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Valentina Alessandria*[a], Ilario Ferrocino[a], Valerio Carta[a], Veronique Zuliani[b], Tim Martin Seibert[c], Jakob Soeltoft-Jensen[d], Kalliopi Rantsiou[a], Luca Cocolin[a]

[a]Dipartimento di Scienze Agrarie, Forestali e Alimentari, Università degli Studi di Torino, Largo Paolo Braccini n°2, 10095 Grugliasco (TO), Italy

[b]Chr. Hansen SAS, Saint-Germain-lès-Arpajon, France

[c]Chr. Hansen GmbH, Pohlheim, Germany

[d]Chr. Hansen A/S, Hoersholm, Denmark

* Corresponding author: Valentina Alessandria, Phone: +39-011-6708873, Fax: +39-011-6708549

Email: valentina.alessandria@unito.it

Abstract

Sliced cooked ham stored in modified atmosphere packaging (MAP) can be spoiled by lactic acid bacteria (LAB) which are dominating under psychrotrophic conditions. Depending on the strains, the colonization can result in a premature spoilage characterized by off-flavors, gas and slime production, discoloration, and acidification. The purpose of this study was the isolation, identification and characterization of potential food culture with protective properties, able to prevent or delay spoilage in cooked-ham. The first step was to identify by means of microbiological analysis, the microbial consortia both in unspoiled and in spoiled lots of sliced cooked ham by the use of media for the detection lactic acid bacteria and total viable count. Counts ranged from values lower than 1 Log CFU/g to 9 Log CFU/g in spoiled and unflawed samples.

The interaction between consortia was then studied in order to screen for strains able to inhibit spoilage consortia. Strains showing antimicrobial activity were identified and characterized by molecular methods and tested for their physiological features. Among a total of 140 strains isolated, nine were selected for their ability to inhibit a large number of spoilage consortia, to grow and ferment at 4°C and to produce bacteriocins. The effectiveness of the fermentation made by food culture was evaluated, through challenge tests *in situ*, analysing the microbial profiles of artificially inoculated cooked-ham slices during storage by high throughput 16S rRNA gene sequencing. The native population *in situ* resulted competitive against the inoculated strains and only one strain was able to significantly reduce the native populations reaching about 46.7 % of the relative abundance. The results obtained in this study provide information about the selection of autochthonous LAB on the base of their action against spoilage consortia, in order to select protective potential cultures able to improve the microbial quality of sliced cooked ham.

Keyword: Sliced cooked ham, Bioprotection, Lactic acid bacteria, Microbial Consortia, Bacteriocins.

1. Introduction

Cooked ham constitutes over 26% of the volume of the delicatessen products sold in Europe, where Spain, France and Italy are the biggest consumers (Casiraghi et al., 2007). The final quality of the products depends both on the raw material used and the processing, which normally includes injection of brine and tumbling and cooking reaching at least 75°C core. These products are very susceptible to bacterial spoilage and several preservation techniques are combined to optimize their microbial quality.

Thermal processing is a critical step in cooked ham production and plays an important role in the microbiota selection. The process is not always effective against thermo-tolerant vegetative bacteria and has no effect on the re-contamination that may occur during slicing and packaging (Franz & von Holy, 1996; Vasilopoulos et al., 2010). The spoilage microorganisms that colonize cooked ham stored in modified atmosphere packaging (MAP) are, amongst other, psychrotrophic lactic acid bacteria (LAB) such as *Leuconostoc*, *Latilactobacillus* and *Carnobacterium* (Vasilopoulos et al.,2008). The use of gas mixture of N_2 and CO_2 in MAP products favors microbiota that withstand microaerophilic conditions, usually LAB species and *Brochothrix thermosphacta* (Casaburi et al., 2011; Geeraerts et al., 2017; la Storia et al., 2012; Samelis et al., 2000; Vasilopoulos et al., 2015). LAB growth is also encouraged by presence of sodium chloride and sodium nitrite and a reduced water activity. The overwhelming colonization may result in premature spoilage and subsequent food waste, characterized by accelerated pH decrease, gas and slime production, discoloration, and/or off-flavor formation. As mentioned above, the contamination can come mainly from post processing contamination. Therefore, apart from cooking and good sanitation practices, the use of various post-cooking or post-slicing technologies such as high pressure, antimicrobial treatments and use of food cultures can be useful for the microbial and sensory quality preservation of cooked meat (Comi et al., 2016; Vermeiren et al., 2004). Biopreservation through controlled fermentation can thus contribute to reduce food waste and extend the shelf life of the products. The mechanism of biopreservation is driven by a multiple mode of actions such as the production of organic acids, competitive exclusion (spatial volume and substrate availability), quorum sensing or by metabolites with antimicrobial properties such as bacteriocins, natural peptides with the potential to inhibit the growth of foodborne pathogens or spoilage bacteria in food products (Deegan et al., 2006). The production of bacteriocins that leads to a better preservation of the product is a characteristic of different starter cultures

(Cleveland et al., 2001). Strains of different genera of LAB have been identified as bacteriocin producers and their action against pathogen bacteria as *Listeria monocytogenes* in fermented meat has been deeply investigated (Franciosa et al., 2018).

The purpose of this study was the isolation and identification of potential strains selected based on their action against spoilage consortia. The potential food cultures with protective properties were tested for their effectiveness against spoilage consortia, isolated from sliced cooked ham samples coming from different processing plants in Europe.

2. Material and methods

2.1 Microbiological analysis of sliced cooked ham samples

A total of 67 sliced cooked ham samples, packed in modified atmosphere, were supplied by 10 producers located in different areas in Europe and sent to the DISAFA laboratory. Companies were named with letters from "A" to "L". Among 67 samples, 30 were indicated as spoiled by the producers and their defects confirmed at the laboratory. Criteria used from the companies to identify samples as spoiled were: package swelling, presence of slime, off-odors and off-flavors and discoloration. Samples were divided in spoiled (30 samples) and unspoiled (37 samples); for each sample several subsamples were supplied. The subsamples of the unspoiled ones were stored at 7°C until the end of the shelf-life defined by the producers. Microbiological analyses were conducted on spoiled samples the day of arrival, while the subsamples of the unspoiled were analyzed also during their shelf life (at the beginning [B], middle [M] and end [E] of shelf life). The three times of sampling were planned on the base of their shelf life (provided by producers), knowing the day of production an the expiry day. Plate counts were adopted in order to isolate the predominant populations. Ten grams of each sample were diluted in 90 mL of sterile Ringer solution (Oxoid, Milan, Italy) and homogenized in a blender (Stomacher 400 Circulator, International PBI S.p.a, Italy) for 2 min at room temperature. For enumeration, appropriate decimal dilutions were prepared and plated, in triplicate, on different media: lactic acid bacteria (LAB) on de Man Rogosa Sharp agar in microaerophilic conditions (MRS, Oxoid), while total viable count on BHI agar (Oxoid), incubated at 30°C and 37°C respectively for 48 h. In both cases samples were spread on the plates.

2.2 Selection of isolates with antimicrobial activity

Colonies from spoiled hams were recovered from representative count plates of MRS and BHI by adding 1 mL of Ringer's solution, scraping and collecting the cell suspension. The recovered cell suspension represented the microbial populations composing the consortia and they were used as indicators in assays performed to detect antimicrobial capabilities. Among the 30 spoiled samples, 5 of them with the most evident defects (mainly gas production/blowing) were selected and their corresponding spoilage consortia were used. To screen a large quantity of LAB colonies for antimicrobial activity, the plates of the sampling of the unspoiled hams, after counting, were overlaid with a layer of MRS or BHI agar containing the consortia recovered from the spoiled samples (used as indicators). Specifically, 1% of the suspension was added to soft-agar media (agar [10 g/l]) and plates of unspoiled samples were covered with a layer of soft agar containing the spoilage consortium and incubated at 30°C for 24 h. After incubation colonies that displayed inhibition halo were marked, collected from the original plates and cultivated in BHI or MRS as appropriate. In the case of unspoiled samples, count plates (with a maximum of 300 colonies) of MRS and BHI were replicated by "replica plates" (Dal Bello et al., 2010) in order to test the same population against all the spoilage consortia selected. All the spoiling consortia were dispensed in Eppendorf tubes with glycerol and stored at -80°C.

A total of 140 colonies were selected for their antimicrobial activity and subjected to Gram staining and catalase test. In order to confirm the antimicrobial activity, each of the 140 isolates was tested against the spoilage consortia through agar-well-diffusion assay (AWDA), as described by Toba et al. (1991). Fifty µL of an overnight broth culture of each isolate were placed into a well of 5 mm diameter in a soft agar plates (agar [10 g/l], either MRS or BHI) containing the spoilage consortium. After incubation for 24 h at 30 and 37°C, respectively for MRS and BHI, they were examined for the presence of inhibition zones. The isolates able to inhibit more than the 60% of spoiled consortia tested, were selected. In order to determine the nature of the inhibition, isolates were growth overnight in MRS and BHI broth and after removal of the cells, through a 0.2 mm filter (Millipore, Italy), and the pH neutralization with KOH (1 M), proteinase K (25 mg/mL) was added to the supernatant and a new AWDA was performed. The absence of inhibition halos after the proteinase K treatment confirmed the proteinaceous origin of the antimicrobial compound.

With the aim of finding potential starter for cooked ham, the isolates exerting antimicrobial activity were also evaluated for their ability to grow at refrigeration temperature. A 1% (v/v) inoculum was added to BHI and MRS broth and incubated at 4°C for 72 h. Tubes that displayed value of 5 compared to the McFarland scale were considered positive.

2.3 DNA extraction from bacteriocin producing isolates and molecular identification and characterization

Genomic DNA of the bacteriocin producing isolates was extracted as described in Cocolin et al. (2001). Isolates were first grouped by their fingerprinting profile by the means of PCR 16S–23S rRNA gene spacer analysis (RSA). The RSA was carried out with primers G1 (GAAGTCGTAACAAGG) and L1 (CAAGGCATCCACCGT) (Dolci et al., 2008). Representative isolates from each group where then identified by amplified the variable V1 and V3 regions of 16S rRNA gene (Cocolin et al., 2001). PCR products were sequenced and the resultant sequences were aligned with those in Gene Bank using the Blastn program (Altschul et al., 1997) to determine the known relatives.

Rep-PCR fingerprinting was performed for molecular characterization with the single oligonucleotide primer (GTG)5, following the protocol described by Gevers et al. (2001). Products of rep-PCR were run in a 2% agarose gel and the fingerprints obtained were visualized under ultraviolet light using UVI pro platinum 1.1 Gel Software (Eppendorf, Germany) and analyzed with BioNumerics 4.6 software package (Applied Maths, Belgium). The similarity among digitalized profiles was calculated using the Pearson correlation and an average linkage (UPGMA) dendrogram was derived from the profiles.

The methods adopted in the next steps of the analyses were chosen on the basis of the results of the identification of strains.

2.4 LAB bacteriocin activity (AU)

The AWDA method was adopted to titrate the bacteriocin produced. Isolates that possessed antimicrobial activity were grown overnight in M17 added either with glucose or lactose in concentration of 0.5 % p/v. M17 medium was chosen because of its suitable composition for the growth of *Lactococcus* spp. In order to quantify

the bacteriocin activity, the cell free overnight supernatants were two-fold serially diluted in PBS (pH 7) solution reaching a dilution of 1: 4096. Fifty µL of each dilution was added to BHI soft agar plate containing 1% inoculum of an overnight culture of *L. monocytogenes* EGDe as an indicator. The bacteriocin activity was expressed in arbitrary units (AU)/ml. One arbitrary unit was defined as the reciprocal of the highest dilution yielding a clear inhibition zone of the indicator strain(Ananou et al., 2005). Four *Lactococcus lactis* strains (strains 41FL18, 41FLL3, 39FL2 and 41FL1) from the DISAFA collection, isolated by artisanal products from North West of Italy (Dal Bello et al., 2010) were included in the work because of their ability to produce bacteriocins. The values of bacteriocin activity shown in this study were the averages of three assays.

Data were subjected to one-way ANOVA, and Duncan test was used to determine differences in the inhibition activity expressed as AU/ml at $p < 0.05$, using the statistical software, STATISTICA 7.0 for Windows (Statsoft, Tulsa, USA).

2.5 Targeting the bacteriocin encoding gene by PCR amplification

On the base of the results of the isolates identification, PCR was used to amplify the following target genes: nisin A (Choi et al., 2000), lactococcin A(M. I. Martínez et al., 1998), lactococcin B (van Belkum et al., 1992), lactococcin 513 (Villani et al., 2001), lactococcin 972 (B. Martínez et al., 1999), lacticin RM (Villani et al., 2001) and lacticin 481(Piard et al., 1993), as performed in Dal Bello et al. (2010). The reaction was performed in a final volume of 25 µL containing 1X PCR buffer, 1.5 mmol/L $MgCl_2$, 0.2 µmol/L dNTP, 0.2 µmol/L of each bacteriocin primer and 1 U of Taq polymerase (Sigma Aldrich). The amplification cycles used were 95°C for 5 min as initial step, 95° C for 1 min, annealing at 42°C (for the primers of Nis A and Nis Z), 56°C (for the primers of lactococcins and lacticins) for 1 min, and 72 °C for 1 min for the next 30 cycles, 72 °C for 5 min concluded the amplification as reported in (Dal Bello et al., 2010). Fragments were visualized on 2% agarose gels added of ethidium bromide using the 100 bp ladder (Promega, Italy) as the molecular weight marker.

2.6 In situ challenge tests

188 Challenge tests were carried out on cooked ham slices packaged in a white room of a Piedmont ham plant the
189 same day of analysis, inoculating the most performing bacteriocin producing isolates. Strains isolated from
190 spoiled samples were also included in the trials to simulate the interaction in the occurrence of spoilage
191 (Raimondi et al., 2019). Four spoilage consortia (SC) were prepared: consortium 1 was composed by strains
192 34, 36, 37 and 38, respectively identified as *Leuconostoc carnosum, Kazachstania servazzi, Candida sake* and
193 *Yarrowia lipolytica,* consortium 2 by strain 46 (*Enterococcus gilvus),* consortium 3 by *Leuconostoc carnosum*
194 1 and *Latilactobacillus curvatus* 11 and consortium 4 by strains 18,19 and 20 belonging to *Latilactobacillus*
195 *sakei* species.
196 The potential food cultures with protective properties (BC) selected and SC were inoculated to a final
197 concentration of 6 Log CFU/g and 4 Log CFU/g, respectively, alone and in interaction. Negative controls with
198 only Ringer solution were carried out for each trial. Two batches were carried out for each trial and biological
199 replicates were carried out for each trial.
200 Briefly, the strains were cultured for 24h and cell counts were performed by the preparation of appropriate
201 decimal dilutions and plated on the appropriate media (MRS and M17 for LAB, Malt Agar for yeasts (Oxoid))
202 and incubated at 30°C for 48 h. Cells were washed twice with Ringer solution and 10 mL of cells suspension
203 were filled in spray bottles in order to reach a final count on ham (sliced the day of the inoculum in a white
204 room) of 4 and 6 Log CFU/g for SC and BC respectively. Four slices were collected sterile and inoculated in
205 both sides using spray bottles. The inoculated hams were packed on modified atmosphere (70% N_2 and 30%
206 CO_2) and stored at 7°C for 10 weeks (abuse time).
207 About 10 g from each sample before the inoculum and after 10 weeks of storage were homogenized with
208 Ringer solution (Oxoid, Milan, Italy) and analyzed as previously described using M17 (Oxoid, Milan) and
209 MRS media in triplicate. Samples inoculated with consortium 1 were also analyzed using WLN for the
210 detection of yeast.
211 The pH was measured by immersing the pH probe of a digital pH meter (micropH2001; Crison, Barcelona,
212 Spain) in the first decimal dilution before inoculation and after the 10 weeks.
213
214 *2.8 DNA extraction from ham samples*

Before the inoculum and after 10 weeks of storage, 1 ml of the first 10-fold serial dilution was collected and directly centrifuged at maximum speed for 5 minutes. Nucleic acid was extracted by pooling two biological replicates from each sampling point from each trial by using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) following the manufacturer's instructions. The nucleic acids were resuspended in 50 µl of sterile water. DNA was quantified by using the Nanodrop 1000 (Thermo Scientific, Milano, Italy) and standardized at 100 ng/µl.

2.9 16S rRNA amplicon target sequencing

DNA sequencing was used to assess the microbiota by amplification of the V3-V4 region of the 16S rRNA gene using the primers and protocols described by Klindworth et al. (2013). The PCR products were purified and tagged by using the Nextera XT index kit (Illumina), according to the manufacturer's instructions. Sequencing was performed with a MiSeq instrument (Illumina) with V3 chemistry and generated 250-bp paired-end reads, according to the manufacturer's instructions.

2.10 Bioinformatics and statistical analysis

After sequencing raw reads were first joined through flash and analyzed with QIIME 1.9.0 software (Caporaso et al., 2010) and the pipeline described by Ferrocino et al. (2017). Briefly after quality filtering OTUs were clustered at 99% of similarity and centroids sequence were mapped against the green genes by means of the RDP Classifier. OTU table was rarefied at the lowest number of sequence and displays the highest taxonomic level. Table was used to build a principal-component analysis (PCA) as a function of the production by using the made4 package of R.

3. Results

3.1 Microbial counts

Among 67 samples, 30, coming from 8 different plants, were defined spoiled on the basis of their main defects: they showed off-flavors formation, gas and slime production, discoloration, and acidification. From each plant

241 different number of samples were supplied. Table 1 reports the microbial counts of the spoiled samples. Both
242 MRS and BHI counts are shown.
243 In spoiled samples, counts ranged from values lower than 1 Log CFU/g to 9 Log CFU/g (the lowest from C
244 and L plants, the highest from I) in both MRS and BHI media. Important differences between the two media
245 were observed for plant F, where LAB counts were higher than population grown on BHI while in the case of
246 plant C, counts resulted higher in BHI for two of the three samples supplied. In the case of plant L, no colonies
247 were observed in the BHI plates (< 1 Log CFU/g).
248 For the samples that were classified as unspoiled when arrived at the laboratory (37 out of 67), the microbial
249 dynamics during shelf life were monitored on MRS and BHI plates. All the samples at the beginning of the
250 shelf life showed a load under 4 Log CFU/g, except samples of plant B where counts reached values of 6 Log
251 CFU/g while samples from plant C and E showed counts under the detection limit (< 1 Log CFU/g). In Figure
252 1, LAB counts on MRS medium at the beginning (B), middle (M) and end (E) of shelf life are reported.
253 In samples from company B, the counts were the highest from the beginning to the end if compared to the
254 other samples. Regarding the other companies, the microbial counts were similar at the middle and at the end
255 of shelf life except for samples produced by company C, where more variability was observed.
256
257 *3.2 Detection of antimicrobial activity and molecular identification*
258 Five spoilage consortia coming from samples with the most evident defects were selected for the inhibition
259 assays. Among 37 consortia from unspoiled samples, 20 showed inhibition against all consortia. A total of 140
260 colonies, with potential antimicrobial activity, were observed. The isolates were then tested against all the
261 spoilage consortia. After this second selection, 17 isolates were chosen according to their ability to inhibit
262 more than 60% of the spoilage consortia. All 17 isolates originated only from samples of the same plant (plant
263 B). The isolates were then identified by RSA and 16S rRNA gene sequencing. Among 17, 16 resulted to be
264 *Lactococcus lactis* subsp. *lactis,* while one was identified as *Enterococcus faecium.* Due to their possible
265 exploitation to preserve sliced cooked ham, the ability to grow at 4°C was investigated for the *L. lactis* strains
266 while the strain identified as *E. faecium* was not used or tested further.

In Table 2 the inhibition activity (expressed as percentage of inhibited consortia tested in MRS and BHI media) of the 16 *L. lactis* strains are reported along with their ability to grow at refrigeration temperatures. Only three strains did not show growth expressed as turbidity in BHI broth. All the strains expressed higher inhibition towards spoilage consortia in MRS compared to those in BHI medium.

In order to identify the nature of the antimicrobial compound, the AWDA was carried out on these 16 strains after neutralization and proteinase K assay. The results highlighted that among 16 isolates, 7 produced halos for acidity while 9 were putative bacteriocin producers. These nine strains were selected and subjected to Rep-PCR followed by cluster analysis where a high level of similarity (90%) was observed (data not shown).

The 9 bacteriocinogenic *L. lactis* strains (named A16, A14, A30, A34, A35, A36, A51 and A52) were subjected to the identification of the bacteriocin genes by PCR. In addition, the fours *L. lactis* strains (41FL18, 41FLL3, 39FL2 and 41FL1) belonging to the culture collection of DISAFA were included from this point forward of the study. The presence of the genes responsible for the nisin A was revealed in all the isolates. Strain 39FL2 resulted positive also for the production of lactoccin Gα, Gβ, Qα and Qβ while A13, A16 and 41FL1 a part from the presence of nisA possessed the gene encoding for lactoccin Gα, Gβ. The bacteriocin activity, expressed as arbitrary units (AU)/ml was also determined (Figure 2). The activity was calculated on M17 medium, with the addition of glucose and lactose.

The results highlighted that the different sugar added to the medium was determinant for the bacteriocin production: all the tested strains showed a higher bacteriocin titer with the presence of glucose (significant differences were observed for strains 39FL2 and A16) and the highest bacteriocin activity was detected for strain A16 with a value of 2346 AU/ml in presence of glucose and for strain 39FL2 with a titer of 7253 AU/ml. The difference in the bacteriocin production was not related to different growth rates in glucose and lactose (data not shown).

3.3 In situ challenge tests

Challenge tests were carried out using the four DISAFA strains as well as strain A16 that was the most performing strain, considering the bacteriocin titer, among the strains isolated from unspoiled sliced cooked ham samples.

294 Non inoculated sliced cooked hams at time zero showed an average of pH of about 6.3 that decreased after 10
295 weeks reaching average value of 5.3. Same trend was observed for all the inoculated samples, with final pH
296 values ranging from 5.06 to 5.3.
297 Microbial counts at time zero showed a maximum of 3 Log CFU/g on both MRS and M17 media, reaching
298 values of 8 Log CFU/g after 10 weeks in all the samples. Regarding yeast population analyzed for samples
299 inoculated with consortium 1, all the samples presented values of about 5 Log CFU/g. No significant
300 differences were observed between the counts of the different trials, including inoculated and control samples
301 (data not shown).
302 Concerning the visual observation, no intense defects were observed after 10 weeks of storage in any of the
303 trials, but discoloration and slime production were randomly observed also when food cultures were
304 inoculated. Only samples inoculated with strain 39FL2 did not show any defects also when in interaction with
305 spoilage consortia (except in the case of consortium 4 where discolorations were observed). In order to follow
306 the development of the microbial populations in the challenge tests performed, amplicon (16S rRNA encoding
307 gene) based sequencing was performed. A total of 90 samples were analyzed and results are shown in Figure
308 3. Not inoculated samples showed a simple microbiota composition across the entire datasets characterized by
309 the presence of *L. sakei*, *Leuc. carnosum* and *Weissella viridescens* that accounted for 68,39%, 26,02% and
310 5,17% of the relative abundance respectively (Figure 3). We observed that *L. sakei* was the dominant OTU
311 also in hams inoculated with the four spoilage consortia together with *Leuc. carnosum.* In samples of
312 consortium 2 *Leu. carnosum* represented above 46,5% of the total OTUs. In addition, *Enterococcus*
313 *pseudoaviuim* was found in samples inoculated with consortia 2 and 3.
314 Comparing the relative abundance of the samples inoculated with the food cultures, it was evident how strain
315 39FL2 was the most competitive against the autochthonous microbiota (Figure 3c) reaching about 46.7 % of
316 the relative abundance. Strains 41FL1, 41FL18 and 41FLL3, despite the inoculum of 6 Log CFU/g, reached
317 respectively 14. 8%, 8% and 14% of the relative abundance after the 10 weeks of storage. Interestingly, by the
318 application of these three food cultures, a reduction of *L. sakei* from 68.4% to about 57% was observed while
319 the % of *Leuc. carnosum* remained constant at about 26% for all the samples. In general, the five starter strains

were more competitive against consortium 1 composed by 4 different species (*Leuc. carnosum, K. servazzi, C. sake* and *Y. lipolytica).*

The trials with the A16 strain (Figure 4) were carried out in a different day and for this reason it had another control compared to the other strains. This control was also composed mainly by *L. sakei*, *Leuc. carnosum* and *Weissella viridescens*. *L. lactis* was detected, with an average abundance of 15 and 23% in trials with consortia 1 and 2 respectively, but it was below the detection limit when co-inoculated with consortia 3 and 4. In the sample with the inoculum of strain A16 alone, a decrease of *L. sakei* and *L. carnosum,* compared to the control, was registered (Figure 4). When co-inoculated with consortium 2, the A16 strain showed a good ability to inhibit *Enterocossus* spp.

Strain 39FL2 resulted the most competitive against consortium 1, 2 and 3 covering respectively 31, 24 and 28% of the relative abundance. Only with consortium 4 (composed by three strains of *L. sakei*), *L. lactis* 39FL2 only reached 3.6% of the relative abundance. By Principal Component Analysis, a significant shift in the microbiota was observed only when strain 39FL2 was used (Figure 5). In this case it is possible to observe a certain degree of separation in the samples inoculated with only the 39FL2 strain compared to the control and to the samples inoculated with the spoilage consortia.

In the case of strain 41FL1, the control and the samples inoculated with food culture showed a high degree of similarity. In the other cases the production with food cultures were not well separated by the ones with spoilage consortia (data not shown).

4. Discussion

Sliced cooked ham is a perishable meat product having low salt content, pH around 6 and water activity higher than 0.945. These physicochemical parameters represent only small hurdles to inhibit microorganisms associated to postprocessing contamination (Aymerich et al., 2005). Re-pasteurization after slicing and packaging is not recommended because of the consequent release of meat juices. In this context the application, after slicing, of alternative hurdles, such as natural antimicrobials, are useful tools in the preservation of this product.

346 Part of the LAB species are generally recognized as safe bacteria and play an important role in preservation of
347 fermented foods. Their ability to produce bacteriocins may be useful also in the preservation of products such
348 as cooked ham (Laursen et al., 2009). The shelf life of processed meat products, if stored under anaerobic
349 conditions or MAP conditions, is generally dictated by LAB growth.
350 In this study, samples of cooked ham were analyzed and microbial counts at the end of shelf life seemed not
351 to be connected to the defects: samples coming from the same plant showed similar microbial counts
352 independently from the fact that they presented or not sensorial defect. Samples from company A and D
353 showed higher counts at the end of shelf life in acceptable samples, if compared to the spoiled ones (Table 1
354 and Figure 1). This underlines that the microbial count is not the only parameter that has to be taken into
355 account to describe or predict spoilage, and it becomes of great importance to properly understand the ecology
356 of the samples when spoilage occurs. Unexpectedly, spoiled samples of plant C (on MRS) and of plant L,
357 showed microbial counts under the detection limit (Table 1). However it has to be considered that spoilage can
358 be driven also by other microorganism that can not be detected by the media and conditions used in this
359 research.
360 As found by Raimondi et al. (2019), the microbiota analysis at the end of the shelf life of unspoiled and spoiled
361 samples of cooked hams, did not unveil any specific taxon that could be surely associated to spoilage,
362 indicating that a negative evolution can be due to strain dependent characteristics (Ercolini et al., 2010).
363 Different microbial loads were observed between companies, underlining how the production plant is
364 determinant in the microbial development (Table 1). Moreover, raw materials and processing steps are of
365 primary importance in the definition of the final microbiota. The hygiene conditions throughout the processing
366 line have great impact on the initial microbial levels, on the microbiota composition (Vihavainen et al., 2007)
367 and consequently influence the onset of spoilage. LAB can contaminate the products during the post-cooking
368 processing. The control of potential spoilage microorganisms through the use of other microorganisms is of
369 great interest. Several studies have demonstrated that autochthonous microbiota can influence the reduction of
370 pathogens such as *L. monocytogenes* in cooked ham (Hwang & Sheen, 2011), while others underlined how
371 autochthonous microbiota do not influence the growth of the pathogens in samples of modified-atmosphere-

372 packaged poultry (Barakat & Harris, 1999). A probable reason for this discrepancy may be attributed to
373 difference in the microbiota investigated in the cited studies.
374 In this study, selection of potential food cultures with protective properties and active towards spoilage
375 microbial consortia, was performed. An advanced experimental design was applied to identify strains with
376 potential to inhibit entire microbial consortia rather than focusing on an isolated, putative spoilage
377 microorganism. The study was focused on the main bacterial spoilage of cooked ham and in particular on LAB
378 that are dominating under psychrotrophic conditions: for this reason, MRS medium, commonly used for LAB
379 detection, was chosen together with BHI, selected for the detection of microbial viable counts. A limitation of
380 the method could be linked to the difficulties of specific microbial groups involved in spoilage of cooked ham
381 as *Carnobacterium* spp. to grow on MRS and BHI media: future studies could adapt the methos including also
382 other specific media. Among 37 consortia coming from unspoiled sliced cooked ham, from 6 plants, 20 showed
383 an inhibition against spoilage consortia. On the basis of the inhibition observed in lab conditions, 140 isolates
384 were selected. These isolates were tested against all the consortia coming from spoiled samples and 17 isolates
385 confirmed a good inhibition against more than 60% of the spoiled consortia tested (Table 2). The selected
386 inhibitor isolates were identified as *L. lactis* (16/17) and *E. faecium* (1/17). Surprisingly the strains selected for
387 their attitude to inhibit the largest number of spoilage consortia came from the ham samples produced in one
388 plant. Only *E. faecium* was not considered further because of the controversial role of this genus *Enterococcus*
389 in food microbiology. Despite the important role in contributing in the taste and flavor of various fermented
390 foods through proteolysis, lipolysis, members of this genus have been associated with human infections
391 (Foulquié Moreno et al., 2006). Furthermore, due to its ability to resist to a wide variety of antibiotic and
392 through the skills to exchange genetic information by conjugation, it may spread antibiotic resistance among
393 non-pathogenic organisms (Cocconcelli et al., 2003). In this light, among the two species, our attention was
394 focused on *L. lactis* species. Comi et al. (2016) used this species to improve the shelf life of cooked bacon
395 where it showed its ability to reduce the risk of *Leuconostoc mesenteroides* spoilage. Comi et al. (2011) also
396 evaluated the oxygen scavenging ability of *L. lactis* ssp. *lactis,* to improve the quality of sliced cooked ham
397 packaged under MAP. Suspensions were spread on sliced cooked ham packed with MAP (oxygen < 0.5%),
398 stored at 4°C for 30 days under light and samples were analyzed during the shelf life to evaluate the quality.

399 *L. lactis ssp. lactis* was able to reduce the oxygen concentration, the lipid oxidation and inhibited the
400 autochthonous LAB, extending the shelf-life of the products to 30–35 days. It was shown that this species
401 influenced the flavor and odor and the stability of the red color of the sliced cooked ham. Despite the high
402 similarity observed by rep-PCR (data not shown), the selected strains showed a different attitude to produce
403 bacteriocins. From the technological point of view, thanks to their provenience from unspoiled samples and,
404 at the same time, to their ability to inhibit a great number of spoilage consortia, plus their attitude to grow at
405 refrigerated temperature, they can be considered in the elaboration of new potential food cultures after the
406 evaluation of their sensory impact on the final product.
407 It has to be considered that the bacteriocin production is strongly dependent on the medium composition
408 (Gänzle et al., 1999) and on the temperature. The results of the present study confirmed glucose as a better
409 carbon source for nisin producing microbes (Guerra et al., 2001). Furthermore comparing the inhibition of the
410 strains against spoiled consortia higher inhibition was detected in MRS than in BHI for all the strains: these
411 phenomena can be explained by the better ability of the LAB to grow in MRS compared to the BHI. The
412 composition of the substrate represented by the food matrix and the surrounding conditions have to be
413 considered also in the understanding of the *in situ* inhibition ability of the strains. As reported by Chollet et
414 al., (2008) also sodium chloride concentration can influence the nisin concentration and its antimicrobial
415 activity.
416 In the challenge tests performed in this study, strain A16 was selected because of its ability to produce the
417 higher titer of bacteriocin compared to the others selected strains. Taking in consideration the bacteriocin titer,
418 strains present in the DISAFA collection were also included in the challenge tests because of their ability to
419 produce bacteriocins. The autochthonous microbiota resulted competitive against the strains selected for their
420 antimicrobial properties inoculated at 6 Log CFU/g. It has to be considered that the analysis of the16S
421 amplicons were performed to have a generic overview: the other population could have covered the presence
422 of the BCs because in a higher concentration. Strain 39FL2 was able to significantly reduce the autochthonous
423 populations, representing the 46.7% of the total OTUs, while in the other cases the inoculated strains covered
424 maximum the 15% of the OTUs (Figure 3). Therefore strain 39FL2 resulted to be the better performing strain

in the inhibition of indigenous cooked ham populations and this could be associated to its ability to produce lactococcins together with nisins.

5. Conclusion

This study provides a detailed analysis of cooked ham coming from plants located in different European countries with own processing procedures. The interaction between a high number of consortia coming from unspoiled and spoiled samples allowed to select strains able to inhibit consortia of different spoiled hams. The power of the study was the selection connected to the inhibition against consortia and not against single or indicator strains. The ability of the strains to inhibit *in vitro* of a high number of spoilage consortia remarks the effectiveness of this methods in the selection of bioprotective cultures. However further investigations need to be conducted on real production where different factors can affect the action of bioprotective cultures, especially for the strong competition of the autochthonous population as resulted by the challenge tests conducted in this study.

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Legend to figures

Figure 1. Microbial evolution of the not spoiled samples during the shelf life on MRS and BHI plates at the beginning (B), middle (M) and end (E) of shelf life. Results were calculated as the means of Log counts for two determinations.

Figure 2. AU/ml of the strains on M17 with glucose or lactose in a concentration of 0.5 % p/v. *L. monocytogenes* EGDe was used as indicator. The results are the mean of three replicates. Values with different letters are significantly different, $P < 0,05$. Capital letters indicate differences among AU/ml in lactose while lowercase letters among AU/ml in glucose.

Figure 3. Relative abundance of the major taxonomic groups. Results are average mean of the biological replicates. Only OTUs with an incidence above 0.5% in at least two samples are shown. Letters a, b, c, d correspond to the production with starters 41FL18, 41FLL3, 39FL2 and 41FL1 respectively. The control (first bar in each histogram) is the same for all trials.

Figure 4. Abundance of the major taxonomic groups of production with A16 strain. Only OTUs with an incidence above 0.5% in at least two samples are shown.

Figure 5. PCA based on the OTU abundance of the strain 39FL2 and its interaction with spoilage consortia after the 10 weeks of storage.

Figure 1

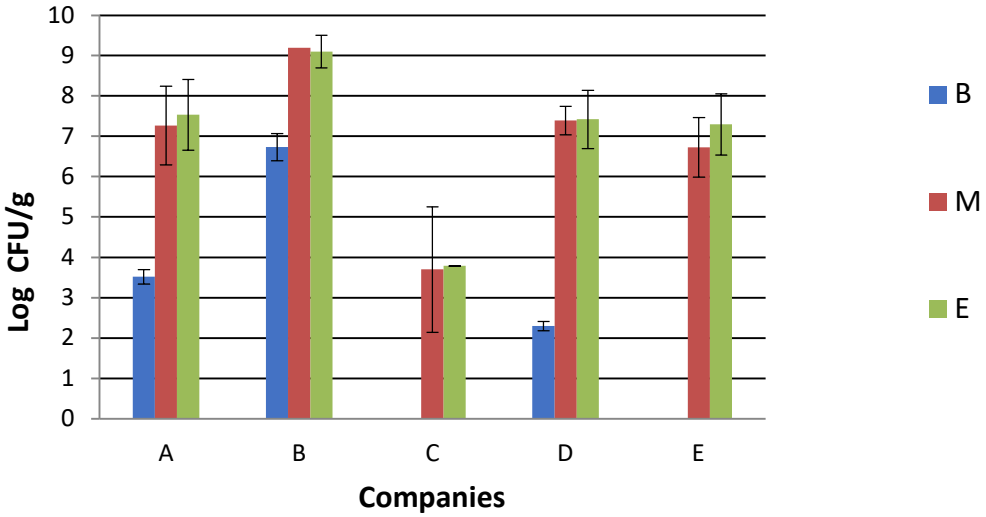

Figure 2

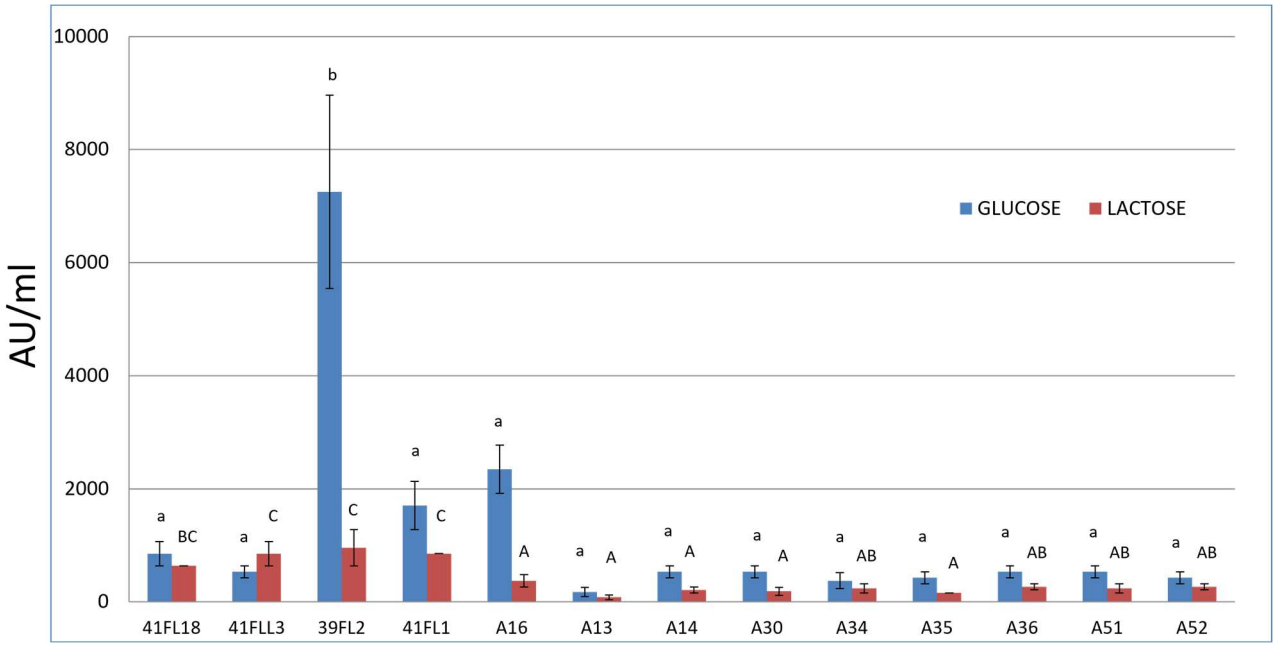

Figure 3

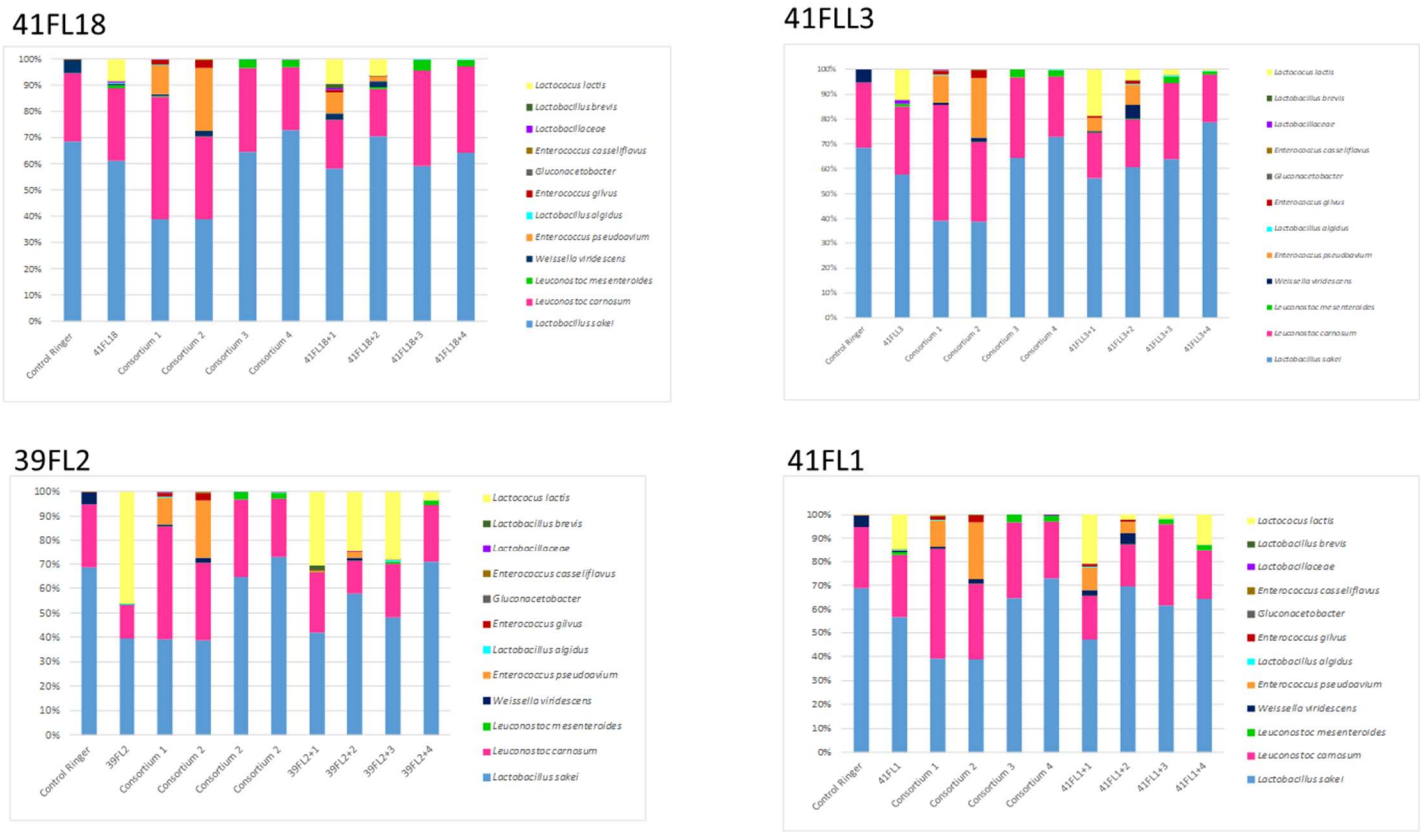

Figure 4

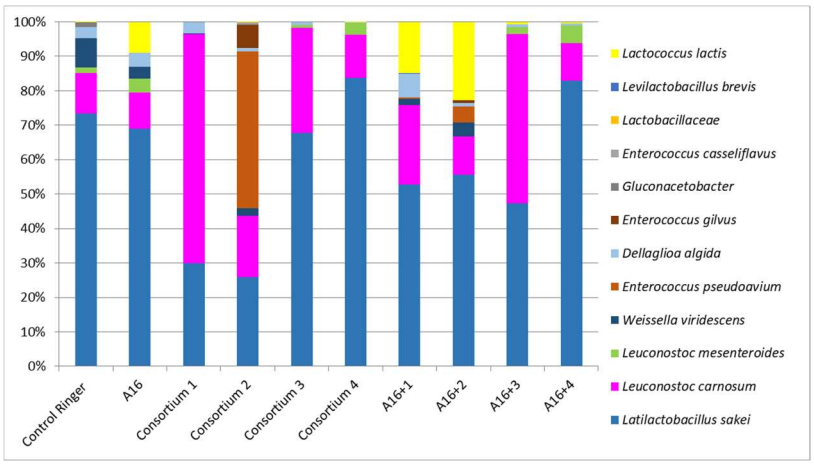

Figure 5

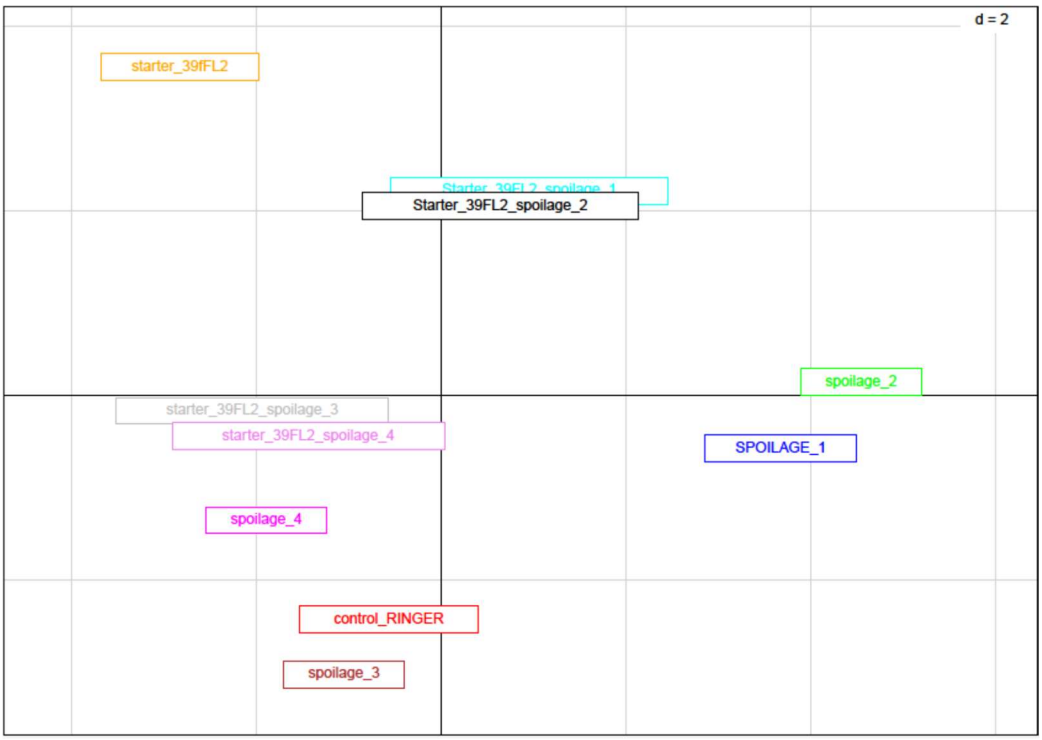
d = 2
starter_39fFL2
Starter_39FL2_spoilage_2
spoilage_2
starter_39FL2_spoilage_3
starter_39FL2_spoilage_4
SPOILAGE_1
spoilage_4
control_RINGER
spoilage_3

Table 1. Microbial count expressed in Log (CFU/g) of the spoiled samples from the different plants (indicated by letters) in MRS and BHI media. Each line corresponds to different ham samples. Results were calculated as the means of Log counts for two determinations.

Producer	BHI counts (CFU/g Log10)	MRS counts (CFU/g Log10)
F	8.12	9.22
	7.53	8.89
	7.68	8.79
	7.44	8.45
	7.05	8.39
	6.65	8.53
	5.9	8.47
	6.06	8.53
	5.58	8.69
G	7.57	7.15
	7.57	7.47
	7.73	7.6
C	5.63	<1
	6.87	6.49
	5.88	2
H	6.96	6.91
	7.1	4.68
	7.36	6.67
	7.64	7.61
D	6.35	6.37
	8.14	7.89
	5.66	7.18
	4.88	8.28
	7.16	6.45
	6.67	8.11
A	5.79	6
I	8.82	9.26
	8.3	9.19
L	<1	5.54
	<1	<1

671 **Table 2.** Inhibition of spoilage consortia (expressed as % of inhibited consortia of the total of 30 tested) both
672 in BHI and MRS by the selected *L. lactis* strains. Ability of the *L. lactis* strains to grow at refrigeration
673 temperature is also shown.
674

Selected strains	% of inhibition against spoilage consortia		*Growth at 4°C
Code	BHI	MRS	4°C.- 72h (BHI)
A3	63.6%	73.9%	+
A4	81.8%	100.0%	-
A5	79.2%	100.0%	-
A6	79.2%	100.0%	-
A12	70.8%	95.7%	+
A13	70.8%	100.0%	+
A14	66.7%	100.0%	+
A16	77.3%	95.7%	+
A30	70.8%	95.7%	+
A34	70.8%	95.7%	+
A35	75.0%	95.7%	+
A36	70.8%	95.7%	+
A37	75.0%	100.0%	+
A50	75.0%	100.0%	+
A51	75.0%	100.0%	+
A52	75.0%	100.0%	+

675
676 * (+/-) indicate the ability (+) and not (-) to grow at 4°C.
677