Lactic acid bacteria as protective cultures in fermented pork meat to prevent Clostridium spp. growth

Lactic acid bacteria as protective cultures in fermented pork meat to prevent *Clostridium* spp. growth

Diana Di Gioia [a,*], Giuseppe Mazzola [a], Ivana Nikodinoska [a,b], Irene Aloisio [a], Tomaz Langerholc [c], Maddalena Rossi [d], Stefano Raimondi [d], Beatriz Melero [b], Jordi Rovira [b]

[a] Department of Agricultural Science, University of Bologna, viale Fanin 42, 40136 Bologna, Italy

[b] Department of Biotechnology and Food Science, University of Burgos, Plaza Misael Bañuelos s/n, 09001 Burgos, Spain

[c] Department of Microbiology, Biochemistry, Molecular Biology and Biotechnology, University of Maribor, Pivola 10, 2311 Hoce, Slovenia

[d] Department of Life Science, University of Modena and Reggio Emilia, Via Campi 183, 41125 Modena, Italy

* Corresponding author: diana.digioia@unibo.it

Abstract In meat fermented foods, Clostridium spp. growth is kept under control by the addition of nitrite. The growing request of consumers for safer products has led to consider alternative bio-based approaches, the use of protective cultures being one of them. This work is aimed at checking the possibility of using two Lactobacillus spp. strains as protective cultures against Clostridium spp. in pork ground meat for fermented salami preparation. Both Lactobacillus strains displayed anti-clostridia activity in vitro using the spot agar test and after co-culturing them in liquid medium with each Clostridium strain. Only one of them, however, namely L. plantarum PCS20, was capable of effectively surviving in ground meat and of performing anti-microbial activity in carnis in a challenge test where meat was inoculated with the Clostridium strain. Therefore, this work pointed out that protective cultures can be a feasible approach for nitrite reduction in fermented meat products.

Keywords: Bioprocesses, Protective cultures, Meat fermentation, Clostridia, Nitrite

1. Introduction

Food safety and preservation are major priorities for consumers and associated industry. In spite of the introduction of advanced technologies and safety concepts (Ojha et al., 2015), the reported number of foodborne illnesses and intoxications is still matter of concern (EFSA, 2015). Fermentation is a hurdle approach which can potentially guarantee food safety (Leroy et al., 2013). However, despite the rigorous hygienic measures and technological safety standards, fermented meats are still not exempted from microbial hazards (EFSA, 2015).

Fermented meat products, such as salami, are partially dehydrated to favor their preservation at ambient temperature. The acidic pH (in the range 5.1–6.0) and water activities lower than 0.94 make microbial growth difficult to occur. Among foodborne pathogens in meat products, a major concern is represented by *Clostridium* species, in particular *Clostridium perfringens* and *Clostridium botulinum* (Akhtar et al., 2009; Golden et al., 2009; Linton et al., 2014). Both can be isolated from raw meat as well as from cooked or uncooked cured meat products (Akhtar et al., 2009; Linton et al., 2014). Regarding *C. perfringens*, almost all outbreaks are the result of temperature abuse, allowing multiplication of clostridia, reaching 6–7 Log CFU/g (Huang, 2003). Spores and, to a certain extent, vegetative cells ingested through contaminated food can survive the acidic conditions of the stomach. In the large intestine, during sporulation and/or germination process, production and release of enterotoxins occur, respectively. Occasionally death may occur, particularly in elderly patients (Songer, 2010). On the other hand, botulism occurs after ingestion of a neurotoxin formed in food when spores germinate and vegetative cells multiply. The toxins of *C. botulinum* are relatively sensitive to heat and are inactivated by heating at 80 °C for 10 min (Mataragas et al., 2008). In dry-processed meat products, nitrites and nitrates are commonly used as curing agents. Their addition in raw meat processing enabled to strongly reduce the risk of clostridia and other foodborne pathogen growth in meat products (Parthasarathy and Bryan, 2012). In particular, the use of these preservatives still remains the most efficient strategy to inhibit the spore germination of *C. botulinum* (Linton et al., 2014). The first half of the 20th century brought a gradual shift from nitrate to nitrite and research studies allowed then to outline that nitrite could result in formation of carcinogenic *N*-nitrosamines (De Mey et al., 2014; Honikel, 2008). Finally, the IARC (International Agency for Research on Cancer) in 2006 concluded that "Ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans" (IARC, 2010).

Nevertheless, the adverse effects of nitrite on health as well as the increasing number of consumer demands for natural, chemical free products (Akhtar et al., 2009; Ojha et al., 2015), has stimulated the research of new strategies to substitute or to reduce the nitrites and other additives, with alternative natural friendly and possibly bio-based methods.

In this context, the approach of using protective cultures, in particular lactic acid bacteria (LAB) against food pathogens appears particularly interesting, as shown by the large number of research works and reviews on the use of protective cultures in food (Chaillou et al., 2014; Comi et al., 2015; Gaggia et al., 2011; Gálvez et al., 2010). A large number of LAB, including those used as protective cultures, are considered safe for human consumption as they possess the GRAS (Generally Recognised As Safe) and/or QPS (Qualified Presumption of Safety) status from the US FDA and the EFSA, respectively. Certain protective LAB strains can produce anti-microbial peptides such as bacteriocins, as well as organic and metabolic compounds formed along the fermentation process, which can inhibit foodborne pathogens growth (Varsha et al., 2015). In addition, a direct competition of protective cultures with potential pathogens is another important mechanism to restrict the growth of undesired organisms. Several works have focused on the use of LAB against foodborne pathogens in meat such as *Listeria* spp., *Salmonella* spp., *Campylobacter jejuni*, *Escherichia coli* (Baffoni et al., 2012; Maragkoudakis et al., 2010; Melero et al., 2012; Osés et al., 2015; Raimondi et al., 2014; Santini et al., 2010), but only a few studies have been specifically focused on *Clostridium* spp. and the possibility of combating this pathogen with the use of protective cultures, as reviewed by Allaart et al. (2013). One of the most relevant application *in carnis* of LAB against clostridia has been described by Nieto-Lozano et al. (2010). This study showed that, during the storage period, *Pediococcus acidilactici* MCH14 pediocin-producing strain was able to reduce *C. perfringens* in Spanish dry-fermented sausages.

Considering the exposed facts, the aim of this study is to evaluate the capability of two *Lactobacillus* strain, *Lactobacillus plantarum* PCS20 and *Lactobacillus delbrueckii* DSM20074, chosen on the basis of their previously demonstrated anti-microbial activity against several pathogens (Santini et al., 2010; Savino et al., 2011), of being protective cultures against *Clostridium* strains in ground meat used for fermented salami production in order to reduce or eliminate the nitrite amount added to meat.

2. Material and methods

2.1. Microorganisms and culture conditions

L. plantarum PCS20(deposited at Microbial Strain Collection of Latvia, accession numbers P 977) and *L. delbrueckii* DSM 20074 were used as protective cultures. *Lactobacillus* strains were grown in Tryptone, Peptone, Yeast Extract medium (TPY, Santini et al., 2010) or in deMan Rogosa Sharpe medium (deMRS, Oxoid, Ltd., Basingstoke, Hampshire, England) incubated in anaerobic conditions, at 37 °C for 48 h, whereas for cell viability evaluation deMRS solid agar (Oxoid) and same incubation conditions were used. Anaerobic conditions were generated using an Anaerocult® A (Merck, Darmstadt, Germany). Where indicated, incubation was performed in aerobic conditions.

C. perfringens DSM 756 and *Clostridium* sp. DSM 1985 were used as target microorganisms. The latter is a surrogate strain, which, based on partial 16S rDNA sequence and as reported on the DSMZ website (https://www.dsmz.de/catalogues/details/culture/DSM-1985.html), is closely related to *Clostridium botulinum* type E, strain ATCC 23387; it does not produce a neurotoxin but a bacteriocin-like substance. Both *Clostridium* strains were grown in TPY medium supplemented with 20% (*v*/*v*) Chopped Meat broth (CM, Oxoid) under anaerobic conditions at 37 °C for 24 h or in Reinforced Clostridial Medium (RCM, Oxoid). For evaluation of viable cell population Reinforced Clostridial Agar (RCA, Oxoid) was used, using the same incubation conditions described above.

2.2. Antagonistic activity of LAB strains against Clostridium spp. strains

2.2.1. Spot agar test

The procedure described in Savino et al. (2011) was used with some modifications as follows. Each *Lactobacillus* strain was grown in MRS broth for 24 h, the culture was neutralized to pH 7 with 1 N NaOH, and centrifuged at 7000*g* for 10 min. Cells were washed and suspended in saline at the concentration of 6 Log CFU/ml. TPY-CM agar plates were inoculated with 50 μl of each *Clostridium* culture at 7 Log CFU/ml. Two sterile paper blank disks (diameter 6 mm) were placed on the agar plate and imbibed with 50 μl of washed *Lactobacillus* cells. After incubation for 24 h at 37 °C in anaerobic conditions, the presence of inhibition zones was evaluated. Each assay was performed in duplicate.

2.2.2. Antimicrobial activity in liquid co-cultures

The capability of the two LAB strains of interfering with the growth of *Clostridium* spp. was evaluated by co-culturing both strains in the same tube and checking survival of both LAB and *clostridium* strains in above mentioned selective agar plates. After verification that LAB growth was not altered in TPY-CM medium with respect to TPY (data not shown), both the LAB and the *Clostridium* strains were pre-grown on TPY-CM medium under anaerobic conditions at 37 °C up to early stationary phase, corresponding to about 8 Log CFU/ml. Tubes containing 10 ml of TPY-CM medium (pH was set to 6.5 with NaON 1 N) were inoculated with: i) 1 ml of each *Lactobacillus* strain (*L. plantarum* PCS20 or *L. delbrueckii* DSM 20074), ii) 1 ml of each *Clostridium* strain (*C. perfringens* DSM 756 and *Clostridium* sp. DSM 1985), iii) 0.5 ml of each *Lactobacillus* strain and 0.5 ml of each *Clostridium* strain (LAB-*Clostridium* co-culture). Subsequently, tubes were incubated in anaerobic conditions at 37 °C. At 4 h intervals, tubes containing cultures were centrifuged for 15 min at 5000*g* and pellets were resuspended in new sterile TPY-CM medium, in order to exclude growth inhibition due to pH variation or nutrient limitation. After this washing step, tubes were incubated in anaerobic conditions as described above. Determination of LAB and *Clostridium* spp. growth was followed after 24 h, 48 h and 72 h of incubation period. TPY agar plates incubated in aerobic conditions at 37 °C was used to allow growth of only LAB strains and in RCA plus mupirocin (100 μg/ml) in anaerobic conditions at 37 °C to allow growth of only *Clostridium* spp. Each experimental condition was set up in triplicate.

2.3. Study design in ground meat

This study was designed to evaluate the potential inhibitory activity of *L. plantarum* PCS20 and *L. delbrueckii* DSM 20074 against a *C. perfringens* strain and a *Clostridium* sp. strain related to *C. botulinum* type E in pork ground meat used for fermented sausage production (salami). The pork ground meat was provided by a local butcher in Bologna (Italy), and contained: ground pork meat containing 30% pork fat, NaCl 2.5% (*w*/*w*), dextrose 0.5% (*w*/*w*) and no other preservatives. The study was performed in the following sequence:

a) evaluation the survival of the two protective cultures in pork ground meat upon inoculation of the strains: with quantitative methods for total lactobacilli evaluation, both in the inoculated and non-inoculated meat at different incubation times, by traditional plate counts and Real time PCR; randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis on MRS grown picked colonies were also performed with the aim to evaluate the percentage of each protective cultures present in the meat, in comparison with native lactic acid bacteria; b) challenge test aimed at studying, *in carnis*, the antimicrobial activity of the protective cultures against *Clostridium* spp., inoculated in pork ground meat in the presence and in the absence of $NaNO_2$ 150 mg/kg in meat.

2.4. Survival of the two protective cultures in pork ground meat

The inocula for the *Lactobacillus* strains were prepared as follows: glycerinated frozen cultures were pre-grown in MRS broth as previously described, 10 ml MRS tubes were then inoculated (2% *v*/*v*) and incubated overnight at 37 °C under anaerobic conditions. The concentration of overnight culture growth was determined through plate counts after serial dilutions and prior their inoculation in pork meat batter.

Subsequently, the cultures were centrifuged and cells resuspended in Ringer solution (Oxoid) in order to achieve 8 Log CFU/ml. Aliquots of this suspension were used to inoculate 30 g of meat, at the theoretical initial concentration of protective culture of about 6 Log CFU/g. The real concentration after inoculation in the meat was evaluated with plate counts.

Three different conditions were set up, each one in triplicate: 1) meat inoculated with *L. plantarum* PCS20, 2) meat inoculated with *L. delbrueckii* DSM 20074, 3) uninoculated control. Each meat portion was placed in a synthetic casing (supplied by Morgan, Florence, Italy). The meat fermentation was developed in 3 days in microaerophilic conditions generated by CampyGen™ Compact (Oxoid) at 22 °C. Meat was sampled after inoculation and after 1, 2 and 3 days of incubation for plate counts and DNA extraction for Real time PCR.

2.5. Challenge test on ground meat

Meat portions prepared as described above (30 g of pork ground meat, added with NaCl at the final concentration of 2.5% and dextrose 0.5%) were challenged either with *C. perfringens* DSM 756 or *Clostridium* sp. DSM 1985 in the presence and absence of $NaNO_2$. *Clostridium* inocula were prepared using the same procedure adopted for *Lactobacillus* strains, except that they were grown in RCM and that the final applied concentration was 4 Log CFU/g of meat. Half of the batches were inoculated with *L. plantarum* PCS20. The batches prepared and conditions used are described in Table 1. Meat fermentation was developed for 9 days in microaerophilic conditions at 22 °C. The amount of lactobacilli and of *Clostridium* spp. was monitored at different incubation times (0, 2, 5 and 9 days).

2.6. Plate counts on meat samples

Meat samples of 2 g were removed aseptically from each tray and homogenized in 18 ml of Buffer Peptone Water (BPW, AESlaboratoire, Bruz, France) for 2 min in 50 ml sterile tubes. Serial dilutions (1:10 in BPW) of the meat homogenate were performed. 1.0 ml aliquot from different dilutions were transferred to petri dishes and subsequently MRS agar medium was poured. Plates were incubated in jars, for 48 h at 37 °C under anaerobic conditions.

2.7. Quantification of Lactobacillus spp. on meat samples with Real time PCR

200 mg of pork meat were used for the DNA extraction. The QIAamp DNA Stool Mini Kit (Qiagen, West Sussex,UK) was used with a slight modification of the standard protocol: an additional incubation at 95 °C for 10 min of the meat sample with the lysis buffer was added to improve the bacterial cell rupture. Extracted DNA was stored at −80 °C. The purity and concentration of extracted DNA were determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite® 200 PRO NanoQuant, Tecan, Mannedorf, Switzerland). The primers used were Lac-F and Lac-R (Castillo et al., 2006). The assays were performed in 20 μl PCR amplification mixture containing 10 μl of Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA,USA), 0.2 mM of each primer, H_2O molecular grade and 2 μl DNA extracted from pork meat at a concentration of 2.5 ng/μl. The amplification was performed using StepOne™ Real-Time PCR Systems (Applied Biosystems). The concentration of both primers was optimized through primer optimization matrices in a 48-well plate and evaluating the best Ct/ΔRn ratio. The primers were also checked for their specificity using the database similarity search program nucleotide-nucleotide BLAST. Moreover, to determine the specificity of amplification, analysis of product melting curve was performed after the last cycle of each amplification. Thermal cycling consisted of an initial denaturation at 95 °C for 20s, followed by 40 cycles at 95 °C 3 s and at 63.5 °C for 30s, respectively for denaturation and annealing temperature. The data obtained from the amplification were then transformed to obtain the number of bacterial Log CFU/g pork meat according to the rRNA copy number available at the rRNA copy number database (Lee et al., 2009). Standard curve was created using 16S rRNA PCR product of *L. brevis* DSM 20054. PCR products were purified with a commercial kit DNA purification system (NucleoSpin® Extract II kit, MACHEREY-NAGEL GmbH & Co. KG, Germany) and the concentration measured at 260 nm. Serial dilutions of PCR product were performed and 10^2, 10^3, 10^4, 10^5, 10^6 copies of the gene were used for calibration.

Table 1
Experimental study design of the Challenge test.

Control batches	Protective culture	*Clostridium* strain	$NaNO_2$ 150 mg/kg
1	−	*C. perfringens*	+
2	−	*C. perfringens*	−
3	−	*Clostridium* sp.	+
4	−	*Clostridium* sp.	−
Challenge test batches			
5	*L. plantarum*	*C. perfringens*	+
6	*L. plantarum*	*C. perfringens*	−
7	*L. plantarum*	*Clostridium* sp.	+
8	*L. plantarum*	*Clostridium* sp.	−

2.8. RAPD PCR

RAPD-PCR was carried out on DNA extracted from randomly selected colonies obtained on MRS agar plates used for counting the total LAB in meat samples. This technique relies on DNA amplification with an arbitrary primer followed by separation on agarose gel. For each experimental conditions (meat inoculated with *L. plantarum* PCS20 and meat inoculated with *L. delbrueckii* DSM 20074), about 100 colonies were picked. DNA extraction from pure colonies was carried out by suspending one colony in 50 μl sterile Tris-EDTA in a single well of a 96 multiwell plate. The plate was subjected to heat treatment at 95 °C for 10 min, cooled at 4 °C, centrifuged at 5000*g* for 5 min to pellet cell debris. DNA containing supernatant was subjected to RAPD-PCR amplification, using primer M13 (5′- GAG GGT GGC GGT TCT) as previously reported in different studies (Cocolin et al., 2009; Rossetti and Giraffa, 2005). Reactions were carried out in a final volume of 20 μl containing 1.2 μl $MgCl_2$ (1.5 mM) (Sigma, Madrid, Spain), 10 μl HotStarTaq Master Mix (Qiagen) 2 μM primer M13 (2 μM), 3.8 μl RNase-free water (Qiagen) and 3 μl template DNA. The amplification cycle was as follows: 45 repetitions at 94 °C for 1 min, 34 °C for 1 min, ramp to 72 °C for 2 min. An initial denaturation at 94 °C for 3 min, and a final extension at 72 °C for 7 min, was also carried out, and subsequently the samples were held at 4 °C in a Mastercycler gradient (Eppendorf, Madrid, Spain). RAPD-PCR products were analyzed by electrophoresis on 2% (*w*/*v*) agarose gels supplied with EtBr, in 1× Tris-Borate-EDTA buffer (TBE, ThermoFisher Scientific, Waltham, MA, USA) at 120 V for 4 h.

2.9. Statistical analysis

A one-way ANOVA was performed to determine significant differences among samples. All the analyses obtained from the Challenge tests were performed using the Statistica 8.0 StatSoft Inc. USA.

3. Results

3.1. Antagonistic activity of LAB strains against Clostridium spp. strains

The results obtained from the spot agar test evidenced that washed cells of both *Lactobacillus* spp. strains were capable of inhibiting the growth of both *Clostridium* strains. The radius of the halos obtained were in the range 2.5–3.5 cm for all the combinations LAB-*Clostridium* assayed. As an example, the inhibitory activity of the two *Lactobacillus*

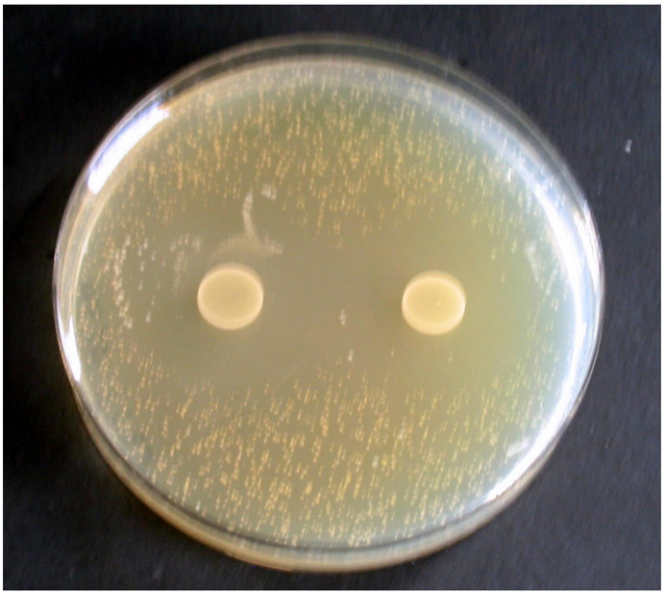


Fig. 1. Spot agar text showing growth inhibition of *C. perfringens* DSM 756 by *L. delbrueckii* DSM 20074 (left) and *L. plantarum* PCS20 (right) strains.

strains assayed against *C. perfringens* DSM756 strains is shown in Fig. 1 (inhibition halo of 3.0 cm).

The results of the anti-microbial activity studies in liquid co-cultures are shown in Figs. 2 and 3, which also report the growth of the *Lactobacillus* and the *Clostridium* strains as single culture. *L. plantarum* PCS20 growth in liquid cultures was not affected by the presence of both *Clostridium* sp. DSM 1985 and *C. perfringens* DSM 756, reaching a 3–4-Log increase after 24 h of incubation and showing a 1-Log decrease at the end of 72 h incubation time both in the presence and in the absence of the *Clostridium* strains (Fig. 2A and B). Both *Clostridium* strains were found to grow well as single culture, but their growth was drastically inhibited by the presence of both *Lactobacillus* strains (Fig. 2A and B). The results obtained were quite similar with the other *Lactobacillus* strain. When *L. delbrueckii* DSM 20074 was cultivated with and without the *Clostridium* strains, the inhibitory activity *versus* the *Clostridium* strains was displayed, although it was lower than that showed by *L. plantarum* PCS20 (Fig. 3A and B). In conclusion, *L. plantarum* PCS20 possess higher inhibitory activity with respect to *L. delbrueckii* DSM 20074 and *Clostridium* sp. DSM 1985 is more sensitive among *Clostridium* strains used in this study.

3.2. Survival of the two protective cultures in pork meat batter

The survival of protective cultures in pork meat batter was evaluated in two batters obtained by the same butcher at two different times. Meat fermentation, developed in laboratory conditions, was performed in microaerophilic conditions at 22 °C, chosen as the closest scenario compared to the realistic one. Plate counts on MRS-agar of meat samples not inoculated and inoculated with PCS20 or DSM 20074 are shown in Table 2. Real time PCR was also performed for the lactobacilli quantification in one of the two meat batters. The initial counts of native bacterial strains capable of growing on MRS was very different in the two meat samples, being 4 Log CFU/g of meat in one of them and 7.15 Log CFU/g in the other one. After two days of incubation, the total count increased in all batches (inoculated with the exogenous

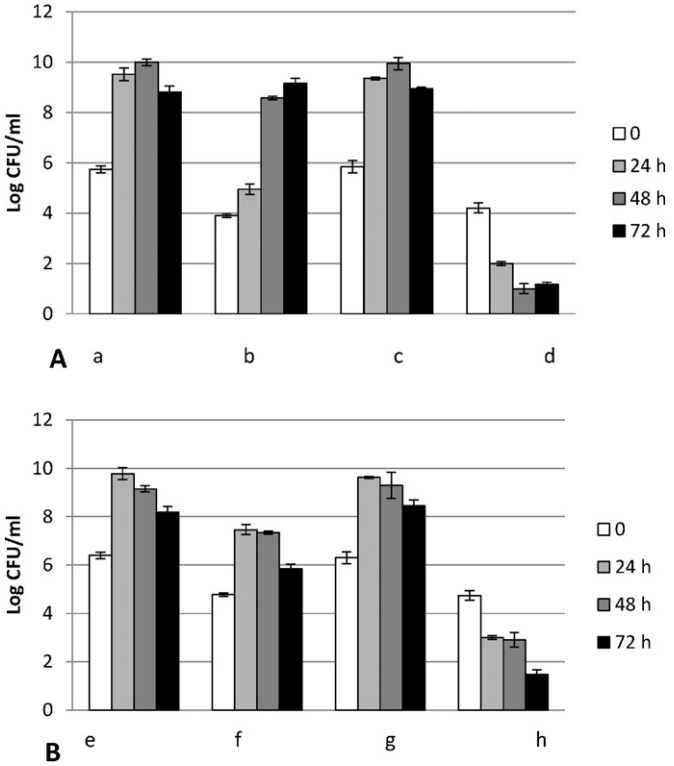


Fig. 2. Antagonistic activity of *L. plantarum* PCS20 in liquid cultures against *Clostridium* sp. DSM 1985 (Fig. 2A): a) *L. plantarum* PCS20 single culture, b) *Clostridium* sp. single culture, c) *L. plantarum* PCS20 co-culture, d) *Clostridium* sp. in co-culture; and *C. perfringens* DSM 756 (Fig. 2B): e) *L. plantarum* PCS20 single culture, f) *C. perfringens* single culture, g) *L. plantarum* PCS20 co-culture, h) *C. perfringens* in co-culture.

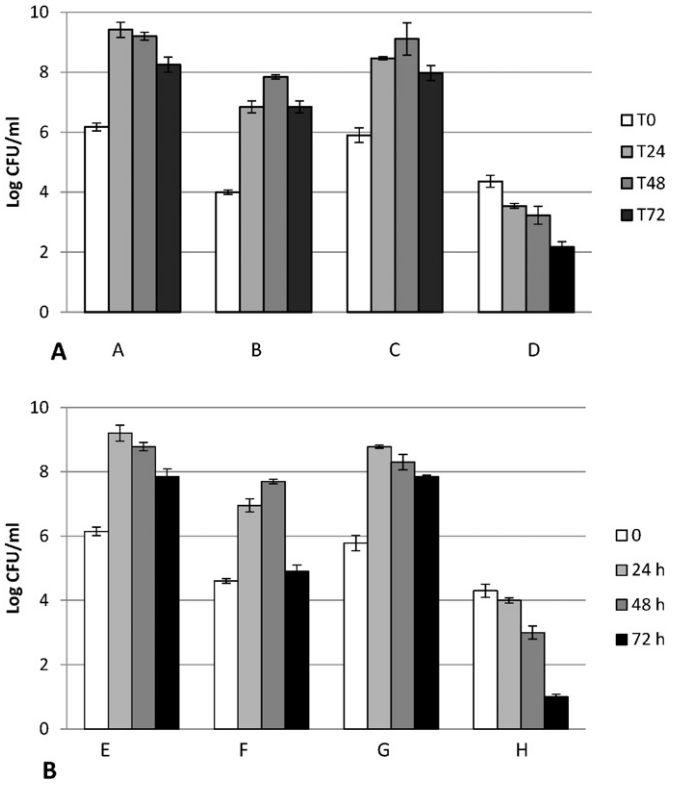
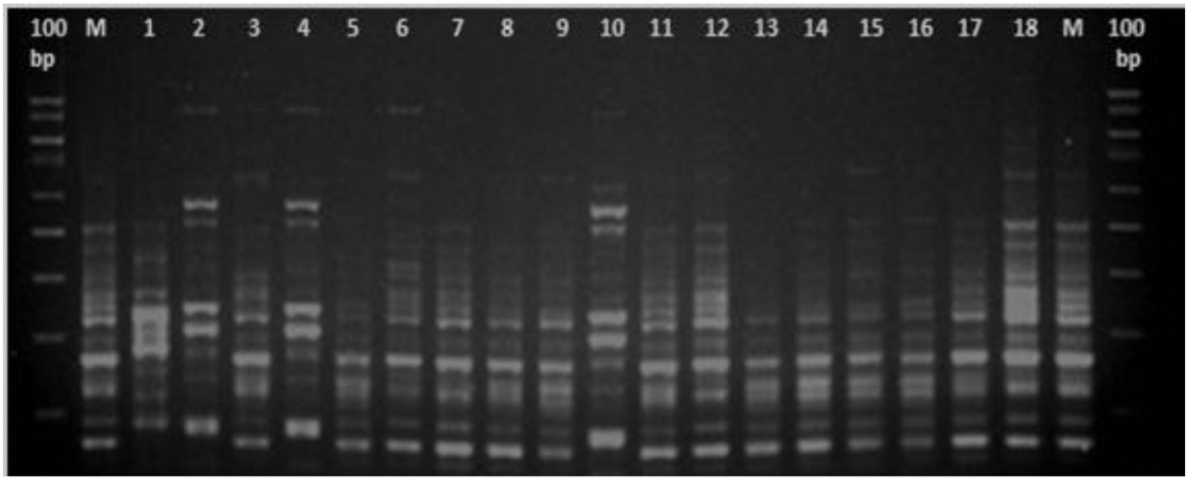


Fig. 3. Antagonistic activity of *L. delbrueckii* DSM 20074 in liquid cultures against *Clostridium* sp. DSM 1985 (Fig. 3A): A) *L. delbrueckii* DSM 20074 single culture, B) *Clostridium* sp. single culture, C) *L. delbrueckii* DSM 20074 co-culture, D) *Clostridium* sp. in co-culture; and *C. perfringens* DSM 756 (Fig. 3B): E) *L. delbrueckii* DSM 20074 single culture, F) *C. perfringens* single culture; G) *L. delbrueckii* DSM 20074 co-culture, H) *C. perfringens* in co-culture.

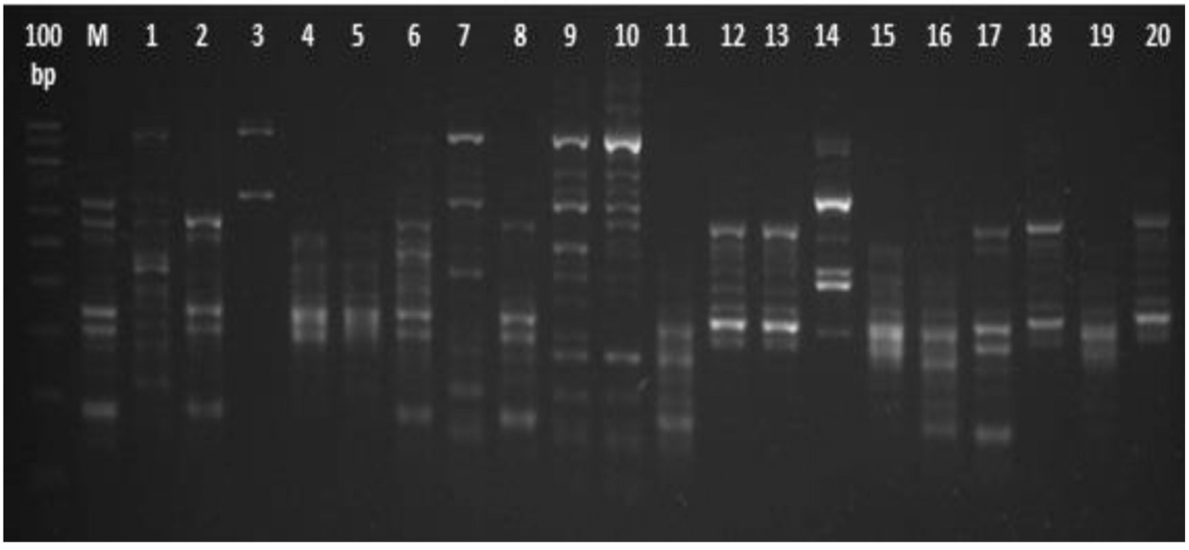
Table 2
Microbial counts on MRS agar plates of the two meat batters considered in the work inoculated with *L. plantarum* PCS20 or with *L. delbrueckii* DSM 200074 and not inoculated with any culture (Control). Counts on meat batter 2 have also been performed with real time PCR using primers targeted to the *Lactobacillus* genus.

Sampling day	Conditions	Log CFU/g		
		Meat batter 1	Meat batter 2	
		Plate counts	Plate counts	Real time PCR
0	+ PCS20	6.10 ± 0.20	7.00 ± 0.35	6.70 ± 0.15
0	+ DSM 200074	6.20 ± 0.15	7.05 ± 0.20	6.80 ± 0.30
0	Control	4.00 ± 0.10	7.15 ± 0.32	6.80 ± 0.15
2	+ PCS20	8.40 ± 0.35	9.15 ± 0.10	8.80 ± 0.25
2	+ DSM 200074	8.50 ± 0.45	8.90 ± 0.25	8.45 ± 0.30
2	Control	8.60 ± 0.25	8.95 ± 0.20	8.70 ± 0.15
4	+ PCS20	8.70 ± 0.25	9.20 ± 0.25	8.50 ± 0.40
4	+ DSM 200074	8.30 ± 0.45	8.80 ± 0.35	7.50 ± 0.30
4	Control	8.40 ± 0.35	8.10 ± 0.20	8.40 ± 0.25
6	+ PCS20	8.50 ± 0.20	9.30 ± 0.35	8.45 ± 0.30
6	+ DSM 200074	8.30 ± 0.35	9.20 ± 0.35	8.80 ± 0.25
6	Control	8.40 ± 0.30	9.15 ± 0.30	9.00 ± 0.45

Lactobacillus spp. and not inoculated) in the range 1.5–2.0 Log CFU/g. At the end of the study period (day 6), MRS agar counts reached the same value of about 9 Log CFU/g in all batches, with and without protective culture inoculation. The morphology of the colonies on MRS plates was variable both in inoculated and not inoculated plates. Therefore, molecular fingerprinting analyses were performed to better understand the survival of each protective culture strain in pork ground meat. As an example, the RAPD pattern profiles obtained from 20 out of the 100 colonies isolated from meat inoculated with *L. plantarum* PCS20, at time zero and after 6 days of incubation, are shown in Fig. 4A and B, respectively. On the whole, the results obtained showed that about 70% of the colonies isolated at time zero had a RAPD profile which could be ascribed to the inoculated strain, whereas 47% had the same profile of the PCS20 strain after 6 days. Conversely, 60% and 11% of the picked colonies could be ascribed to *L. delbrueckii* DSM 20074 at time zero and 6 days of incubation, respectively (data not shown). RAPD analyses of the control sample did not show any colonies with a profile typical of both *L. plantarum* PCS20 and *L. delbrueckii* DSM 20074 strains (data not shown).



A



B

Fig. 4. RAPD profiles of the DNA obtained from 18 = colonies isolated from batch inoculated with *L. plantarum* PCS20 to 20 = colonies isolated from batch inoculated with *L. delbrueckii* DSM 20074 picked colonies. Colonies were obtained from plates resulting from microbial counts of the meat inoculated with protective cultures *L. plantarum* PCS 20 (Fig. 4A) and *L. delbrueckii* DSM 20074 (Fig. 4B). Molecular weight ladder (100 bp); protective culture profile (M); the other lines indicate the profile obtained from the isolated colonies.

3.3. Challenge tests on meat batter

The meat batter used for the challenge tests was checked for absence of detectable clostridia before starting the experiments (data not shown). The effect of *L. plantarum* PCS20 on the growth and survival of *C. perfrigens* DSM 756, both inoculated in pork meat batter in the presence or absence of 150 mg/kg $NaNO_2$, is shown in Fig. 5A. The count of inoculated *C. perfrigens* DSM756 in the meat was 4.2 Log CFU/g at the beginning of the incubation. The inoculated *C. perfrigens* strain was found to survive well in the batter, whereas a significant ($p < 0.05$) reduction of its concentration was observed when $NaNO_2$ was added. When *L. plantarum* PCS20 (initial concentration 9 Log CFU/g, as determined by real time PCR counts) was added to the batter without nitrite treatment, a significant reduction ($p < 0.05$) of *C. perfrigens* was observed between day 2 and 5 in comparison with the batch containing only the pathogen. The results from the last sampling day showed that, after 9 days of the fermentation process, *C. perfrigens* growth was inhibited to 1.5 Log CFU/g in the batches inoculated with the protective culture.

An initial increase of 1 Log CFU/g of the inoculated *Clostridium* sp. DSM 1985 strain was observed up to day 5, followed by a decrease of the strain counts at the last sampling time (Fig. 5B). The same trend was observed when nitrite was added. A reduction of the *Clostridium* strain counts was observed after 5 days of incubation in the batches inoculated with the pathogen and *L. plantarum* PCS20 without nitrite treatment in comparison with the batch with only the pathogen. The results from the last sampling day showed that *Clostridium* counts were reduced to 1 Log CFU/g in meat samples inoculated with *L. plantarum* PCS20, both in the presence and absence of nitrite.

Furthermore, at the end of the incubation time, Real time PCR counts of lactic acid bacteria on the eight batches was in the range 6.8–7.5 Log CFU/g.

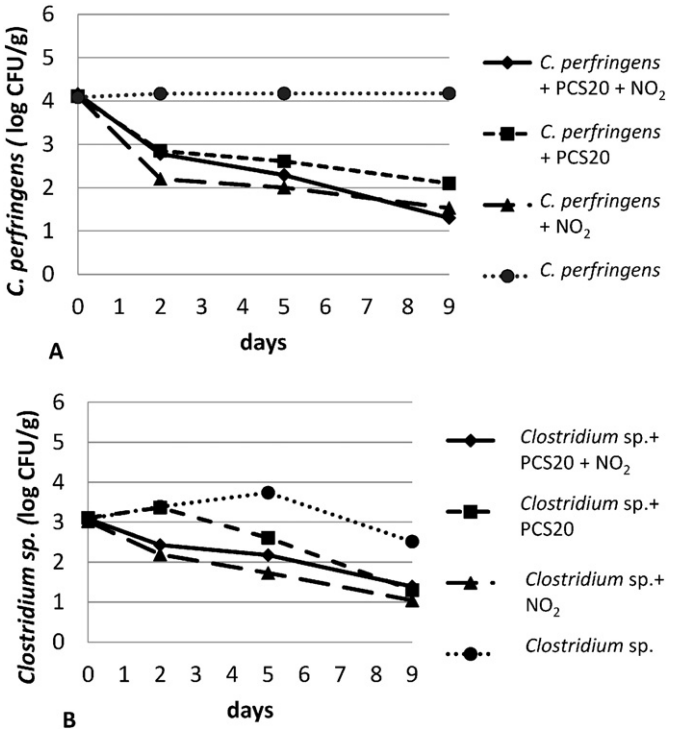


Fig. 5. *Clostridium* counts of pork ground meat challenged with *C. perfringens* DSM 756 (Fig. 5A) and with *Clostridium* sp. DSM 1985 (Fig. 5B) in the presence and absence of *L. plantarum* PCS20 and 150 mg/kg nitrite.

4. Discussion

Clostridium spp. are a serious risk of disease in meat products (Akhtar et al., 2009; Golden et al., 2009; Linton et al., 2014). However, they are kept under control by the addition of nitrite or nitrate as antimicrobial agents, which at present make the risks of *Clostridium* diseases close to zero (Toldrà, 2010). The growing request of consumers for safer products and the concern due to nitrosamine formation have led to the consideration by the food industry of chemical-free alternative approaches (Akhtar et al., 2009; Ergonul, 2013; Ojha et al., 2015), the use of protective cultures being one of them. However, although biopreservation studies have led to reports of efficient protective cultures, some bottlenecks actually limit their application in meat products. In particular, *in vitro* observed antagonistic effects have often been described as abolished *in carnis*, where the adaptation and metabolic activity of inoculated protective cultures can be impaired (Chaillou et al., 2014; Jones et al., 2008, 2009; Linton et al., 2014).

The aim of this work is to study the possibility of using *Lactobacillus* strains as protective cultures against *Clostridium* spp. in meat. The effectiveness of protective cultures in meat products has been studied against *L. monocytogenes*, *C. jejunii* (Melero et al., 2013; Raimondi et al., 2014), and, in meat sausage model systems, against *L. monocytogenes* and *E. coli* (Diaz-Ruiz et al., 2012; Osés et al., 2015) but, to the best of our knowledge, only one application regarding the use of a bacteriocin producing *Pediococcus* strain against *Clostridium* spp. has been described in fermented meat products.

Both *Lactobacillus* strains displayed high antimicrobial activity *in vitro* against the two *Clostridium* strains used in this study. However, RAPD analysis applied to colonies isolated from meat allowed to outline the better adaptation of facultative heterofermentative lactobacilli, such as the *L. plantarum* PCS20 strain with respect to the homofermentative *L. delbruecki* DSM 20074 strain. The growth of the latter was overcome by native lactic bacteria, which are one of the predominant microbial groups in fermented sausages (Rantsiou and Cocolin, 2006). In addition, the temperature used for salami fermentation is not in the range of optimal temperatures for *L. delbrueckii* growth (Arioli et al., 2016).

Meat for salami production is a harsh environment, as it contains a high amount of fat and an initial amount of 2.5% NaCl which increases during the ripening process due to water evaporation. PCS20 is a well studied *L. plantarum* strain (Cho et al., 2010; Maragkoudakis et al., 2010; Nissen et al., 2009; Dimitrovski et al., 2014), known to possess the *pnl* gene for plantaricin production and capable of surviving *in vitro* to low water activity conditions and starvation stresses (Santini et al., 2010). Moreover, it was also found to possess gut health promoting action (Nissen et al., 2009). As Gaggia et al. (2011) pointed out, foods inoculated with LAB protective cultures may have additional functional and beneficial properties for the consumers; thus the concept of "protective cultures" is a broad one and it is not strictly related to food safety concept.

This study shows that the activity of *L. plantarum* PCS20 against *Clostridium* strains was retained *in carnis*. The reduction of the two *Clostridium* strains is not as efficient as the use of the chemical agents; however, an important *Clostridium* reduction (2.0 and 1.5 Log CFU/g of *C.perfringens* and *Clostridium* sp., respectively) was observed after 9 days of fermentation.

The obtained results are important in the perspective of reducing or eliminating the amount of nitrite added to meat products. Even though a number of studies have focused on alternatives to nitrite in meat products, a single compound that performs all its functions has not been found yet (Marco et al., 2006; Alahakoon et al., 2015). Considering that nitrite is also used to maintain a bright red colour in the meat and to obtain the typical "cured meat" taste (Hospital et al., 2015), a reduced amount of nitrite with respect to the traditionally used 150 mg/kg of meat is, hitherto, a more feasible approach for the food processing industry point of view than the complete elimination. Further studies will be therefore focused on a combined approach based on the use of

a reduced amount of nitrite plus the addition of targeted microbial cultures.

This paper also pointed out that real time PCR is an effective and time-saving method for *Lactobacillus* counts in fermented meat products, allowing to obtain results comparable to the officially used plate count procedures. There are several ongoing debates regarding the accuracy of qPCR based approach *versus* traditional count methods in food matrixes (Castillo et al., 2006; Rantsiou and Cocolin, 2006). However, the study here described confirms that qPCR is a reliable method for this purpose.

In conclusion, this work has primarily pointed out that a biopreservative approach, based on the use of protective cultures, can be a feasible solution for the reduction of nitrite in fermented meat products.

Acknowledgements

The research described in this paper was funded by the project LONGLIFE, Joint Action Food Processing for Health of the Joint Programming Initiative a Healthy Diet for a Healthy Life (JPI HDHL). IN was the recipient of a grant funded by the University of Bologna, School of Sciences.

References

Akhtar, S., Paredes-Sabja, D., Torres, J.A., Sarker, M.R., 2009. Strategy to inactivate *Clostridium perfringens* spores in meat products. Food Microbiol. 26, 272–277.

Alahakoon, A.U., Jayasena, D.D., Ramachandra, S., Jo, C., 2015. Alternatives to nitrite in processed meat: Up to date. Trends Food Sci. Technol. 45, 37–49.

Allaart, J.G., van Asten, A.J.A.M., 2013. Gröne A. Comp Immunol Microbiol Infect Dis. 36, 449–464.

Arioli, S., Della, S.G., Remagni, M.C., Stuknyte, M., Colombo, S., Guglielmetti, S., De Noni, I., Ragg, E., Mora, D., 2016. *Streptococcus thermophilus* urease activity boosts *Lactobacillus delbrueckii* subsp. *bulgaricus* homolacticfermentation. Int. J. Food Microbiol. (In Press).

Baffoni, L., Mogna, L., Santini, C., Gaggìa, F., Biavati, B., Di Gioia, D., 2012. A *Bifidobacterium*-based synbiotic product to reduce the transmission of *C. jejuni* along the poultry food chain. Int. J. Food Microbiol. 157, 156–161.

Castillo, M., Martín-Orúe, S.M., Manzanilla, E.G., Badiola, I., Martín, M., Gasa, J., 2006. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. Vet. Microbiol. 114, 165–170.

Chaillou, S., Christieans, S., Rivollier, M., Lucquin, I., Champomier-Vergès, M.C., Zagorec, M., 2014. Quantification and efficiency of *Lactobacillus sakei* strain mixtures used as protective cultures in ground beef. Meat Sci. 97, 332–338.

Cho, J.S., Pietras, E.M., Garcia, N.C., Ramos, R.I., Farzam, D.M., Monroe, H.R., Miller, L.S., 2010. IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. J. Clin. Invest. 120, 1762–1773.

Cocolin, L., Dolci, P., Rantsiou, K., Urso, R., Cantoni, C., Comi, G., 2009. Lactic acid bacteria ecology of three traditional fermented sausages produced in the north of Italy as determined by molecular methods. Meat Sci. 82, 125–132.

Comi, G., Tirloni, E., Andyanto, D., Manzano, M., Iacumin, L., 2015. Use of bio-protective cultures to improve the shelf-life and the sensorial characteristics of commercial hamburgers. LWT Food Sci. Technol. 62, 1198–1202.

De Mey, E., De Klerck, K., De Maere, H., Dewulf, L., Derdelinckx, G., Peeters, M.C., Fraeye, I., Vander Heyden, Y., Paelinck, H., 2014. The occurrence of *N*-nitrosamines, residual nitrite and biogenic amines in commercial dry fermented sausages and evaluation of their occasional relation. Meat Sci. 96, 821–828.

Diaz-Ruiz, G., Ben Omar, N., Abriouel, H., Martínez Cañamero, M., Gálvez, A., 2012. Inhibition of *Listeria monocytogenes* and *Escherichia coli* by bacteriocin-producing *Lactobacillus plantarum* EC52 in a meat sausage model system. Afr. J. Microbiol. Res. 6, 1103–1108.

Dimitrovski, D., Cencic, A., Winkelhausen, E., Langerholc, T., 2014. *Lactobacillus plantarum* extracellular metabolites: in vitro assessment of probiotic effects on normal and cancerogenic human cells. Int. Dairy J. 39, 293–300.

EFSA, 2015. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. URL http://www.efsa.europa.eu/it/efsajournal/pub/3991.

Ergonul, B., 2013. Consumer awareness and perception to food safety: a consumer analysis. Food Control 32, 461–471.

Gaggia, F., Di Gioia, D., Baffoni, L., Biavati, B., 2011. The role of protective and probiotic cultures in food and feed and their impact in food safety. Trends Food Sci. Technol. 22, S58–S66.

Gálvez, A., Abriouel, H., Benomar, N., Lucas, R., 2010. Microbial antagonists to food-borne pathogens and biocontrol. Curr. Opin. Biotechnol. 21, 142–148.

Golden, N.J., Crouch, E.A., Latimer, H., Kadry, A.R., Kause, J., 2009. Risk assessment for *Clostridium perfringens* in ready-to-eat and partially cooked meat and poultry products. J. Food Prot. 7, 1376–1384.

Honikel, K.O., 2008. The use and control of nitrate and nitrite for the processing of meat products. Meat Sci. 78, 68–76.

Hospital, X.F., Carballo, J., Fernández, M., Arnau, J., Gratacós, M., Hierro, E., 2015. Technological implications of reducing nitrate and nitrite levels in dry-fermented sausages: typical microbiota, residual nitrate and nitrite and volatile profile. Food Control 57, 275–281.

Huang, L., 2003. Dynamic computer simulation of *Clostridium perfringens* growth in cooked ground beef. Int. J. Food Microbiol. 87, 217–227.

Ingested nitrate and nitrite, and cyanobacterial peptide toxins. In: IARC (Ed.), Monographs on the Evaluation of Carcinogenic Risks to Humans. Volume 94, p. 450.

Jones, R.J., Hussein, H.M., Zagorec, M., Brightwell, G., Tagg, J.R., 2008. Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. Food Microbiol. 25, 228–234.

Jones, R.J., Zagorec, M., Brightwell, G., Tagg, J.R., 2009. Inhibition by *Lactobacillus sakei* of other species in the flora of vacuum packaged raw meats during prolonged storage. Food Microbiol. 26, 876–881.

Lee, Z.M., Bussema, C., Schmidt, T.M., 2009. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Res. 37, 489–493.

Leroy, F., Geyzen, A., Janssens, M., De Vuyst, L., Scholliers, P., 2013. Meat fermentation at the crossroads of innovation and tradition: a historical outlook. Trends Food Sci. Technol. 31, 130–137.

Linton, M., Connolly, M., Houston, L., Patterson, M.F., 2014. The control of *Clostridium botulinum* during extended storage of pressure-treated, cooked chicken. Food Control 37, 104–108.

Maragkoudakis, P., Chingwaru, W., Gradisnik, L., Tsakalidou, E., Cencic, A., 2010. Lactic acid bacteria efficiently protect human and animal intestinal epithelial and immune cells from enteric virus infection. Int. J. Food Microbiol. 141, S91–S97.

Marco, A., Navarro, J.L., Flores, M., 2006. The influence of nitrite and nitrate on microbial, chemical and sensory parameters of slow dry fermented sausage. Meat Sci. 73, 660–673.

Mataragas, M., Skandamis, P.N., Drosinos, E.H., 2008. Risk profiles of pork and poultry meat and risk ratings of various pathogen/product combinations. Int. J. Food Microbiol. 126, 1–12.

Melero, B., Diez, A.M., Rajkovic, A., Jaime, I., Rovira, J., 2012. Behaviour of non-stressed and stressed *Listeria monocytogenes* and *Campylobacter jejuni* cells on fresh chicken burger meat packaged under modified atmosphere and inoculated with protective culture. Int. J. Food Microbiol. 158, 107–112.

Melero, B., Vinuesa, R., Diez, A.M., Jaime, I., Rovira, J., 2013. Application of protective cultures against *Listeria monocytogenes* and *Campylobacter jejuni* in chicken products packaged under modified atmosphere. Poult. Sci. 92, 1108–1116. Nieto-Lozano J.C., Reguera-Useros J.I., Peláez-Martínez M.d.C., Sacristán-Pérez-Minayo G., Gutiérrez-Fernández A.J. la Torre A.H.d. The effect of the pediocin PA-1 produced by *Pediococcus acidilactici* against *Listeria monocytogenes* and *Clostridium perfringens* in Spanish dry-fermented sausages and frankfurters. *Food Control* **21**, 2010, 679–685.

Nieto-Lozano, J.C., Reguera-Useros, J.I., Peláez-Martínez, M.d.C., Sacristán-Pérez-Minayo, G., Gutiérrez-Fernández, A.J., la Torre, A.H.d., 2010. The effect of the pediocin PA-1 produced by *Pediococcus* acidilactici against *Listeria monocytogenes* and *Clostridium perfringens* in Spanish dry-fermented sausages and frankfurters. Food Control 21, 679–685.

Nissen, L., Chingwaru, W., Sgorbati, B., Biavati, B., Cencic, A., 2009. Gut health promoting activity of new putative probiotic/protective *Lactobacillus* spp. strains: a functional study in the small intestinal cell model. Int. J. Food Microbiol. 135, 288–294.

Ojha, K.S., Kerry, J.P., Duffy, G., Beresford, T., Tiwari, B.K., 2015. Technological advances for enhancing quality and safety of fermented meat products. Trends Food Sci. Technol. 44, 105–116.

Osés, S.M., Diez, A.M., Gómez, E.M., Wilches-Pérez, D., Luning, P.A., Jaime, I., Rovira, J., 2015. Control of *Escherichia coli* and *Listeria monocytogenes* in suckling-lamb meat evaluated using microbial challenge tests. Meat Sci. 110, 262–269.

Parthasarathy, D.K., Bryan, N.S., 2012. Sodium nitrite: the “cure” for nitric oxide insufficiency. Meat Sci. 92, 274–279.

Raimondi, S., Popovic, M., Amaretti, A., Di Gioia, D., Rossi, M., 2014. Anti-*Listeria* starters: *in vitro* selection and production plant evaluation. J. Food Prot. 77, 837–842.

Rantsiou, K., Cocolin, L., 2006. New developments in the study of the microbiota of naturally fermented sausages as determined by molecular methods: a review. Int. J. Food Microbiol. 108, 255–267.

Rossetti, L., Giraffa, G., 2005. Rapid identification of dairy lactic acid bacteria by M13-generated, RAPD-PCR fingerprint databases. J. Microbiol. Methods 63, 135–144.

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioia, D., Biavati, B., 2010. Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. Int. J. Food Microbiol. 141, S98–S108.

Savino, F., Cordisco, L., Tarasco, V., Locatelli, E., Di Gioia, D., Oggero, R., Matteuzzi, D., 2011. Antagonistic effect of *Lactobacillus* strains against gas-producing coliforms isolated from colicky infants. BMC Microbiol. 11, 157.

Songer, J.G., 2010. Clostridia as agents of zoonotic disease. Vet. Microbiol. 140, 399–404.

Toldrà, F., 2010. Handbook of Meat Processing, ed. Wiley-Blackwell, USA.

Varsha, K.K., Devendra, L., Shilpa, G., Priya, S., Pandey, A., Nampoothiri, K.M., 2015. 2,4-Di-*tert*-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated *Lactococcus* sp. Int. J. Food Microbiol. 211, 44–50.