The direct identification of the bacteria by a culture independent method  
255 (PCR-DGGE and sequencing of the DGGE band) confirmed the traditional identification of the  
256 isolates. The DGGE profile of the sample treated using the culture independent method was

257 composed by one only band on the gel, which corresponded to *Leuc. mesenteroides*. *Leuconostocs*  
258 and *L. sakei* are considered to be the bacteria responsible for the spoilage of the cooked meat  
259 product stored at 4 °C (Samelis et al., 2000, Metaxopoulos et al., 2002; Cantoni et al., 2008;  
260 Vasilopoulos et al., 2010; Comi et al., 2012; Comi and Iacumin, 2012). In this study, the spoiled  
261 bacon was contaminated with up to 8 log CFU/g LAB, almost 4 log CFU/g more than the unspoiled  
262 cooked bacon (p < 0.05). The levels of contamination in spoiled and unspoiled cooked bacon were  
263 similar to those present in vacuum and Modified Atmosphere Packaged (MAP) cooked meats from  
264 other countries (Nielsen et al., 1983; Samelis et al., 2000). Samelis et al., (2000) found a  
265 concentration of LAB up to 7 log CFU/g in sliced cooked ham packaged under vacuum after 15-30  
266 days of storage at 4 °C. In our study, *Leuc. mesenteroides* was the only species present in the  
267 unspoiled cooked bacon at a concentration level of 4 log CFU/g. Our data are not in agreement with  
268 the ones of other authors (Bjorkroth et al., 1998; Metaxopoulos et al., 2002; Cantoni et al., 2008;  
269 Audenaert et al., 2010; Vasilopoulos et al., 2008, 2010a, 2010b; Comi et al., 2012), that found  
270 different LAB. Probably *Leuc. mesenteroides* was the predominant species that was selected in that  
271 plant and consequently was the main responsible of the spoilage. However, *Leuconostocs* were  
272 highly concentrated in the spoiled cooked bacon. Our data demonstrate that *Leuconostocs* were  
273 more profuse because these bacteria grew faster than other LAB at 4 °C, as demonstrated by Comi  
274 and Iacumin (2012). It is well-known that in cooked meat products packaged under vacuum,  
275 *Leuconostocs*, as well as some others LAB species, are the most prevalent because of the combined  
276 effects of pH, Aw (water activity), salinity of the brine and smoke (Samelis et al., 2000; Comi and  
277 Iacumin, 2012). During the spoilage, *Leuconostocs* produce lactic acid, acetic acid, ethanol, CO<sub>2</sub>  
278 and various other compounds that can lead to off-flavours (Cantoni et al., 2008). *Leuc.*  
279 *mesenteroides* produced hydrogen peroxide in vitro. This compound is responsible for the greening  
280 in meat products because hydrogen peroxide oxidises myoglobin to cholemyoglobin (Collins et al.,  
281 1993; Comi and Iacumin, 2012; Jääskeläinen et al., 2013). In the spoiled bacon samples considered  
282 in the present study, greening areas were observed by visual analysis.

283 Three main genotypic groups were observed after the identification of the *Leuc. mesenteroides* at  
284 strain level. One group isolated from unspoiled bacon had low fermentation speed and growth at 4  
285 °C. Conversely one group had high and one medium fermentation and growth speed. The group  
286 with higher fermentation and growth speed was isolated from the spoiled bacon. The group with a  
287 medium fermentation and growth speed belonged to either unspoiled or spoiled bacon (data not  
288 shown). It was assumed that the *Leuconostoc* strain of the spoiled bacon had a greater vitality than  
289 the unspoiled one, consequently they were able to produce the spoilage within 90 days of storage. It  
290 was assumed that the strains of the unspoiled bacon were less effective and consequently were not  
291 able to spoil the bacon within the end of their shelf-life. The lower activity was demonstrated by the  
292 concentration reached at 90 days that did not change in respect to 30 days in unspoiled bacon.  
293 *Listeria monocytogenes* spp. and *Salmonella* spp. were not detected in any of the tested samples,  
294 Clostridia were not detected (less than 10 CFU/g).

295

### 296 3.3 Analysis of the volatile compounds

297 The analyses of the volatile fractions from the spoiled and unspoiled cooked bacon packaged under  
298 vacuum are presented in Table 2. This table shows the mean retention times, the individual  
299 compounds and the concentrations of these compounds expressed in µg/kg product from ten  
300 analytical runs. The data suggest that the differences observed between the levels of volatile  
301 compounds in the spoiled and unspoiled bacon were due to *Leuconostoc* activity. In particular, we  
302 found an abundance of heterolactic fermentation products, such as acetic acid, ethanol other  
303 carboxylic acids and ketones; aldehydes were not identified because these compounds were most  
304 likely transformed into carboxylic acids. Alcohols were mainly present in the spoiled cooked bacon.  
305 The analysis was performed on both spoiled and unspoiled cooked bacon after 30 days of storage,  
306 because the greening and the slime presence in spoiled appeared after 30 days of storage. To better  
307 interpret the results obtained from the analysis of the headspace, the 23 identified substances were  
308 split into 5 classes: ketones (4), carboxylic acids (5), alcohols (9), terpenes (2), and miscellanea (3).

309 According to our data, *Leuc. mesenteroides* produce various compounds by fermenting sugars and  
310 metabolised amino acids. The concentrations of some volatile compounds were higher in the  
311 spoiled bacon than in the unspoiled bacons. Several of the compounds were ascribed to the glucose  
312 and amino acid metabolism of the LAB, to the oxidation and auto-oxidation of lipids (Montel et al.,  
313 1998) and to the endogenous reactions that occur during cooking (Mottram, 1998). The five  
314 identified acids are all typical metabolites from LAB, coliforms and *E.coli* fermentation of sugars  
315 and the degradation of amino acids (Leroy et al., 2009). The presence of certain organic acids,  
316 including acetic, propanoic, 2-Methyl-propanoic, 3-Methyl-butanoic and Hexanoic acid, is due to  
317 the degrading activity of leuconostoc (Comi and Iacumin, 2012; Diez et al., 2009). *Leuc.*  
318 *mesenteroides* can increase the concentration of acetic acid and aldehydes in meat product and in  
319 blood sausage (Diez et al., 2009). Our data confirm the increasing of the acetic acid concentration  
320 but do not show the presence of aldehydes. It could be concluded that the aldehydes were not  
321 present because these compounds were most likely transformed into carboxylic acids by *Leuc.*  
322 *mesenteroides*, such as hexanal transformed in hexanoic acid. It is also possible that the acids with  
323 more than three carbon atoms can be derived by the lipolytic enzymes in the leuconostocs and in the  
324 meat or by lipid oxidation (Chiesa et al., 2006; Comi et al., 2000). Because the brine injected into  
325 the meat before production contained up to 0.5% sugars (w/v), we assumed that the organic acids  
326 found in the bacon were produced by LAB fermentation. Ethanol was the main alcohol produced  
327 and was mainly present in spoiled product. The total concentration of alcohol in the spoiled product  
328 was higher than that in the unspoiled product. Leuconostocs most likely produced fewer ketones  
329 and carboxylic acids than alcohols. Alcohols can be derived from sugar fermentation (Kandler,  
330 1983; Diez et al., 2009), from aldehydes, ketones or from amino acid (leucine, valine and  
331 phenylalanine) catabolism and all these activities are typical of *W. viridescens* and leuconostocs  
332 (Comi et al., 2014a,b,2011; Deetae et al., 2007; Smit et al., 2005; Bedaguè *et al.*, 1993). In  
333 particular, the higher alcohol levels found in the spoiled bacon were produced by the *Leuc.*  
334 *mesenteroides* conversion of aldehydes, ketones and amino acids. In addition, the higher alcohol

335 amounts can be also demonstrated by the higher level of *Leuc. mesenteroides* concentration found  
336 in spoiled products, considering that the strains isolated from them had a growth and fermentation  
337 speed faster than the strains isolated in unspoiled product. The increase of alcohol in spoiled bacon  
338 is associated with the increase of the leuconostoc concentrations (heterofermentative  
339 microorganisms). In meat products, the aldehyde levels usually increase with the fermentative  
340 activity of a starter consisting of LAB and Coagulase Negative Catalase Positive Cocci or after  
341 degradation through the Strecker reaction (Comi et al., 2000). Unlike the results obtained by other  
342 authors (Comi et al., 2000; Tjener et al., 2003), no aldehydes were identified in our study. This  
343 result can be explained by the degradation and reduction of these compounds by leuconostocs,  
344 which converted them in alcohols (Bedarguè et al., 1993; Comi and Iacumin, 2012; Comi et al.,  
345 2014a,b).

346 The identified ketones were primarily derived from the oxidation of fatty acids (2-propanone, 2-  
347 butanone, 3-hydroxy-2-butanone, 2-pentanone) and by LAB activities (Berdague et al.,1993;  
348 Jääskeläinen et al., 2013). Unlike the ketones identified in other studies (Comi et al., 2000; Tjener et  
349 al., 2003, Comi and Iacumin, 2012), diacetyl (2,3-butandione) was not found in our study. This was  
350 probably due on the absence of oxygen because the cooked bacon was under vacuum packaged.  
351 Jääskeläinen et al., (2013) found that in *Leuc. gasicomitatum* the diacetyl production is minimal in  
352 meat packaged under anaerobic condition and on the contrary the respiration enormously increased  
353 the accumulation of acetoin and diacetyl under a high-oxygen modified atmosphere. It is also  
354 possible that diacetyl may have been reduced to 3-hydroxy-butanone (acetoin), which was found in  
355 our study. The absence of diacetyl did not allow to impart a buttery aroma and flavor to spoiled  
356 cooked bacon. However, considering the microbial population identified in spoiled cooked hams, it  
357 is also possible that the ketones were derived from the degradation of alkanes (Montel et al.,1998).  
358 Three out of 4 ketones detected were found in higher concentration in unspoiled than in spoiled.  
359 This was explained by a greater activity of the leuconostocs in spoiled bacon that consequently  
360 produced higher amount of alcohol and less ketones (Bedarguè et al., 1993; Comi et al., 2014a,b).

361 Among the aromatic hydrocarbons (terpenes), two compounds were identified both in the unspoiled  
362 and spoiled cooked bacon. These compounds are typically found in raw materials and most likely  
363 originated from various contaminations in the animal feedstuffs and spices (nutmeg, black pepper)  
364 because these compounds can be found in plants (Van Straten, 1977; Comi et al., 2000) that are  
365 eaten by animals. For these reasons, a large difference was not observed between the terpene  
366 concentrations of the spoiled and unspoiled cooked bacon.

367

### 368 3.4 Challenge test

369 Table 3 shows the results obtained using the bioprotective culture: their use can eliminate the  
370 spoilage produced by leuconostocs. In fact, cooked bacon with bio-protective cultures did not  
371 present greening, slime or inflated packaging at the end of the shelf life. In contrast, a sticky-white  
372 slime and a greening colour were observed in the cooked bacon inoculated only with *Leuc.*  
373 *mesenteroides*. The bio-protective cultures grew during the storage of the products, and at the end  
374 of the shelf-life, the ratio *Leuc. mesenteroides*/*Lc. lactis* subsp. *lactis* was about 1/100. In fact the  
375 DNA of *Leuc. mesenteroides* was retrieved by PCR-DGGE at a sample decimal dilution of  $10^{-3}$ ,  
376 whereas DNA of *Lc. lactis* subsp. *lactis* at a decimal dilution of  $10^{-5}$  (Table 4). On the other hand,  
377 at the end of the shelf-life, the ratio of *Leuc. mesenteroides*/*L. sakei*, was about 1/1000. In fact the  
378 DNA of *Leuc. mesenteroides* was retrieved by PCR-DGGE at a decimal dilution of  $10^{-3}$ , whereas  
379 the DNA of *L. sakei* at a dilution level of  $10^{-6}$  (Table 4). On the basis of the obtained results, can be  
380 assumed that the bioprotective cultures, competing for the substrate with *Leuc. mesenteroides*,  
381 inhibited their growth. This assumption is also confirmed by the results obtained for the control  
382 sample (Lot 1), where the DNA of *Leuc. mesenteroides* was identified at a dilution level of  $10^{-6}$   
383 (Table 4). *Leuc. mesenteroides* concentration in Lot 1 was  $6.0 \pm 0.3$  UFC/g.  
384 Consequently, the growth of the bio-protective cultures eliminated the spoilage caused by  
385 leuconostocs, and the products resulted acceptable at the end of the shelf life (90 days).



386 The use of LAB as bio-protective cultures to prolong the shelf life of the meat products, including  
387 cooked meat product, is a new concept that has been suggested by many authors (Metaxopoulos et  
388 al.,2002; Vermeiren et al.,2004; Comi et al.,2011). Kotzekidou and Bloukas (1996) noticed that  
389 cooked ham supplemented with protective cultures had lower total aerobic bacteria, micrococci,  
390 staphylococci and *B. thermosphacta* counts than control hams, which had higher populations of  
391 LAB and lower populations of pseudomonads. Additionally, Metaxopoulos et al., (2002) and  
392 Vermeiren et al., (2006) concluded that LAB affected the spoilage microflora growth and did not  
393 negatively affect the organoleptic properties of the products. Comi et al., (2012) demonstrated that  
394 starter cultures (*L. sakei*, *L. curvatus* and *Lc. lactis* subsp. *lactis*) extend the shelf life of cooked ham  
395 slices packed in MAP. The microorganisms influenced the flavour, odour and stability of the colour  
396 of the sliced cooked ham.

397

### 398 3.5 Sensorial analysis

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

410

## 411 4. Conclusion

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

417

418

419

#### 420 **Conflict of interest**

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

423

#### 424 **References**

425

426 Audenaert, K., D'Haene, K., Ruysen, T., Vandamme, P., Huys, G., 2010. Diversity of lactic acid  
427 bacteria from modified atmosphere packaged sliced cooked meat products at sell-by date  
428 assessed by PCR-denaturing gradient gel electrophoresis. *Food Microbiol.* 27, 12-18.

429 Björkroth, K.J., Vandamme, P., Korkeala, H.J., 1998. Identification and characterization of  
430 *Leuconostoc carnosum*, associated with production and spoilage of vacuum-packaged, sliced,  
431 cooked ham. *Appl. Environ. Microbiol.* 64, 3313-3319.

432 Cantoni, C., Balzaretto, C., Marzano, M.A., Iacumin, L., 2008. Vecchie e recenti alterazioni di  
433 prodotti carnei cotti. *Ing. Alim.* 3, 23-28.

434 Chiesa, L., Duchini, M., Iacumin, L., Boscolo, D., Comi, G., Cantoni, C., 2006. Caratteristiche  
435 chimiche e batteriologiche di salami della Lomellina. *Arch. Vet. It.* 57 (5) 209-224.

436 Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 2001. Denaturing gradient gel electrophoresis  
437 analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial  
438 population during fermentation of Italian sausages. *Appl. Environ. Microbiol.* 67, 5113–5121.

439 Collins, M.D., Samelis, J., Metaxopoulos, J., Wallbanks, S., 1993. Taxonomic studies on some  
440 *Leuconostoc*-like organisms from fermented sausages: description of a new genus *Weissella*  
441 for the *Leuconostoc paramesenteroides* group of species. *J. Appl. Bacteriol.* 75, 595-603.

442 Comi, G., Andyanto, D., Manzano, M., Iacumin, L., 2014a. A case of spoiled in Wurstel sold in  
443 Italian supermarket. *Food Control* 43, 245-250.

444 Comi, G., Manzano, M., Bricchese, R., Iacumin, L., 2014b. New case of spoilage in San Daniele  
445 Dry Cured Hams. *J. Food Safety*. Volume 34, Issue 4, 263-269.

446 Comi, G., Iacumin, L., 2012. Identification and process origin of microorganisms responsible for  
447 cavities and volatile off-flavor compounds in artisanal cooked ham. *Int. J. Food Sci. Techn.*  
448 47, 114-121.

449 Comi, G., Ottaviani, S., Patthey, C., Mitri, E., Menardi, G., Andyanto, D., Iacumin, L., 2011.  
450 Miglioramento della qualità di prosciutti cotti affettati e confezionati in MAP. *Ing. Alim.* 12,  
451 14-16.

452 Comi, G., Procida, G., Aggio, D., Pittia, P., Cantoni, C., Cocolin, L., 2000. Valutazione di uno  
453 starter microbico per la produzione di salame tradizionale friulano. *Ing. Alim.* 4, 9-25.

454 Deetae, P., Bonnarme, P., Spinnler, H.E., Helinck, S., 2007. Production of volatile aroma  
455 compounds by bacterial strains isolated from different surface-ripened French cheeses. *Appl.*  
456 *Microbiol. Biotechn.* 76, 1161-1171.

457 Diez, A.M., Björkroth, J., Jaime, I., Rovira, J., 2009. Microbial sensory and volatile changes during  
458 anaerobic cold storage of *morcilla de Burgos* previously inoculated with *Weissella*  
459 *viridescens* and *Leuconostoc mesenteroides*. *Int. J. Food Microbiol.* 131, 168-177.

460 Franz, C.M.A.P., von Holy, A., 1996. Thermotolerance of meat spoilage lactic acid bacteria and  
461 their inactivation in vacuum-packaged Vienna sausages. *Int. J. Food Microbiol.* 29, 59-73.

462 Iacumin, L., Manzano, M., Comi, G., 2012. Coagulase-negative catalase-positive cocci in  
463 fermented sausage: variability due to different pork breeds, breeding systems and sausage  
464 production technology. *Food Microbiol.* 29 (2), 178-186.

465 Iacumin, L., Cecchini, F., Manzano, M., Osualdini, M., Boscolo, D., Orlic, S., Comi, G., 2009.  
466 Description of the microflora of sourdoughs by culture-dependent and culture independent  
467 methods. *Food Microbiol.* 26, 128-135.

468 Jääskeläinen, E., Johansson, P., Kostianen, O., Schmidt, G., Somervuo, P., Mohsina, M., Vanninen,  
469 P., Auvinen, P., Björkroth, J., 2013. Significance of Heme-Based respiration in meat spoilage  
470 caused by *Leuconostoc gasicomitatum*. *Appl. Environm. Microbiol.* 79(4), 1078-1085.

471 Kandler, O., 1983. Carbohydrate metabolism in lactic acid bacteria. *Ant. van Leeuv.* 49, 209-224.

472 Kotzekidou, P., Bloukas, J.G., (1996) Effect of protective cultures and packaging film permeability  
473 on shelf life of sliced vacuum cooked ham. *Meat Sci.* 42, 333-345.

474 Leroy, F., Vasinopoulos, C., Van Hemelryck, S., Falony, G., De Vuyst, L., 2009. Volatiles analysis  
475 of spoiled, artisan-type, modified-atmosphere-packaged cooked ham stored under different  
476 temperature. *Food Microbiol.* 26, 94-102.

477 Metaxopoulos, J., Mataragas, M., Drosinos, E.H., 2002. Microbial interaction in cooked cured meat  
478 products under vacuum or modified atmosphere at 4°C. *J. Appl. Microbiol.* 93 (3), 363-373.

479 Montel, M.C., Masson, F., Talon, R., 1998. Bacterial role in flavour development. *Meat Sci.* 49,  
480 suppl. 1, s111-s123.

481 Mottram, D.S., 1998. Flavour formation in meat and meat products: a review. *Food Chem.* 66, 845-  
482 854.

483 Nielsen, H.J.S., 1983. Influence of temperature and gas permeability of packaging film on  
484 development and composition of microbial flora in vacuum-packed Bologna-type sausage. *J.*  
485 *Food Protect.* 46, 693-698.

486 Rantsiou, K., Urso, R., Iacumin, L., Cantoni, C., Cattaneo, P., Comi, G., Cocolin, L., 2005. Culture  
487 dependent and independent methods to investigate the microbial ecology of Italian fermented  
488 sausages. *Appl. Environ. Microbiol.* 71, 1977–1986.

489 Samelis, J., Kakouri, A., Rementzis, J., 2000. Selective effect of the product type and the packaging  
490 conditions on the species of lactic acid bacteria dominating the spoilage microbial association  
491 of cooked meats at 4°C. *Food Microbiol.* 17, 329-340.

492 Smit, G., Smit, B.A., Engels, W.J.M., 2005. Flavour formation by lactic acid bacteria and  
493 biochemical flavour. *FEMS Microbiol. Rev.* 29, 591-610.

494 Tjener, K., Stahnke, L.H., Andersen, L., Martinussen, J., 2003. A fermented meat model system for  
495 studies of microbial aroma formation. *Meat Sci.* 66, 211-218.

496 Vasilopoulos, C., Ravyts, F., De Maere, H., De Mey, E., Paelinck, H., Leroy, F., 2008. Evaluation  
497 of the spoilage lactic acid bacteria in modified-atmosphere-packaged artisan-type cooked ham  
498 using culture-dependent and culture-independent approaches. *J. Appl. Microbiol.* 104, 1341-  
499 1353.

500 Vasilopoulos, C., De Maere, H., De Mey, E., Paelinck, H., 2010a. Technology-induced selection  
501 towards the spoilage microbiota of artisan-type cooked ham packed under modified  
502 atmosphere. *Food Microbiol.* 27, 77-84.

503 Vasilopoulos, C., De Mey, E., Dewulf, L., Paelinck, H., De Smedt, A., Vandendriessche, F., De  
504 Vuyst, L., Leroy, F., 2010b. Interaction between bacterial isolates from modified-atmosphere-  
505 packaged artisan-type cooked ham in view of the development of a bio-protective culture.  
506 *Food Microbiol.* 27, 1086-1094.

507 Van Straten, S., 1977. *Volatile Compounds in Food*, 4th ed. Central Institute for Nutrition and  
508 Food Research TNO, Zeist, The Netherlands.

509 Vermeiren, L., Devlieghere, F., Debevere, J., 2004. Evaluation of meat born lactic acid bacteria as  
510 protective cultures for the biopreservation of cooked meat products. *Int. J. Food Microbiol.* 96  
511 (2), 149-164.

512

513

514 **Table 1:** Process steps of traditional cooked bacon production

<b>Step</b>	<b>Temperature/time</b>
Raw material	4 ± 2 °C
Trimming, Squaring	12 °C
Brine injection	7 °C
Churning	7 °C – 8 hours
Resting	2-4 °C – 7 days
Drying	44-55 °C – 1 hour
Smoking	66 °C – 24 hours
Cooking	72 – 78 °C
Chilling	2-4 °C
Packaging	4 °C
Pasteurization	85 °C – 15 minutes
Storing	4 ± 2 °C

515

516

517

518

519

520

521

522

523

524

525

526 **Table 2:** Volatile compounds in unspoiled and spoiled bacon

527

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

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)

528

529

530

531

533 **Table 3:** Physico-chemical results of the bio-protective effect of the LAB starter used,  
 534 versus *Leuconostoc mesenteroides*  
 535

Lot	Microorganisms	Greening	Slime	pH
1	<i>Leuc. mesenteroides</i>	+	+	5.3 ± 0.2* a
2	<i>Leuc. mesenteroides</i> vs <i>Lc. lactis</i>	-	-	5.4 ± 0.1* a
3	<i>Leuc. mesenteroides</i> vs <i>L. sakei</i>	-	-	5.4 ± 0.2* a

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)



536 **Table 4:** Plate dilution at which the identified species were detected

Lot	Microorganism inoculated	Serial decimal dilution				
		10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-6</sup>	10 <sup>-7</sup>
1	<i>Leuc. mesenteroides</i>	+	+	+	+	-
2	<i>Leuc. mesenteroides</i>	+	-	-	-	-
	<i>Lc. lactis</i>	+	+	+	-	-
3	<i>Leuc. mesenteroides</i>	+	-	-	-	-
	<i>L. sakei</i>	+	+	+	+	-

537