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Effective survival of immobilized *Lactobacillus casei* during ripening and heat treatment of probiotic dry-fermented sausages and investigation of the microbial dynamics

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ABSTRACT

The aim was the assessment of immobilized *Lactobacillus casei* ATCC 393 on wheat in the production of probiotic dry-fermented sausages and the investigation of the microbial dynamics. For comparison, sausages containing either free *L. casei* ATCC 393 or no starter culture were also prepared. During ripening, the numbers of lactobacilli exceeded 7 log cfu/g, while a drastic decrease was observed in enterobacteria, staphylococci and pseudomonas counts. Microbial diversity was further studied applying a PCR-DGGE protocol. Members of *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Carnobacterium*, *Brochothrix*, *Bacillus* and *Debaryomyces* were the main microbial populations detected. Microbiological and strain-specific multiplex PCR analysis confirmed that the levels of *L. casei* ATCC 393 in the samples after 66 days of ripening were above the minimum concentration for conferring a probiotic effect (≥6 log cfu/g). However, after heat treatment, this strain was detected at the above levels, only in sausages containing immobilized cells.

1. Introduction

Nowadays there is a growing interest in developing novel foods containing probiotic microorganisms, such as bifidobacteria and lactic acid bacteria (LAB). Such functional cultures may offer organoleptic, technological and nutritional advantages, but more importantly confer a health benefit on the host. Indeed, administration of probiotics has been linked to the treatment of various diseases, including viral or bacterial diarrhoea, gastroenteritis, irritable bowel syndrome, inflammatory bowel disease, Crohn's disease and ulcerative colitis, depressed immune function, lactose intolerance, infant allergies, *Helicobacter pylori* infections, and others (Deshpandea, Rao, & Patolea, 2011). The mechanisms and the efficacy of a probiotic effect often depend on the interactions with the host microbiota or with the immuno-competent cells of the intestinal mucosa (Saad, Delattre, Urdaci, Schmitter, & Bressollier, 2013).

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 probiotic food, has been suggested as the minimum intake to provide a probiotic effect. However, monitoring the survival of the probiotic cultures in foods is hampered by the lack of accurate, reliable, convenient and sensitive methods of identification, with the ability to distinguish the strains of interest among other closely related microorganisms present in the products.

A properly designed strategy for the incorporation of probiotic microorganisms into foods (formulation strategies, processing, stability and organoleptic quality issues) is a key factor in the development of functional products. Although encapsulation systems have largely been exploited in the pharmaceutical (e.g. drug and vaccine delivery) and agricultural sector (e.g. fertilizers), the food industry has only recently become aware of the immense benefits that these technologies are able to offer (Champagne, Lee, & Saucier, 2010). Insertion of beneficial bacteria into a food matrix presents a new challenge, not only because of their interactions with other constituents, but also because of the severe conditions often employed during food processing and storage, which might lead to important losses in viability, as probiotic strains are very often thermally labile (on heating and/or freezing) and sensitive to acidity, oxygen or other food constituents (e.g. salts). To overcome such deficiencies, immobilization techniques are usually applied in order to maintain cell viability, activity and functionality. Thus, many studies have focused on the immobilization of probiotic bacteria in various supports, such as starch (Mattila-Sandholm et al., 2002), fruit pieces (Kourkoutas, Ksolis, Kallis, Bezirtzoglou, & Kanellaki, 2005, Kourkoutas

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et al., 2006), casein (Dimitrellou, Kourkoutas, Koutinas, & Kanellaki, 2009) and wheat grains (Bosnea et al., 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. 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, *Lactobacillus casei* ATCC 393 strain has been extensively added into food products to confer probiotic properties (Kourkoutas et al., 2006, 2005; Li, Chen, Cha, Park, & Liu, 2009). Recently, a new method for rapid detection and identification of the above strain based on multiplex PCR was demonstrated (Karapetsas, Vavoulidis, Galanis, Sandaltzopoulos, & Kourkoutas, 2010). The presented methodology proved an efficient tool for accurate, convenient and reliable identification of the *L. casei* ATCC 393 in food products.

Dry-fermented sausages are typical Mediterranean meat-products, the acceptability of which is strongly influenced by their quality. There are a few attempts at incorporating probiotic cultures in dry-fermented sausages (De Vuyst, Falony, & Leroy, 2008; Muthukumarasamy & Holley, 2006) and the results are still considered preliminary for evaluating the effect of probiotic fermented meats on human health. Most concerns are associated with the survival of the probiotic strains during the manufacturing process and detection in high numbers in the end-product (De Vuyst et al., 2008; Leroy, Verluyten, & De Vuyst, 2006). Thus, commercial application of probiotic microorganisms in fermented sausages is not available yet.

Hence, the aim of the present study was the assessment of immobilized *L. casei* ATCC 393 on wheat in probiotic dry-fermented sausage production and the investigation of the microbial dynamics. The results documented the survival of *L. casei* ATCC 393 cells at the required levels for providing the health benefits during manufacture and ripening, as well as after mild heat treatment, repression of spoilage and pathogenic bacteria, improvement of quality characteristics and extension of products' shelf-life.

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 the 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 1/4 strength Ringers solution (Sigma-Aldrich, UK).

2.3. Production of probiotic dry-fermented sausages

Dry-fermented sausages were prepared according to traditional techniques and recipes. 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), sodium chloride (50.0 g), black 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 immobilized *L. casei* on wheat. After mixing, the stuffing of natural casings produced fresh sausages (25 cm length, 3.0–3.5 cm thick).

To investigate the effect of the initial concentration of immobilized *L. casei*, dry-fermented sausages were produced containing 300 (sample I—300), 100 (sample I—100) or 30 g (wet weight) (sample I—30) of immobilized cells/kg of the above stuffing mixture. For comparison, dry-fermented sausages containing free cells of *L. casei* (1.0 g wet weight/kg of stuffing mixture) (sample Fr) and with no starter culture (sample NC) were also produced, as described above. The initial cell counts of *L. casei* ATCC 393 in the probiotic products was >6 log cfu/g in all cases (when incorporated in either immobilized or free form). Ripening was carried out at room temperature (19–23 °C with a relative humidity between 40 and 85%) for 10–12 days and then the temperature was decreased to 4–6 °C at 2–4 °C/day with a relative humidity between 50 and 75% for up to 66 days.

All experiments were carried out in triplicate. 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 pH meter (WTW, pH-330i pH meter, 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 11th day. The differences in weight were expressed as a 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. Therefore, representative 10 g portions of dry-fermented sausage samples taken from the interior were blended with 90 ml of sterilized 1/4 Ringers solution (Sigma-Aldrich) and subjected to serial dilutions.

The following tests were performed: (i) total aerobic counts on plate count agar (Fluka, Switzerland) at 30 °C for 48 h, (ii) lactobacilli [Gram (+), catalase (−)] on acidified MRS agar (Fluka) at 37 °C for 48 h anaerobically (Anaerobic jar, Anerocult C, Merck), (iii) enterobacteria on violet red bile glucose agar (Fluka) at 37 °C for 24 h, (iv) pseudomonads on pseudomonas CFC selective agar (Merck) at 30 °C for 72 h, (v) staphylococci on Baird Parker egg yolk tellurite medium (Fluka) at 37 °C for 48 h and confirmed by a positive coagulase test, (vi) yeasts and moulds on malt agar (Fluka) (pH was adjusted to 4.5 by sterile solution of 10% lactic acid) at 30 °C for 48 h and (vii) clostridia on TSC Agar (Fluka) at 37 °C for 24 h anaerobically (Anaerobic jar, Anerocult C, Merck). All incubations were further extended up to 120 h, but no extra colonies were observed. Gram staining and catalase tests were performed for lactic acid bacteria confirmation. Results are presented as log of mean colony-forming units on solid media culture plates containing between 30 and 300 colonies per gramme of dry-fermented sausage.

2.6. PCR-DGGE analysis

10 g portions of sausages samples taken from the interior were blended with 40 ml of sterilized 1/4 Ringers 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)), in a reaction mixture containing

2 μl (about 50 ng) of template DNA, 0.4 mM deoxynucleoside triphosphates (dNTPs), each primer at a concentration of 0.2 μM, 2.5 mM $MgCl_2$, 5 μl of 10× PCR reaction buffer [1×: 67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20], and 5 units of Taq DNA polymerase (HyTest Ltd, Finland). Amplifications were carried out in a Thermal Cycler (Mastercycler, Eppendorf, Germany) in a final volume of 50 μl by using an amplification cycle characterized by an initial touchdown step in which the annealing temperature was lowered from 60 °C to 50 °C in intervals of 2 °C every two cycles, and 20 additional cycles were done with annealing at 50 °C. The template DNA was denatured for 5 min at 94 °C and extension was performed at 72 °C for 3 min. A final extension of 72 °C for 5 min ended the amplification cycle.

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. PCR was performed in a final volume of 50 μl containing 2 μl (about 50 ng) of template DNA, 0.4 mM deoxynucleoside triphosphates (dNTPs), each primer at a concentration of 0.2 μM, 2.5 mM $MgCl_2$, 5 μl of 10× PCR buffer [1×: 67 mM Tris-HCl, pH 8.8, 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20], and 5 units of Taq DNA polymerase (HyTest Ltd). Amplifications were carried out in a Thermal Cycler (Mastercycler, Eppendorf) in a final volume of 50 μl under the following conditions: 94 °C (5 min), followed by 30 cycles of 94 °C (1 min), 55 °C (45 s), 72 °C (1 min), followed by a final extension step at 72 °C (7 min). Five microliters of each PCR product was separated on 1.5% w/v agarose gels, visualized under UV illumination and photographed with a digital camera (Gel Doc EQ System Biorad, Italy). A GC clamp (5′ CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G 3′) was added to primers P1 and NL1 when used for DGGE analysis. The reverse primers P2 and LS2 were fluorophore-labelled with the addition of a 5′-terminal 6-carboxyfluorescein (FAM).

The PCR products were subjected to DGGE analysis using an INGENYphorU DGGE system (Ingeny, The Netherlands). Electrophoresis was performed in a 0.8-mm-thick polyacrylamide gel [8% (wt/vol) acrylamide–bisacrylamide (37.5:1)] with a denaturant gradient from 40 to 60% [100% corresponded to 7 M urea and 40% (wt/vol) formamide] increasing in the direction of the electrophoretic run. The gels were subjected to a constant voltage of 120 V, 100 mA for 8 h at 60 °C. Followed the electrophoresis, the gels were scanned with a fluorescent imager (Molecular Imager FX, BioRad, Italy) and the bands of interest excised.

2.7. Sequencing of DGGE fragments and data analysis

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 was reamplified with primers P1 and P2 for bacteria and NL1 and LS2 for eukaryotes, respectively. The forward primers P1 and NL1 lack the GC clamp, whereas the reverse primers P2 and LS2 were used without the addition of a 5′-terminal 6-carboxyfluorescein (FAM). Amplification was carried out in a Thermal Cycler (Mastercycler, Eppendorf) under the following conditions: 94 °C (5 min), followed by 30 cycles of 94 °C (1 min), 55 °C (1 min), 72 °C (2 min), followed by a final extension step at 72 °C (5 min). The PCR products were purified, and sent for sequencing to VBC-Biotech, Austria. Searches in the GenBank with the BLAST programme 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 the strain in dry-fermented sausages, a recent methodology was followed (Saxami et al., 2012; Sidira

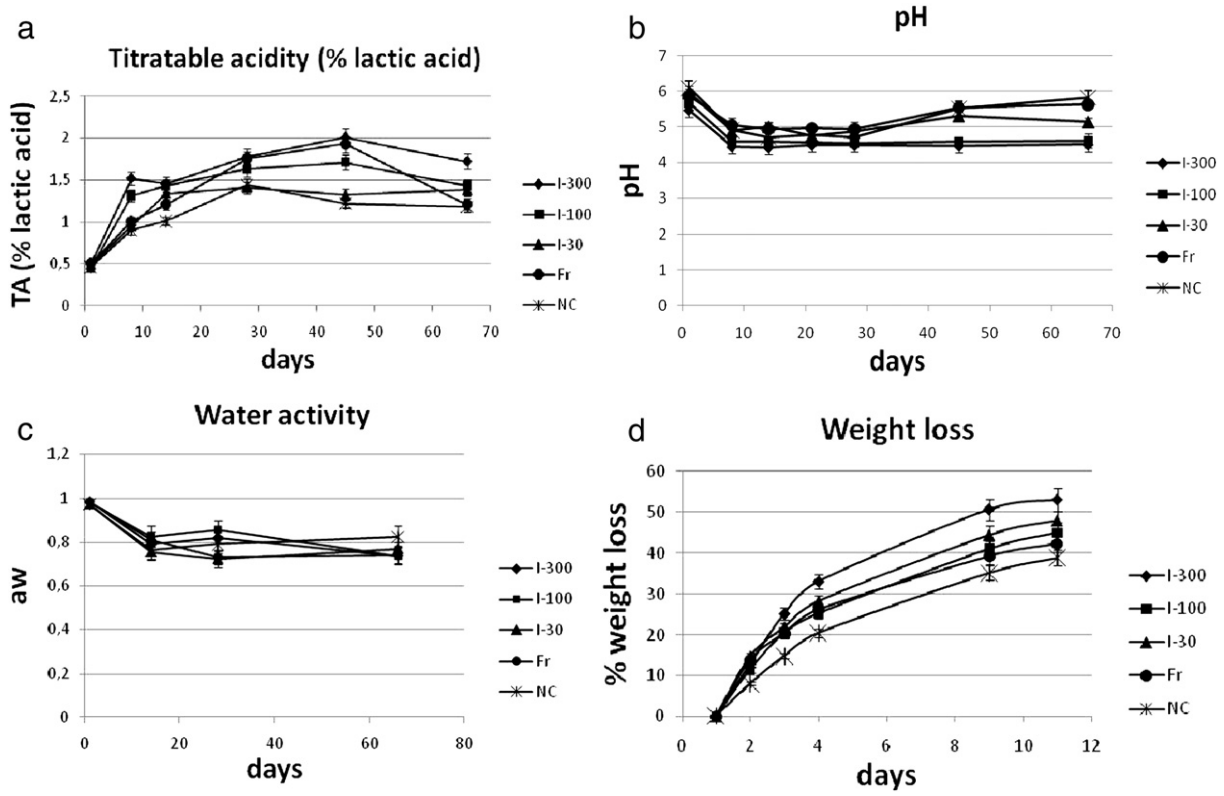

Fig. 1. Effect of immobilized and free *L. casei* ATCC 393 on physicochemical parameters during ripening of probiotic dry-fermented sausages: a) titratable acidity (TA), b) pH, c) water activity (aw), and d) weight loss. I–300: sausages containing 300 g immobilized *L. casei*/kg of stuffing mixture, I–100: sausages containing 100 g immobilized *L. casei*/kg of stuffing mixture, I–30: sausages containing 30 g immobilized *L. casei*/kg of stuffing mixture, Fr: sausages produced with free *L. casei*, NC: sausages produced by no starter culture. Error bars indicate standard deviations ($n = 3$).

et al., 2010). Briefly, after enumeration of lactobacilli on MRS agar, the plates corresponding to all dilutions were washed with 10 mL sterilized 1/4 Ringers 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 et al., 2010). Genomic DNA from the lactobacilli suspensions was extracted using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocol. Multiplex PCR reactions were carried out in a total volume of 50 μl, containing 5 units Taq DNA polymerase (HyTest Ltd), 400 μM each dNTPs (Promega, UK), 1.5 mM $MgCl_2$ (HyTest Ltd) and 100 ng template DNA. The set of specific for *L. casei* ATCC 393 primers used consisted of GGCGACCAAGGCAGCG (10 pmol), CTTCGGTTTCATCTTCC (50 pmol) and GGCCAACTTTTTCCA TA (50 pmol) (Karapetsas et al., 2010). Additionally, a set universal for lactobacilli primers were used as positive controls, which consisted of AGCAGTAGGGAATCTTCCA (10 pmol) and ATTYCACCGCTACACATG (10 pmol) (Walter et al., 2001). Amplification was carried out in a Thermal Cycler (Mastercycler, Eppendorf) under the following conditions: 94 °C (2 min), followed by 25 cycles of 94 °C (15 s), 51 °C (15 s), 72 °C (30 s), followed by a final extension step at 72 °C (1 min). The PCR products were separated on 1.5% w/v agarose gels, visualized under UV illumination and photographed with a digital camera (Gel Doc EQ System, Biorad).

Molecular identification of *L. casei* ATCC 393 was also carried out after mild heat treatment (70–72 °C for 8–10 min; the temperature at the interior of the product did not exceed 45 °C) of the probiotic dry-fermented sausages ripened for at least 8 days.

2.9. Preliminary sensory evaluation

The fermented sausages produced with immobilized or free *L. casei* and without starter culture were heated at 70–72 °C for 8–10 min (the temperature at the interior of the product did not exceed 45 °C) and then compared to a similar type commercial product for their sensory characteristics. Samples of approximately 25 g were served in random order at room temperature. Sensory evaluation was conducted in duplicate by 15 laboratory members using locally approved protocols. The panel was asked to give scores on a 0–5 scale (0: unacceptable, 5: exceptional) for attributes grouped into four categories: appearance, colour, taste and aroma. Panellists used water to clean their palates between samples and were unaware of the identity of the samples they tasted.

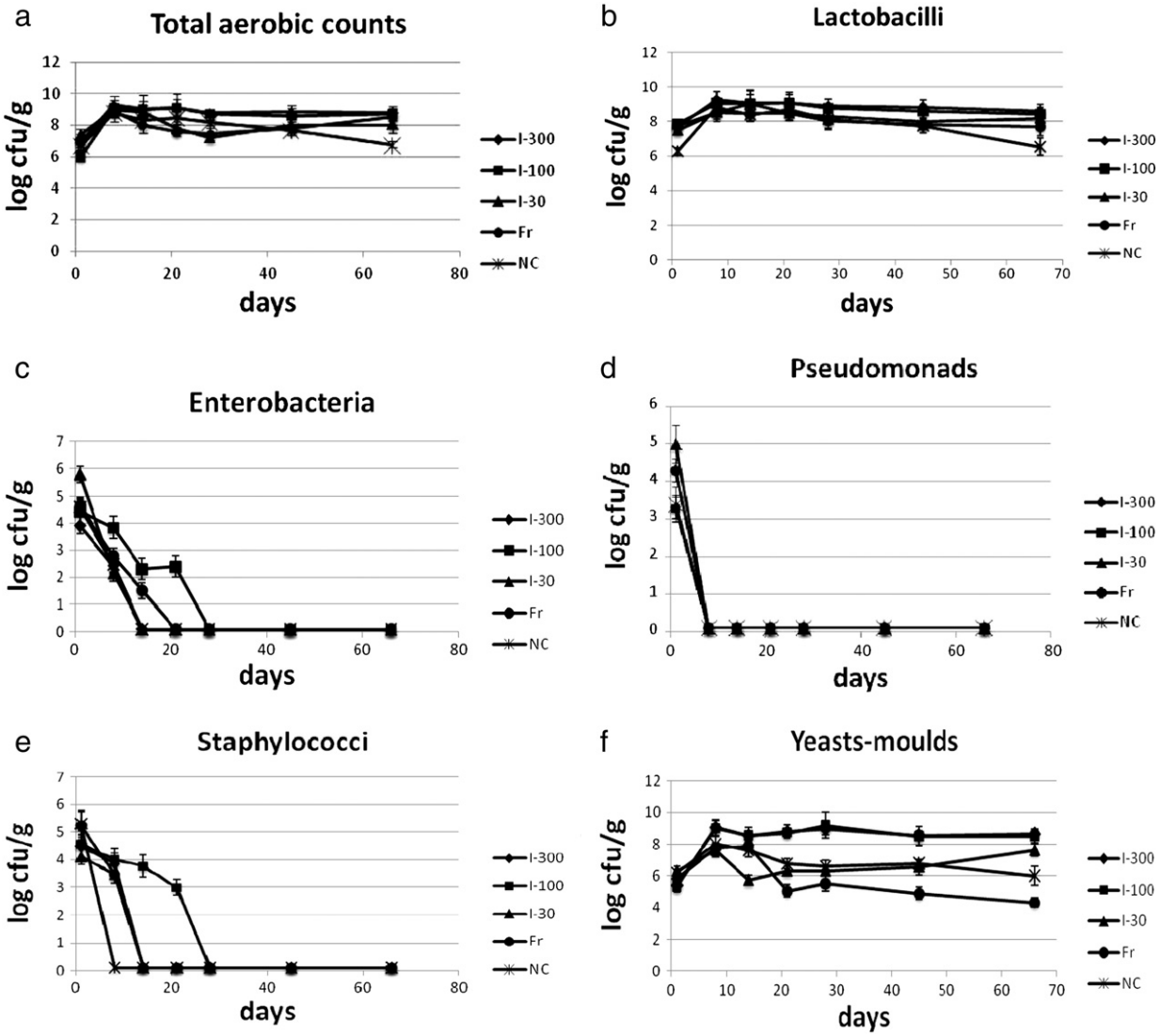

Fig. 2. Effect of immobilized and free *L. casei* ATCC 393 on microbial associations during ripening of probiotic dry-fermented sausages: a) total aerobic counts, b) lactobacilli, c) enterobacteria, d) pseudomonas, e) staphylococci and f) yeasts/moulds. I—300: sausages containing 300 g immobilized *L. casei*/kg of stuffing mixture, I—100: sausages containing 100 g immobilized *L. casei*/kg of stuffing mixture, I—30: sausages containing 30 g immobilized *L. casei*/kg of stuffing mixture, Fr: sausages produced with free *L. casei*, NC: sausages produced by no starter culture; points at zero represent levels below the detection limit (<1.50 log cfu/g for enterobacteria and <2.50 log cfu/g for pseudomonas and staphylococci). Error bars indicate standard deviations ($n = 3$).

Spoilage was determined macroscopically and by using sensory tests. A scoring scale with three categories was used: class 1 corresponded to high quality product without any off odours or off flavours, class 2 corresponded to product with slight off odours or off flavours 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. The experiments were designed and analyzed statistically by ANOVA. Duncan's multiple range test was used to determine the 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 results of the physicochemical parameters concerning the probiotic dry-fermented sausages during ripening are summarized in Fig. 1. Both the probiotic culture and the ripening time affected significantly all physicochemical parameters tested ($P < 0.05$). A significant increase in titratable acidity and a significant decrease in pH were noted during ripening, which were more pronounced in probiotic dry-fermented sausages containing immobilized *L. casei* ATCC 393. Similarly, water activity (aw) and weight loss were significantly reduced during maturation.

3.2. Microbiological analysis

The association of the microbial groups examined during ripening of the probiotic dry-fermented sausages is presented in Fig. 2.

Briefly, no spoilage was observed in sausages containing immobilized or free *L. casei* cells, whereas in sausages produced using no culture, spoilage was obvious on the 43th day of ripening. Spoilage was mainly due to white spots on the sausage surface attributed to yeast/mould overgrowth, resulting also in slight formation of off-odours and off-flavours, but the product was still acceptable.

All microbial counts were significantly affected by both the probiotic culture and the ripening time ($P < 0.05$). While total aerobic counts and levels of lactobacilli and yeasts/moulds remained high during ripening, a drastic decrease was observed in enterobacteria, pseudomonas and staphylococci counts, which dropped to undetectable levels during maturation. Likewise, *Clostridium* spp. were not detected in any sample during the whole ripening period or after mild heat treatment (70–72 °C for 8–10 min; the temperature at the interior of the product did not exceed 45 °C).

3.3. Determination of microbial biodiversity using molecular techniques

Probiotic dry-fermented sausages produced with immobilized (sample I—100), free cells (sample Fr) and with no starter culture (sample NC) were subjected to PCR-DGGE analysis after 1 and 66 days of ripening, in order to examine the microbial ecology. The results are presented in Tables 1 and 2 and Fig. 3. Assays were conducted in triplicate and identical profiles were obtained for the same sausage type. Sequence determination of the separated bands revealed similar microbial patterns in all samples. The probiotic strain *L. casei* ATCC 393 was confirmed in I-100 and Fr-sausages but not in NC sample, as expected. Other species of *Lactobacillus*, *Brochothrix thermosphacta* and members of the genera *Leuconostoc*, *Lactococcus*, *Carnobacterium* and *Bacillus* were detected as the main bacterial populations.

On the other hand, *Debaryomyces hansenii* and *Candida zeylanoides* or *Schwanniomyces polymorphus* were present only in samples ripened for 66 days. Of note, *Allium* species, added during production as ground leek, was also identified in all samples.

Table 1
Phylogenetic affiliations of bacterial dynamics in probiotic dry-fermented sausages and sausages produced with no starter culture after 1 and 66 days of ripening based on DNA analyses and the corresponding band(s) in the DGGE profile.

Band[a]	Most closely related species	Identity (%)	Accession number[b]
1	*Bacillus thuringiensis*	98	JF512478.1
	Bacillus cereus	98	HQ683796.1
2	*Lactobacillus casei* ATCC 393	97	NR_041893.1
	Lactobacillus zeae	97	AB008212.1
3	*Brochothrix thermosphacta*	98	AF318169.1
4	*Leuconostoc pseudomesenteroides*	98	HE646416.1
	Leuconostoc citreum	98	AB602811.1
	Leuconostoc mesenteroides	98	AF318164.1
	Leuconostoc kimchii	98	CP001758.1
5	*Leuconostoc* sp. B 244	98	GU998869.1
	Leuconostoc mesenteroides	95	AF318164.1
6	*Lactococcus lactis* subsp. *cremoris*	96	AB681219.1
	Lactococcus lactis subsp. *lactis*	96	JF922121.1
7	*Lactobacillus casei* ATCC 393	97	NR_041893.1
	Lactobacillus zeae	97	AB008212.1
	Uncultured *Lactobacillus* sp.	97	AY857628.1
8	*Carnobacterium divergens*	98	AB598939.1
9	*Carnobacterium maltaromaticum*	96	GQ304931.1
10	*Brochothrix thermosphacta*	98	AF318169.1
11	*Lactococcus lactis*	100	AB571485.1
	Uncultured *Streptococcaceae* sp.	100	AB234513.1
	Lactococcus fujiensis	100	AB485959.1
12	*Lactococcus lactis*	100	EU091434.1
	Uncultured bacterium	98	JN884004.1
13	*Carnobacterium divergens*	98	AB598939.1
14	*Carnobacterium maltaromaticum*	96	GQ304931.1

[a] Bands are lettered as indicated on DGGE gel shown in Fig. 3a.
[b] Accession numbers of sequences of most closely related species found with Blast search.

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 primers that generated two unique PCR products of 67 bp and 144 bp respectively (Karapetsas et al., 2010). A set universal for lactobacilli primers generating a PCR product of 340 bp was used as positive control (Walter et al., 2001).

Table 2
Phylogenetic affiliations of eukaryotic dynamics in probiotic dry-fermented sausages and sausages produced with no starter culture after 1 and 66 days of ripening based on DNA analyses and the corresponding band(s) in the DGGE profile.

Band[a]	Most closely related species	Identity (%)	Accession number[b]
A	*Candida zeylanoides*	91	EU131538.1
	Schwanniomyces polymorphus	91	HQ637561.1
B	Uncultured *Saccharomycetes* sp.	97	FJ195798.1
	Debaryomyces hansenii	96	EU250075.1
C	*Debaryomyces hansenii*	99	HM627066.1
	Debaryomyces nepalensis	98	JN604535.1
	Debaryomyces coudertii	98	JN940508.1
	Debaryomyces subglobosus	98	JN940506.1
	Debaryomyces maramus	98	JN940502.1
D	*Allium fistulosum*	96	JQ283850.1
	Allium cepa	96	JQ283849.1
E	*Debaryomyces hansenii*	90	HQ641266.1
	Debaryomyces fabryi	89	JQ266358.1
	Schwanniomyces polymorphus	89	HQ637561.1
F	*Debaryomyces hansenii*	97	EU250075.1

[a] Bands are lettered as indicated on DGGE gel shown in Fig. 3b.
[b] Accession numbers of sequences of most closely related species found with Blast search.

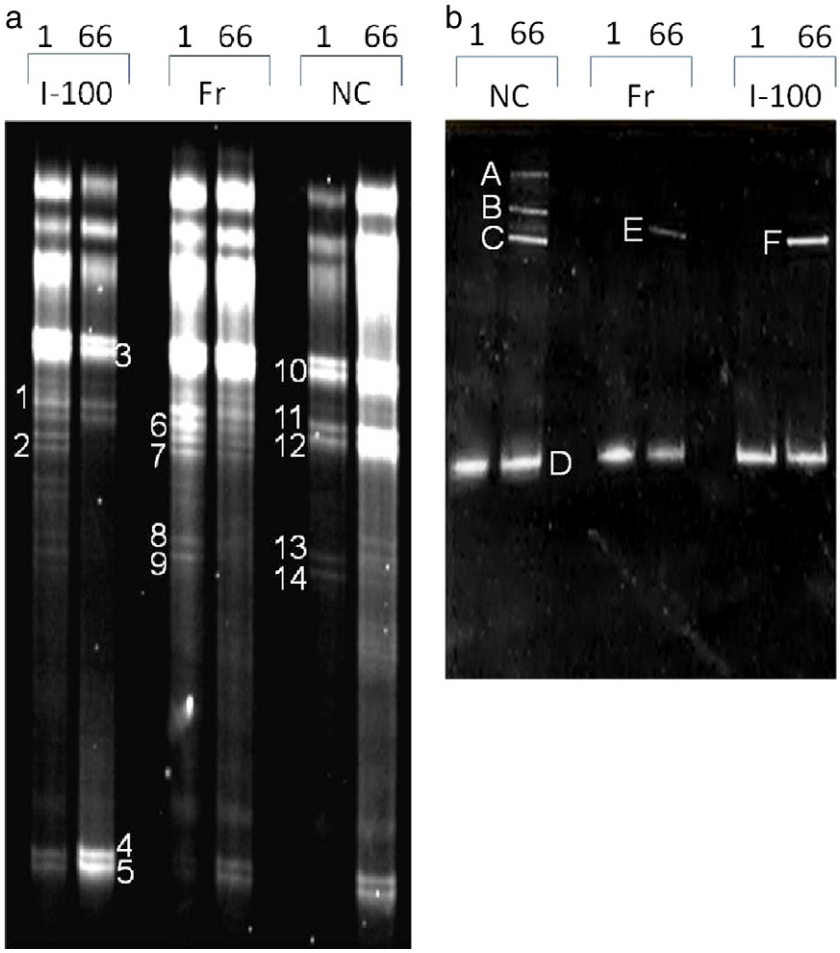

Fig. 3. DGGE bacterial (a) and eukaryotic (b) fingerprint representing PCR-amplified 16S rRNA and 26S rRNA fragments respectively from total community DNA derived from probiotic dry-fermented sausages and sausages produced with no starter culture after 1 and 66 days of ripening. For each type of sausage, two replicate profiles from two independent nucleic acid extracts were analyzed. All bands marked by numbers or letters were subjected to sequence determination. I—100: sausages containing 100 g immobilized *L. casei*/kg of stuffing mixture, Fr: sausages produced with free *L. casei*, NC: sausages produced by no starter culture.

L. casei ATCC 393 was identified at levels ≥6 log cfu/g in all samples containing immobilized or free cells after 66 days of ripening (Fig. 4a). However, the above strain was detected at the same levels (≥6 log cfu/g) in samples I-300 and I-100, but not in samples I-30 and Fr after heating at 70-72 °C for 8-10 min (the temperature at the interior of the product did not exceed 45 °C) (Fig. 4b). Of note, *L. casei* ATCC 393 was >8 log cfu/g prior heat treatment in all probiotic products (containing either immobilized or free *L. casei* ATCC 393 cells). As expected, *L. casei* ATCC 393 was undetectable in sausages produced using no culture (samples NC).

3.5. Preliminary sensory evaluation

Dry-fermented sausages are usually consumed raw or after mild heat treatment. Therefore, the sensory characteristics of the probiotic dry-fermented sausages produced with immobilized or free *L. casei* were compared to sausages produced without starter culture and to a similar commercial product after heat treatment (data not shown). As the addition of wheat grains may significantly affect product quality, the effect of the initial concentration of immobilized *L. casei* on the sensory characteristics was investigated. Although the tasters showed a preference ($P < 0.05$) for the commercial product over sausages produced in the laboratory, except I-300 concerning the aroma, no significant differences ($P > 0.05$) were observed for the other sensory attributes. Of note, untreated sausages produced using immobilized cells had a distinctive, characteristic aroma, which lasted until the end of the ripening period (66 days). In general, dry-fermented sausages produced with immobilized *L. casei* ATCC 393 were approved and accepted by the panel and the preliminary sensory tests ascertained the fine taste and overall high quality.

4. Discussion

Lactobacillus casei ATCC 393 has been proposed for the production of dairy products due to its excellent technological properties (Bosnea et al., 2009; Kourkoutas et al., 2006, 2005), while cell immobilization has shown significantly increased survival rates over free cells during food manufacture and storage (Kourkoutas et al., 2006, 2005). Recently, the probiotic properties of free and immobilized *L. casei* ATCC 393 were assessed by documenting survival after transit through the gastro-intestinal tract, adhesion at the large intestine and regulation of the intestinal microbial flora in rats (Saxami et al., 2012; Sidira et al., 2010). Thus, the scope of the present study was the assessment of immobilized *Lactobacillus casei* ATCC 393 on wheat as probiotic starter culture in dry-fermented sausages and the investigation of the microbial interactions in the novel products. Although the use of probiotics is already common in dairy products, the food industry is seeking to produce functional meat products with potential health benefits. The goals were firstly to maintain survival of the probiotic strain at suitable levels for conferring health effects at the time of consumption (during ripening and after mild heat treatment), at a second stage to improve the quality characteristics of the new products and finally to extend the products' shelf life.

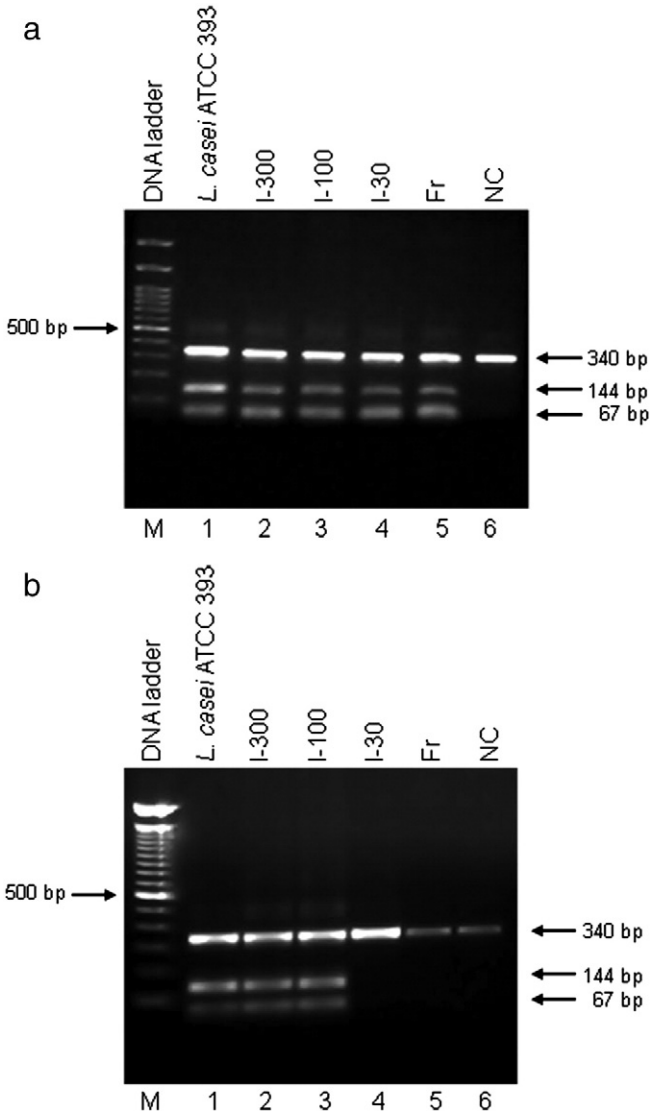

Fig. 4. Molecular identification of *L. casei* ATCC 393 in probiotic dry-fermented sausages. After lactobacilli enumeration on MRS agar plates, the presence/absence of *L. casei* ATCC 393 at levels ≥6 log cfu/g after a) 66 days of ripening and b) mild heat treatment (70–72 °C for 8–10 min; the temperature at the interior of the product did not exceed 45 °C) was confirmed by strain-specific multiplex PCR. Pure culture of *L. casei* ATCC 393 served as a positive control (lane 1). I—300: sausages containing 300 g immobilized *L. casei*/kg of stuffing mixture, I—100: sausages containing 100 g immobilized *L. casei*/kg of stuffing mixture, I—30: sausages containing 30 g immobilized *L. casei*/kg of stuffing mixture, Fr: sausages produced with free *L. casei*, NC: sausages produced by no starter culture. PCR products of 67 bp and 144 bp are unique for *L. casei* ATCC 393, whereas the PCR product of 340 bp is universal for lactobacilli.

The physicochemical parameters of the novel probiotic dry-fermented sausages were at levels usually observed in dry-fermented sausages (Fernández-L pez, Sendra, Sayas-Barberá, Navarro, & Pérez-Alvarez, 2008), except titratable acidity which was slightly increased. Although, overacidity caused by certain *Lactobacillus* strains used as starter cultures may result in sensory quality deterioration, such a trend was not obvious in these samples.

The extension of preservation time obtained in probiotic sausages compared to sausages produced with no culture, for an additional 23 days supported the initial hypothesis for extending the shelf-life of the novel products and could be attributed to the higher titratable acidity and generally lower pH and aw.

The drastic decrease of enterobacteria, pseudomonas and staphylococci counts during maturation (Fig. 2) has been reported previously (Drosinos et al., 2005; Fernández-L pez et al., 2008). On the other hand, the fast growth of LAB was favoured by the sugar added during sausage production and by the high temperature at the early ripening stages (Rantsiou et al., 2005). Counts of yeasts and moulds also remained high, in contrast to previous studies reporting a reduction in the yeast population during ripening (Encinas, L pez Díaz, García L pez, Otero, & Moreno, 2000; Olesen & Stahnke, 2000). Yeasts are considered essential for sausage flavour formation (Flores, Durá, Marco, & Toldrá, 2004), although their role is not well characterized.

An interesting picture of the dry-fermented sausages ecology was obtained when molecular methods were applied to DNA extracted directly from the samples. Two sets of primers, one for the amplification of bacterial DNA and a second for the amplification of eukaryotic nucleic acids, were used to monitor the dynamic changes of the microbial populations during ripening. The main bacterial species identified are usually present in traditional sausages (Cocolin et al., 2001; Rantsiou et al., 2005). No *L. plantarum*, a LAB commonly associated with fermented meat products, was present in the present samples, thereby confirming the results reported in a previous study (Rantsiou et al., 2005). Concerning the eukaryotic ecology, the predominant species were in accordance with previous studies (Cocolin, Urso, Rantsiou, Cantoni, & Comi, 2006; Rantsiou et al., 2005). *D. hansenii* is known for its ability to hydrolyze pork muscle sarcoplasmic proteins, thereby influencing the aroma formation and sensory quality of dry-fermented sausages (Flores et al., 2004), while *C. zeylanoides* is usually involved in sausage fermentation (Cocolin et al., 2006; Encinas et al., 2000). Taking into account that the detection limit for PCR-DGGE identification in mixed cultures is 3–4 log cfu/g (Cocolin et al., 2001) when the predominant populations are above 8 log cfu/g and may reach up to 7 log cfu/g (Cocolin et al., 2006), the results of the plate counts correlated well with the profile obtained by the PCR-DGGE. Hence, no members of *Enterobacteria*, *Pseudomonas* and *Staphylococcus* were identified, as their counts were below the threshold levels. Other potential reasons might have been masking by DNA belonging to the major populations present or poor amplification of the above species by the protocol applied (Rantsiou et al., 2005). Different PCR conditions and primers also failed in acquiring a diverse pattern (data not shown), most likely because only the V1 region allows DGGE differentiation (Cocolin et al., 2001). Similarly, no signal for yeast DNA was detected at the beginning of the ripening process (day 1), probably due to the low levels in addition to the prevalence of *Allium* DNA (Cocolin et al., 2006). Thus, signal for yeast DNA was obvious only when yeast population reached levels of 7 log cfu/g (Cocolin et al., 2006).

In dry-fermented sausages, bacterial viability may be reduced due to the high salt content, the low water activity and pH (De Vuyst et al., 2008). In the present study, both immobilized and free *L. casei* ATCC 393 were detected in sausages at levels required for conferring a probiotic effect (at least 6 log cfu/g) using a combined microbiological and molecular approach (Karapetsas et al., 2010). Significantly, only immobilized *L. casei* ATCC 393 was identified at the above levels after mild heat treatment of the samples, documenting the protective role of immobilization on cell survival. Dry-fermented sausages are very often consumed uncooked, however, application of mild heat treatment did not affect the probiotic properties of sausages produced with immobilized cells (samples I–300 and I–100). Similar results reporting survival of free probiotic cultures at the end of the ripening period (Klingberg, Axelsson, Naterstad, Elsser, & Budde, 2005), as well as significantly enhanced survival of alginate-microencapsulated *Lactobacillus reuteri* over unencapsulated cells used for the production of probiotic dry-fermented sausages have been published (Muthukumarasamy & Holley, 2006). However, in these studies, determination of the levels of the probiotic cultures was based on assays unable to identify the probiotic among other LAB strains, due to the lack of an accurate, reliable, convenient and sensitive detection method. Therefore, the probiotic characterization of the products was dubious. In addition, no data concerning survival of the cells after heat treatment were presented. Although improved resistance to moderate heat processes due to cell

immobilization has been suggested (Champagne et al., 2010), to the best of our knowledge, this is the first time that detection and identification of the probiotic strain at levels providing health benefits after mild heat treatment of meat products are reported. Thus, the application of cell immobilization in the meat industry is expected to result in maintenance of high cell viability, which is an essential requirement in the production of probiotic foods, but it is not always achieved. The proposed cost effective technology could be expanded to other processed products to ensure the survival of the probiotic microorganisms.

5. Conclusions

Immobilized *L. casei* ATCC 393 on wheat proved to be a suitable starter culture for probiotic dry-fermented sausage production. Immobilized *L. casei* ATCC 393 was detected at the essential concentration for providing the health benefits after maturation and after mild heat treatment, while the novel products had an improved profile of aroma-related compounds and were accepted by the panel during the preliminary sensory evaluation. From a technological point of view, the promising results presented in the current work, as well as the simplicity, efficacy and cost effectiveness of cell immobilization on wheat grains, is expected to lead to rapid adoption of the proposed technology by industry. Additionally, since the 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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