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Evaluation of *Lactobacillus casei* ATCC 393 protective effect against spoilage of probiotic dry-fermented sausages

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ABSTRACT

The aim of the present study was to investigate potential protective effects of both free and immobilized *Lactobacillus casei* ATCC 393 on wheat grains against spoilage and pathogenic microbes in probiotic dry-fermented sausages containing reduced or negligible amounts of curing salts. The results showed that the probiotic cultures resulted in significant increase of self-life and the resistance to spoilage was more prominent in samples containing immobilized cells. Although spoilage was mainly due to yeasts/moulds overgrowth, a drastic decrease was observed in enterobacteria, staphylococci and pseudomonads counts. Microbial diversity was further studied applying a PCR-DGGE protocol. Members of *Lactobacillus*, *Lactococcus*, *Saccharomyces* and *Kluyveromyces* and *Debaryomyces hansenii* or *Priceomyces carsonii* were the main microbial populations detected. Noticeably, members of *Rhodococcus*, *Saccharothrix* or *Micromonospora* were only detected in sausages produced with no starter culture. Microbiological and strain-specific multiplex PCR analysis confirmed that the levels of *L. casei* ATCC 393 in all probiotic samples after 71 days of ripening ranged above the minimum concentration for conferring a probiotic effect (≥6 log cfu/g).

1. Introduction

Over the past years, microbial spoilage has reached a growing importance in food safety. Meat products are excellent food substrates for spoilage and pathogenic bacterial contamination. It is well known that nitrite and nitrate are required in sausage fermentation technology as curing agents for microbial stability and colour formation (Leroy, Verluyten, & De Vuyst, 2006). Nitrites are reduced to nitric oxide, which reacts with the myoglobin to form nitrosomyoglobin, the compound responsible for the typical pink color of cured sausage (Ammor & Mayo, 2007). Although food preservatives are widely used to suppress undesired microorganisms and prolong shelf-life, nowadays there has been an upsurge of interest in the use of protective lactic acid cultures. Hence, biopreservation has been proved a helpful tool to avoid overuse of food additives (Vermeiren, Devlieghere, & Debevere, 2004).

Apart from a product resistant to spoilage, consumers are also seeking for innovative foods able to provide potential health benefits. Probiotic products receive market interest as health-promoting, functional foods, because intake of probiotics may stimulate the growth of beneficial microorganisms, reduce the amount of pathogens, boost the immune system, prevent food allergies and intolerances, relieve the symptoms of inflammatory bowel diseases, irritable bowel syndrome, colitis, alcoholic liver disease, and may reduce the risk for colon, liver and breast cancers (Cross, 2002; Mitropoulou, Nedovic, Goyal, & Kourkoutas, 2013; Prado, Parada, Pandey, & Soccol, 2008).

To deliver the health benefits, probiotics need to contain an adequate amount of live bacteria (at least 10^6–10^7 cfu/g) (Boylston, Vinderola, Ghoddusi, & Reinheimer, 2004), able to survive the acidic conditions of the upper GI tract and proliferate in the intestine, a requirement that is not always fulfilled (Boylston et al., 2004). In general, the food industry has adopted the recommended level of 10^6 cfu/g of probiotic bacteria at the time of consumption. Thus, a daily intake of at least 10^8–10^9 viable cells, which could be achieved with a daily consumption of at least 100 g of

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probiotic food, has been suggested as the minimum intake to provide a probiotic effect.

Immobilization techniques are usually applied in order to maintain cell viability, activity and functionality. Thus, many studies have focused on immobilization of probiotic bacteria in various supports, such as starch (Mattila-Sandholm et al., 2002), fruit pieces (Kourkoutas, Xolias, Kallis, Bezirtzoglou, & Kanellaki, 2005; Kourkoutas et al., 2006) and casein (Dimitrellou, Kourkoutas, Koutinas, & Kanellaki, 2009). These efforts aimed at stabilization of cells and formulation of new types of foods fortified with immobilized health-promoting bacteria that are only released upon reaching the human gut. Likewise, wheat grains (Bosnea et al., 2009) were previously used as immobilization supports of *Lactobacillus casei* and the freeze-dried immobilized biocatalysts were found suitable for production of mild and low pH dairy products. Cereals, such as wheat, are expected not only to have the ability to deliver immobilized probiotic LAB to the human gut when used as immobilization supports, but they also contain potential prebiotic compounds, the functional properties of which should be explored (Charalampopoulos, Pandiella, & Webb, 2003).

Among LAB, *L. casei* ATCC 393 strain has been extensively added into food products (Kourkoutas et al., 2005; Kourkoutas et al., 2006; Li, Chen, Cha, Park, & Liu, 2009) to confer probiotic properties (Choi et al., 2006; Lye, Rusul, & Liong, 2010; Sidira et al., 2010; Saxami et al., 2012). Recently, immobilized *L. casei* ATCC 393 cells on wheat were successfully used in the production of probiotic dry-fermented sausages and their effective survival during ripening and heat-treatment was documented (Sidira, Karapetsas, Galanis, Kanellaki, & Kourkoutas, 2014).

Therefore, the aim of the present study was to investigate potential protective effects of both free and immobilized *L. casei* ATCC 393 on wheat grains against spoilage and pathogenic microbes in probiotic dry-fermented sausages. Data supporting prolongation of shelf-life of products containing significantly reduced or negligible amounts of nitrate, nitrites and sodium chloride are presented.

2. Materials and methods

2.1. Bacterial strain and culture conditions

L. casei ATCC 393 (DSMZ, Germany) was grown at 37 °C for 72 h on MRS Broth. Pressed wet weight cells (≈0.5–1.0 g dry weight) were prepared and used directly for production of probiotic dry-fermented sausages.

2.2. Preparation of support and cell immobilization

Wheat grains were boiled and sterilized at 130 °C for 15 min. Cell immobilization was carried out as described previously (Bosnea et al., 2009). In brief, 500 g of wheat grains along with 8 g (wet weight) of *L. casei* cells were introduced in 2 L cell culture. The mixture was allowed to ferment at 37 °C for 48 h without agitation. When immobilization was complete, the fermented liquid was decanted and the immobilized biocatalyst was washed twice with sterile ¼ strength Ringer's solution (Sigma–Aldrich, UK).

2.3. Production of probiotic dry-fermented sausages

Dry-fermented sausages were prepared according to traditional techniques and recipes (Sidira et al., 2014). In brief, a batch consisting of ground pork meat (2.0 kg), lard (0.5 kg), ground orange peel (25.0 g), ground leek (412.5 g), white pepper (3.75 g), red pepper (3.75 g), cumin (3.75 g), ground garlic (1.25 g), oregano (10.0 g), sucrose (15.0 g) and lactose (5.0 g) was inoculated with free (2.5 g wet weight; F-samples) or immobilized cells of *L. casei* (250 g wet weight, I-samples) on wheat. After mixing, the stuffing of natural casings produced fresh sausages. For comparison reasons, sausages with no culture (samples NC) were also produced. Of note, the initial cell counts of *L. casei* ATCC 393 in probiotic products ranged in levels >6 log cfu/g in all cases (when incorporated in either immobilized or free form).

To investigate the effect of preservatives and salt, dry-fermented sausages were produced containing 0.02% $NaNO_2$, 0.02% $NaNO_3$ and 2% NaCl (P-samples) or 0.01% $NaNO_2$, 0.01% $NaNO_3$ and 1% NaCl (RP-samples). Finally, dry-fermented sausages with no preservatives (NP-samples) were prepared. Hence, in total nine samples were produced: sample I–P containing immobilized cells of *L. casei* and preservatives; sample F–P containing free cells of *L. casei* and preservatives; sample NC–P containing no starter culture and preservatives; sample I-RP containing immobilized cells of *L. casei* and reduced amount of preservatives; sample F–RP containing free cells of *L. casei* and reduced amount of preservatives; sample NC–RP containing no starter culture and reduced amount of preservatives; sample I–NP containing immobilized cells of *L. casei* and no preservatives; sample F–NP containing free cells of *L. casei* and no preservatives; sample NC–NP containing no starter culture and no preservatives.

Ripening was carried out at room temperature (19–23 °C with a relative humidity between 40 and 85%) for 12 days and then the temperature was decreased to 4–6 °C at a rate of 2–4 °C/day with a relative humidity between 50 and 75% for up to 71 days.

All experiments were carried out in triplicate (three independent batches of sausages were prepared). Samples from each treatment were collected at various intervals and subjected to chemical, microbiological and molecular analysis.

2.4. Chemical analysis

Titratable acidity (TA) was determined as described previously (Zaika, Zell, Smith, Palumbo, & Kissinger, 1976), while pH was determined with a pHmeter (WTW, pH-330i pHmeter, Germany) by direct insertion into the samples. Water activity (aw) determination was carried out with a calibrated electric hygrometer (HygroLab, Rotronic, Switzerland) according to manufacturer's instructions. Weight loss was calculated by weighing the sausages just after stuffing (day 1) and by reweighing on the 2nd, 3rd, 4th, 9th and 12th day. The differences in weight were expressed as % percentage of the initial weight.

2.5. Microbiological analysis

The samples were subjected to microbiological analysis to monitor the dynamic changes in the population responsible for ripening of fermented sausages and their hygienic quality. The analysis was carried out as described previously (Sidira et al., 2014).

2.6. PCR-DGGE analysis

PCR-DGGE analysis was performed as previously described (Sidira et al., 2014). Briefly, 10 g portions of sausages samples taken from the interior were blended with 40 ml of sterilized ¼ Ringer's solution (Sigma–Aldrich). Big debris was allowed to deposit for 1 min and 1 mL of supernatant was used for DNA extraction using a DNeasy Tissue Kit (Qiagen, Germany) according to the manufacturer's protocol.

Bacterial DNA was amplified with primers P1 (5′ GCG GCG TGC CTA ATA CAT GC 3′) and P2 (5′ TTC CCC ACG CGT TAC TCA CC 3′) (Cocolin, Manzano, Cantoni, & Comi, 2001), while for eukaryotic DNA amplification, primers NL1 (5′ GCC ATA TCA ATA AGC GGA GGA

AAA G3′) and LS2 (5′ ATT CCC AAA CAA CTC GAC TC 3′) (Cocolin, Bisson, & Mills, 2000) were used.

The PCR products were subjected to DGGE analysis by using an INGENYphorU DGGE system (Ingeny, The Netherlands) (Sidira et al., 2014). Followed the electrophoresis, the gels were scanned with a fluorescent imager (Molecular Imager FX, BioRad, Italy) and the bands of interest were excised.

2.7. Sequencing of DGGE fragments and data analysis

Sequencing of DGGE fragments and data analysis was carried out according to Sidira et al. (2014). In brief, DGGE fragments to be sequenced were excised from the gel with a sterile scalpel. The fragments were then transferred in 100 μl of sterile water and the DNA of the bands were left to diffuse overnight at 4 °C. Ten microliters of the eluted DNA from each band were reamplified with primers P1 and P2 for bacteria and NL1 and LS2 for eukaryotes, respectively. The PCR products were purified, and sent for sequencing to VBC-Biotech, Austria. Searches in the GenBank with the BLAST program were performed to determine the closest known relatives of the partial rRNA sequences obtained. DGGE analyses were performed at least twice.

2.8. Identification of L. casei ATCC 393 by multiplex PCR

To confirm the presence/absence of our strain in dry-fermented sausages, a recently described methodology was followed (Saxami et al., 2012; Sidira et al., 2010, 2014). After enumeration of lactobacilli on MRS agar, the plates corresponding to all dilutions were washed with 10 mL sterilized ¼ Ringer's solution (Sigma–Aldrich), and then the cell suspensions were subjected to molecular analysis based on multiplex PCR for the detection of *L. casei* ATCC 393 (Karapetsas, Vavoulidis, Galanis, Sandaltzopoulos, & Kourkoutas, 2010).

2.9. Spoilage evaluation

Spoilage was determined macroscopically and by sensory tests. A scoring scale with three categories was used: class 1 corresponded to high quality product without any off odor or off flavor, class 2 corresponded to product with slight off odors or off flavors but still acceptable and class 3 corresponded to product of unacceptable quality. The shelf-life limit was defined as the point when 50% of the panellists rejected the sausage samples.

2.10. Experimental design and statistical analysis

All treatments were carried out in triplicate (three independent batches of sausages were prepared). The experiments were designed and analyzed statistically by ANOVA. Duncan's multiple range test was used to determine significant differences among results (coefficients, ANOVA tables and significance ($P < 0.05$) were computed using Statistica v.5.0).

3. Results

3.1. Physicochemical analysis

The effect of the probiotic culture, the addition of preservatives and the ripening time on physicochemical characteristics was studied (data not shown). All parameters were significantly affected by the above factors ($P < 0.05$). A significant increase in titratable acidity and a significant decrease in weight loss, pH and water activity (aw) were noted during maturation in all samples.

3.2. Microbiological analysis

Data concerning spoilage of the dry-fermented sausages are presented in Table 1. The probiotic culture and addition of preservatives (nitrates, nitrites and sodium chloride) resulted in significant increase of self-life, as expected. Noticeably, the resistance to spoilage was more prominent in samples containing immobilized cells. Spoilage was mainly due to white spots on the sausage surface and to white thin fibers in the interior attributed to yeasts/moulds overgrowth, resulting also in slight formation of off-odors and off-flavors, but the products were still acceptable.

All microbial counts were significantly affected by the probiotic culture, the addition of preservatives and the ripening time ($P < 0.05$) (data not shown). While total aerobic counts and levels of lactobacilli and yeasts/moulds remained high during ripening, a drastic decrease was observed in enterobacteria, pseudomonads and staphylococci counts, which dropped to undetectable levels. Likewise, *Clostridium* spp. was not detected in any sample during the whole ripening period.

3.3. Determination of microbial biodiversity using molecular techniques

Probiotic dry-fermented sausages produced with immobilized (I-samples), free cells (F-samples) and with no starter culture (NC-samples) containing different content of preservatives (samples P, RP and NP) were subjected to PCR-DGGE analysis, in order to examine the microbial ecology. The results are presented in Tables 2 and 3 and in Fig. 1. Assays were conducted in triplicate and identical profiles were obtained for the same sausage type. The probiotic strain *L. casei* ATCC 393 was confirmed in I- and Fr-sausages, but not in NC-samples, as expected. Other species of *Lactobacillus* and members of the genera *Lactococcus* were detected as the main bacterial populations. Noticeably, members of *Rhodococcus*, *Saccharothrix* or *Micromonospora* were only detected in sausages produced with no starter culture (NC-samples).

Concerning the yeast/moulds ecology, *Debaryomyces hansenii* or *Priceomyces carsonii* and members of *Saccharomyces* and *Kluyveromyces* dominated. Of note, *Allium* species, added during production as ground leek, was also identified.

3.4. Molecular identification of L. casei ATCC 393

After cell enumeration, the presence/absence of *L. casei* ATCC 393 in petri dishes corresponding to all dilutions was confirmed by multiplex PCR. Identification was carried out by multiplex PCR assay using strain-specific (Karapetsas et al., 2010) and universal for lactobacilli (Walter et al., 2001) primers.

Table 1
Effect of preservatives and probiotic starter culture on shelf-life of dry-fermented sausages.

Sample code	Preservatives	Starter culture	Day of spoilage
I–P	0.02% $NaNO_2$,	Immobilized	No
F–P	0.02% $NaNO_3$, 2% NaCl	Free	No
NC–P		No culture	No
I–RP	0.01% $NaNO_2$,	Immobilized	71
F–RP	0.01% $NaNO_3$, 1% NaCl	Free	54
NC–RP		No culture	48
I–NP	No preservatives	Immobilized	63
F–NP		Free	52
NC–NP		No culture	38

Table 2
Phylogenetic affiliations of bacterial dynamics in probiotic dry-fermented sausages and sausages produced with no starter culture based on DNA analyses and the corresponding band(s) in the DGGE profile.

Band[a]	Most closely related species	Identity (%)	Accession number[b]
1	*Lactococcus* sp. 14A	100	HQ289889.1
	Uncultured bacterium clone B5	100	GU977202.1
2	*Lactobacillus casei* ATCC 393	99	NR_041893.1
	Lactobacillus rhamnosus strain V92	99	JF444753.1
3	*Lactobacillus casei* ATCC 393	99	NR_041893.1
	Lactobacillus rhamnosus clone WWC_C4AKM117	99	GU429394.1
4	*Lactobacillus casei* ATCC 393	99	NR_041893.1
	Lactobacillus paracasei clone WWC_C4MLM108	99	GU425011.1
5	*Lactobacillus casei* ATCC 393	98	NR_041893.1
	Lactobacillus casei LOCK919	98	CP005486.1
6	*Rhodococcus erythropolis*	94	KF313553.1
	Rhodococcus globerulus	94	AB828263.1
7	*Saccharothrix* sp. EGI 80154	94	KF040433.1
	Micromonospora sp. EGI 80045	94	KF040413.1
8	*Lactobacillus casei* ATCC 393	98	NR_041893.1
	Lactobacillus casei LOCK919	98	CP005486.1
9	*Lactobacillus sakei* strain EC7	99	JN851763.1
10	*Lactobacillus sakei* strain N2MR5	98	KF193896.1
	Lactobacillus fuchuensis strain MFPC41A28-08	98	JF756333.1
11	*Lactobacillus casei* ATCC 393	99	NR_041893.1
	Lactobacillus rhamnosus clone WWC_C4MKM113	99	JF444753.1

[a] Bands are numbered as indicated on DGGE gel shown in Fig. 1a.
[b] Accession numbers of sequences of most closely related species found with Blast search.

L. casei ATCC 393 was identified at levels ≥6 log cfu/g in all samples containing immobilized or free cells after 71 days of ripening (data not shown). As expected, *L. casei* ATCC 393 was undetectable in sausages produced using no culture (samples NC–P, NC–RP and NC–NP).

4. Discussion

The addition of nitrite and nitrate salts is of great importance for inhibiting the undesirable bacteria in combination to pH reductions as it occurs in fermented sausages and for color formation. On the other hand, their use is under discussion because of their contribution to the formation of health affecting nitrosamines. Functional starter cultures in dry-fermented sausage production should be designed in order to meet health promoting, food safety, shelf-life, technological effectiveness and economic feasibility criteria and may be useful in reducing levels of nitrites and nitrates. Thus, the objective of the present study was to evaluate *L. casei* ATCC 393 as protective culture in prolonging the shelf-life of probiotic dry-fermented sausages containing reduced or negligible amounts of nitrite, nitrates and sodium chloride.

The physicochemical parameters of the probiotic sausages ranged in levels usually observed in dry-fermented sausages (Fernández-López, Sendra, Sayas-Barberá, Navarro, & Pérez-Alvarez, 2008; Roseiro et al., 2008), except titratable acidity which was significantly increased (Bolumar, Nieto, & Flores, 2001; Castaño, García-Fontán, Fresno, Tornadijo, & Carballo, 2002), probably due to overgrowth of LAB. However, no sensory quality deterioration was noted, usually attributed to overacidity caused by certain *Lactobacillus* starter cultures (Sidira et al., 2014).

The extension of preservation time observed in probiotic sausages compared to sausages produced with no culture (Table 1) supported our initial hypothesis for extending the shelf-life of the novel products, and could be attributed to the higher titratable acidity and lower pH and aw recorded.

To study the microbial diversity during maturation, storage and spoilage, a multiphasic approach was employed, characterized by the application of culture-dependent and -independent methods. In this context, samples were analyzed on agar plates, and counts were determined after incubation. DNA was directly extracted from the sausages, amplified with universal bacterial and eukaryotic primers, and after PCR-DGGE analysis, the profiling of the ecology was revealed.

A significant result concerning the microbial dynamics as determined by plating analysis was the drastic decrease of

Table 3
Phylogenetic affiliations of eukaryotic dynamics in probiotic dry-fermented sausages and sausages produced with no starter culture based on DNA analyses and the corresponding band(s) in the DGGE profile.

Band[a]	Most closely related species	Identity (%)	Accession number[b]
1	*Saccharomyces cerevisiae* strain NL32	97	JX141338.1
2	*Kluyveromyces marxianus*	89	KC512907.1
	Kluyveromyces lactis	89	HE799667.1
3	*Debaryomyces hansenii*	80	JQ916047.1
	Priceomyces carsonii	80	JX456534.1
4	*Saccharomyces cerevisiae* strain NL21	96	HM191652.1
5	*Alium fistulosum*	93	JQ283850.1
6	*Alium fistulosum*	93	JQ283850.1
7	*Saccharomyces cerevisiae* strain NL38	95	HM191669.1
8	*Candida ethanolica*	95	EF550225.1
	Pichia deserticola	95	GQ222353.1

[a] Bands are numbered as indicated on DGGE gel shown in Fig. 1b.
[b] Accession numbers of sequences of most closely related species found with Blast search.

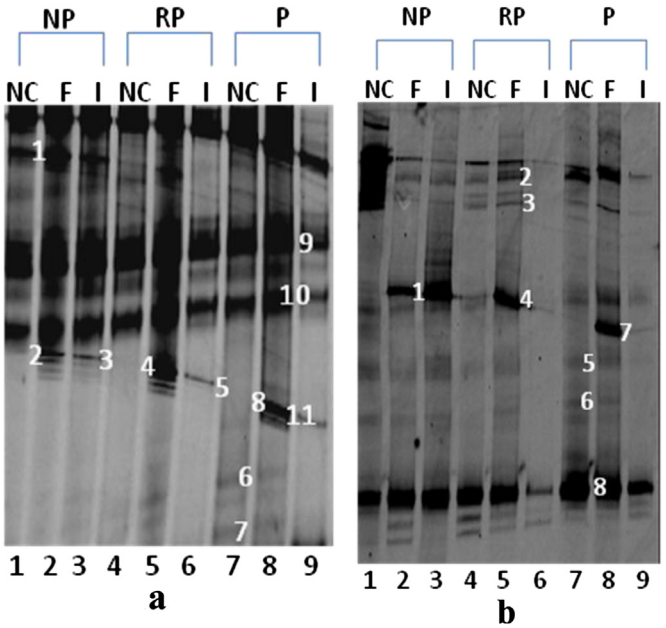

Fig. 1. DGGE bacterial (a) and eukaryotic (b) fingerprint representing PCR-amplified 16S rRNA and 26S rRNA fragments respectively from total community DNA derived from probiotic dry-fermented sausages and sausages produced with no starter culture after 71 days of ripening. For each sample, two replicate profiles from two independent nucleic acid extracts were analyzed. All bands marked by numbers or letters were subjected to sequence determination. I: sausages containing immobilized cells of *L. casei*; F: sausages containing free cells of *L. casei*; NC: sausages containing no starter culture; P: sausages with preservatives (0.02% $NaNO_2$, 0.02% $NaNO_3$, 2% NaCl); RP: sausages with reduced amount of preservatives (0.01% $NaNO_2$, 0.01% $NaNO_3$, 1% NaCl); NP: sausages with no preservatives.

enterobacteria, pseudomonads and staphylococci counts in all cases during maturation (data not shown). Of note, they remained in undetected levels even when spoilage occurred. Although similar reductions have been reported previously (Drosinos et al., 2005; Fernández-López et al., 2008; Sidira et al., 2014), it still remains an important observation related to the safety aspect of the products. On the other hand, LAB dominated the microflora. Their fast growth was favored by the sugar added during sausage production and by the high temperature at the early stages of ripening (Rantsiou et al., 2005), while spoilage in samples containing reduced amounts or no preservatives was partly attributed to their uncontrolled metabolic activity. Similarly, counts of yeasts and moulds also remained in high levels and an overgrowth was observed during storage which resulted in spoilage.

Microbial diversity of the novel probiotic products was further studied using a PCR-DGGE protocol. The main bacterial species identified are usually present in traditional sausages (Cocolin et al., 2001; Rantsiou et al., 2005; Sidira et al., 2014). However, members of *Rhodococcus*, *Saccharothrix* or *Micromonospora* detected only in NC-samples usually isolating from soil (Asturias, Eltis, Prucha, & Timmis, 1994; Cserháti et al., 2013; Hirsch & Valdés, 2010; Labeda, Testa, Lechevalier, & Lechevalier, 1984) may derived from leek or herbs added.

Concerning the yeast ecology, the predominant species detected was *D. hansenii*, which was in accordance with previous studies (Cocolin, Urso, Rantsiou, Cantoni, & Comi, 2006; Rantsiou et al., 2005; Sidira et al., 2014).

Microbiological and strain-specific multiplex PCR analysis confirmed that the levels of both free and immobilized *L. casei* ATCC 393 in the samples after 71 days of ripening were above the minimum concentration for conferring a probiotic effect ($\geq$6 log cfu/g). These results were in accordance to our recently published study (Sidira et al., 2014) and documented the survival of the probiotic strain even when spoilage occurred.

5. Conclusions

It is important in the meat industry to know the likely occurrence of microbial hazards and the means needed to obtain a desirable shelf life. The main factors affecting microbial growth are undoubtedly physical parameters such as temperature, pH, and water activity, together with nutrients (oxygen, carbohydrates, protein content, etc.) and preservatives (sodium chloride, nitrate and nitrite, etc). While these factors, in association with food structure, constitute the basis of predictive microbiology, the presence of certain microbial populations can result in unexpected interactions and therefore in unpredicted microbial compositions. The present study demonstrated the use of *L. casei* ATCC 393 as a potential means to extend the shelf life of probiotic dry-fermented sausages in association with the repression of certain microbial species and suggested its use for the production of healthy meat products, addressed to people with high blood pressure. Additionally, since consumption of probiotic sausages has already been associated with beneficial effects to consumers, future clinical trials will give more insight into the role of probiotic dry-fermented sausages along with the potential prebiotic characteristics of wheat on promotion of human health.

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