Research Article

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Production of probiotic bovine salami using *Lactobacillus plantarum* 299v as adjunct

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Abstract

BACKGROUND: Five probiotic lactobacilli were tested, alone or in combination with two commercial starters, to select the most suitable strain for a probiotic bovine salami production. *Lactobacillus plantarum* 299v was used with both starters, to make salami according to a traditional recipe. Salami obtained by using just the starters and by spontaneous fermentation, served as control. Microbial dynamics, as well as the main physico-chemical parameters, were monitored throughout ripening. The survival of probiotic 299v was confirmed by strains' tracking by means of RAPD-PCR coupled to a culture-independent approach PCR-DGGE-based.

RESULTS: The results showed a remarkable viability of the probiotic strain even after 60 days of storage. Experimental salami exhibited the same level of sensory acceptance of control salami, were hygienically safe, and characterised by pH, weight loss and microbiological loads within the ranges conventionally advocated for optimal fermented sausages.

CONCLUSION: Outcomes indicate the workable possibility of using second-quality beef cuts for probiotic salami production.

Keywords: bovine salami; probiotic cultures; PCR-DGGE; RAPD-PCR; sensory analysis

INTRODUCTION

Fermented meats are traditional foods par excellence, having originated as the result of empirical methods for meat preservation in a distant, probably post-Palaeolithic, past.[1] Nevertheless, over the last 10 years, meat products have generally been correlated with health problems such as coronary diseases, hypertension, cancer, etc.[2] However, meat and meat products are an important group of highly nutritious foods, being part of the diet of many consumers around the world.[2]

In recent years, much effort has been made to develop meat-based functional foods by using strategies aimed to increase the presence of beneficial compounds and/or to limit those with negative health implications.[3] So, besides achieving higher food safety levels, novel technologies include the use of non-traditional ingredients to reduce costs and the adoption of carefully selected industrial starter cultures with specific functionalities to create benefits to both producers and consumers.[1] Nowadays, a crucial increase in functional foods is related to food containing probiotic cultures, mainly lactic acid bacteria (LAB) or bifidobacteria which proved to have beneficial effects on the health and well-being of consumers by maintaining the intestinal microbial balance when administered in adequate amounts.[4]

During salami production, microorganisms naturally present on raw materials and/or eventually inoculated as starters are responsible for the fermentation process. This process involves a succession of events in which all conditions characterising the dripping, drying and ripening phases need to be monitored to assure high level of hygienic safety and sensory quality of the final product.[5] Therefore, numerous studies were aimed to improve the survival of pro-technological cultures during salami manufacture and ripening. Starter cultures are generally used to control the growth of pathogens[6] and to standardise the quality and the safety of the final product.[5]

Generally, salami are made of swine meat and fat; however, the use of other animal species, i.e. cattle or donkey, raised great interest and appreciation.[7] Bovine salami are typically produced in northern Italy but, to the best of authors' knowledge, just one survey[8] has been carried out to monitor the technological/chemical/microbiological parameters during this kind of process, though the search for a commercial alternative for the meat of autochthonous breeds with prevailing aptitude to milk production is a goal of paramount importance in Italy.

In this regard, the aim of this study was to develop fermented bovine meat-based salami enriched with probiotics. Once individuated the most suitable probiotic strain, functional salami were produced and monitored along ripening up to 60 days. The techniques of random amplification of polymorphic DNA – polymerase

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chain reaction (RAPD-PCR) and PCR–denaturing gradient gel electrophoresis (PCR-DGGE) were used to assess the survival of the probiotic strain during salami ripening and storage.

MATERIALS AND METHODS

Starter cultures used in the study

Two commercial starter cultures were used: (A) Euroferment Medio (Europrodotti S.p.a., Concorezzo, Italy); (B) Startec TCSS 1/150 (Tec-Al S.r.l., Traversetolo, Italy). Starter A was a combination of a *Staphylococcus* (*St.*) *xylosus* and a *Lactobacillus* (*Lb.*) *plantarum* strain, while starter B was a combination of a *St. xylosus* and a *Pediococcus* (*Pd.*) *pentosaceus* strain. 16S rDNA sequencing was performed to confirm the taxon of strains occurring in both starters. Primers (FD1 and RD1), firstly designed by Weisburg *et al.*[9] were used according to a routine procedure previously described.[10]

Probiotic strains tested in the study

Commercial probiotics initially screened were: *Lb. plantarum* 299v or DSM 9843 (Probi AB, Lund, Sweden); *Lb. rhamnosus* LbGG or ATCC 53103 (Valio Dairy Ltd, Helsinki, Finland); *Lb. casei* Shirota YIT 9029 (Yakult Honsha Co., Ltd, Tokyo, Japan); *Lb. reuteri* DSM 17938 (BioGaia AB, Lund, Sweden) and *Lb. casei* ATCC 393.

Acidification rate of starter and probiotic cultures

The acidification rate of probiotic lactobacilli was evaluated in a medium developed *ad hoc* to simulate conditions prevailing in the sausages at the beginning of fermentation. The ECM-M medium was prepared by boiling for 20 min, 500 g of minced bovine fresh meat in 1 L of deionised water; after paper filtering, dextrose (1.5 g L^{-1}), sodium chloride (30 g L^{-1}), sodium nitrate (0.2 g L^{-1}), sodium nitrite (0.05 g L^{-1}), and 2.8 mL of a hydro-alcoholic solution of bromocresol purple (17 g L^{-1}) were added. pH was adjusted to 6.20–6.30.

Before use, LAB and staphylococci strains were cultured twice in MRS (Oxoid, Basingstoke, UK) and BHI (Oxoid), respectively. Strains were inoculated at an initial level of about 6 Log CFU mL^{-1}. The acidification rate was evaluated for single strains, as well as for combination of both starter cultures, with or without probiotics addition. At time 0, as well as after 24, 48 and 120 h of incubation at 22 °C, pH and LAB loads were assessed. LAB were enumerated on differential mMRS, modified according to Ricciardi *et al.*[11] After incubation at 30 °C for 24 h, different types of colonies were selectively counted and tentatively classified by cell observation under light microscopy.

Bovine salami preparation

Fermented bovine salami were produced by using 80% bovine meat (second-quality beef cuts: thick rib, thin rib, brisket) and 20% swine lard. Meat and lard were minced with a plate of 6 mm to obtain the mixture and sodium chloride (30 g kg^{-1}), pepper (3 g kg^{-1}) and ascorbic acid (2 g kg^{-1}) were added. The dough was divided into five batches of 1.5 kg. Two batches were inoculated with the two starter cultures; two were inoculated with the starter cultures plus the probiotic strain *Lb. plantarum* 299v, while the last batch, without any bacterial addition, served as control. Doughs were then stuffed into artificial casings and kept for 24 h at 19–20 °C (relative humidity of 90–92%). Hereafter, salami were transferred for further 20 days at 18–19 °C (relative humidity of 75–80%) and finally at 4 °C for 2 months. Two salami were analysed for each sampling point. Weight loss, pH and microbial loads were determined at time 0 and after 6, 12, 20, 32 and 60 days of ripening. For microbial counts, peptone water (225 mL) was added to 25 g of product, after removal of the casing in aseptic conditions, and homogenised by Stomacher Lab-Blender 400 (Seward Medical, London, UK) for 1 min at low speed, followed by 1 min at high speed. After decimal dilutions, samples were plated on: plate count agar (30 °C, 72 h) for total aerobic microflora; MRS agar at pH 5.50 for LAB (30 °C, 72 h, anaerobiosis); MRS agar with bile salts (3 g kg^{-1}, Oxgall) pH 8.00 for probiotic lactobacilli (30 °C, 72 h, anaerobiosis); Slanetz and Bartley agar for enterococci (37 °C, 48 h); mannitol salt agar for Micrococcaceae (37 °C, 48 h); Baird–Parker agar with egg yolk tellurite emulsion for coagulase-positive staphylococci (37 °C, 48 h); violet red bile glucose agar for Enterobacteriaceae (37 °C, 24 h; overlay); violet red bile lactose agar for members of the *Coli-aerogenes* group (37 °C, 24 h; overlay); *Pseudomonas* agar base with CFC supplement for *Pseudomonas* spp. (25 °C, 48 h); WL nutrient agar with chloramphenicol (100 mg L^{-1}) for yeasts and moulds (28 °C, 72 h). Counts were carried out in duplicate. All media and supplements were purchased from Oxoid, except for bile salts, which were purchased from Sigma–Aldrich (Milan, Italy).

Lactobacilli monitoring by RAPD-PCR

Putative lactobacilli were isolated throughout salami ripening and storage by MRS and mMRS agar plates. Three colonies were randomly picked by counting plates seeded with the highest dilutions, purified by repetitive streaking on MRS agar, and checked for catalase, Gram reaction and cell morphology. A total of 72 presumptive lactobacilli were submitted to DNA extraction according to the protocol proposed by Aponte *et al.*[12] and analysed by RAPD-PCR. Three random primers, M13,[13] M13-R2[14] and BOX[15] were tested for the ability to discriminate within isolated putative *Lactobacillus* spp. strains and for reproducibility. Primer M13-R2 showed the highest discrimination capacity and was then selected for RAPD-PCR analysis.

Evaluation of microbial dynamics by PCR-DGGE

DNA was directly extracted from salami samples at 0, 32 and 60 days of ripening according to the procedure proposed by Aponte *et al.*[16] In order to increase the amount of amplicons of hypervariable region V3 within 16S rDNA, nested PCRs were performed. In other words, the entire 16S rRNA was amplified by means of universal primers (FD1 and RD1) designed by Weisburg *et al.*[9] and used as template for PCR amplification of hypervariable regions V3 according to Aponte *et al.*[16] Conditions, temperature profile and reaction mixture, for amplifications were the same reported by Blaiotta *et al.*[10] PCR products were analysed by DGGE using a Bio-Rad D-code apparatus (Bio-Rad, XXXXX, XXXXX) and the procedure described by Aponte *et al.*[16] PCR-DGGE patterns were grouped by means of cluster analysis with the Pearson's product moment correlation coefficient and the unweighted pair group method using arithmetic averages (UPGMA). The profiles were analysed by means of Bionumerics software (XXXXX, XXXXX) to obtain a dendrogram.

Sensory analysis

Sensory analysis was performed on 2.5 mm thick slices of bovine salami after 20 days of ripening by a descriptive panel of 10 assessors (seven males and three females, aged between 25 and 55 years) with experience in sensory analysis.[17] Unsalted biscuits and warm water were used to rinse the mouth between evaluations of the samples. The judges were trained in three

preliminary sessions using different samples of commercial salami, to develop a common vocabulary for the description of the sensory attributes and to familiarise themselves with scales and procedures. The judges generated sensory terms individually. Each attribute term was extensively described and explained to avoid any doubt about the relevant meaning. Finally, 14 attributes were selected by consensus (frequency of citation >60%) to describe the salami: colour intensity, ease of peeling (aspect), odour intensity, acid odour, ripened odour, butter odour (flavour sensorial profile), sweet, salt, acid, bitter, ripened flavour (gustative sensorial profile), hard, gummy (tactile) and overall impression.

The panelists used a 10-cm structured scale to rate the intensity of each attribute. Random samples were evaluated by assigning a score between 0 (absence of the sensation) and 9 (extremely intense).

Samples were coded with three-digit random numbers and were presented to the assessors balancing the first-order and the carry-over effects. The average score given by the ten experts for each sample and session was recorded and used in the statistical analysis, which was performed using SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA). The Duncan's multiple comparison test was applied to discriminate amongst the means of descriptive sensory data at the 95% confidence level ($P \leq 0.05$). Results were expressed as means and standard deviation of at least three independent experiments.

RESULTS AND DISCUSSION

Selection of probiotic strains

Five probiotic lactobacilli were evaluated for the ability to acidify – alone or in combination with the two starter cultures – a broth *ad hoc* formulated to mimic conditions initially prevailing in salami at the onset of fermentation. pH monitoring confirmed the effectiveness of both starters to lead a fermentation in simulated conditions, while a high variability was recorded for probiotic strains when alone. Strains *Lb. casei* ATCC393 and *Lb. reuteri* DSM 17938 showed a poor adaptation to the medium: populations never exceeded the 7 Log mL^{-1} population level (Fig. 1).

Microbial counts highlighted potential synergic interactions between probiotic and starter strains. LAB counts on MRS were quite similar for the starter A, while significant differences, likely to be related to the presence of the probiotic strains, occurred for the starter B. In detail, counts were undeniably higher when the starter was combined with *Lb. plantarum* 299v and, at less extent, with *Lb. rhamnosus* LbGG (Fig. 1). Actually, members of the *Lb. plantarum* group have already been proved to find their elective habitat in cured meat,[5] while LbGG is well known for its ability to survive and to tolerate adverse storage conditions in dairy,[18] and non-dairy products,[19] as well as in dry-fermented sausages.[20–22]

Physico-chemical monitoring of salami

On the basis of outcomes, bovine salami were prepared by using the probiotic strain *Lb. plantarum* 299v in combination with both starters (AP and BP). Salami were also produced without the probiotic strain (A and B) and without added bacteria (C). The choice of the probiotic strain 299v was even due to the widely recognised tolerance to sodium chloride of members of the *Lb. plantarum* species. According to Papamanoli *et al.*[23] all *Lb. plantarum* strains, isolated by Greek dry-fermented sausages, were able to grow in presence of 6.5–10% sodium chloride. Moreover, *Lb. plantarum* 299v has already been used once, as starter culture to produce probiotic fuets, Spanish low-acid fermented sausages.[21,22]

Weight loss mainly occurred during the first week of ripening and almost stopped when salami were transferred at 4 °C. Moreover, weight loss was significantly lower for salami produced without starter and this could be related to the pH values that in this trial remained a bit higher (Fig. 2). Inoculated salami, particularly those obtained by using the starter B, experienced a higher weight loss, if compared to salami achieved by spontaneous fermentation. This event may be related to the increased acidification of such raw sausages, which facilitated the weight loss. As matter of fact, pH is one of the main factors influencing the diffusion of water from the inside to the surface of salami.[23] Other authors reported higher values, achieving weight losses of 40% in sausages fermented by *Lb. rhamnosus* after 28 days of processing,[20] between 40% and 45% in some Italian salami[24] and of 59% in salami fermented by *Lb. plantarum* cultures after 20 days of processing.[24]

At the end of the first week, pH was around 5.40 in salami obtained by starters inoculation, then a slower increase could be noticed (Fig. 2). Such a pH rise, during the second half of the ripening, could be related to basic compounds resulting from proteolysis and likely due to both indigenous proteinases and aminopeptidase and deaminase activities of the microbiota added for technological purposes.[22]

A pH lower than 5.2 is conventionally considered essential to obtain 'shelf-stable' meat products.[8] Actually, several studies performed on other meat foods reported a pH higher than 5.2.[14,25] Indeed, non-acid salami productions, characterised by pH endpoints above 6.0, are quite common within Mediterranean salami and the safety of the product is assured by inhibitory compounds other than acids.[26] As matter of fact, the reduction in the pH of fermented meat products also poses a challenge for the survival of inoculated probiotics. A reduction in pH from 5.6 to 4.9 after fermentation was proved to affect the probiotics survival (*Lb. rhamnosus* GG and E-97800) in fermented sausages.[20]

Microbial monitoring of salami

Total aerobic microflora, enterococci, lactobacilli and bile salts resistant lactobacilli (Table 1), micro-staphylococci, coagulase positive staphylococci, pseudomonads, yeasts and moulds (Table 2), *Enterobacteriaceae* and *Coli-aerogenes* bacteria (data not shown) were monitored during salami ripening. After 12 days at 18–19 °C, salami produced by spontaneous fermentation appeared characterised by an unpleasant smell of rotting meat and were so excluded from further analyses.

Total microflora reached a maximum after 6 days, independently by the trial. At 32 days, microbial levels in salami with starter A, either alone or combined with 299v (AP), were even 1 log higher if compared to the others. After 2 months, microbial loads were decreased, but were higher than 7 Log CFU g^{-1} in all trials (Table 1).

Lactobacilli were monitored on MRS agar at pH 5.50 and on MRS agar added of bile salts to attempt a selective count of the probiotic strain 299v, which was verified to be resistant to the adopted bile salts concentration in former try-outs. On the day of stuffing, salami contained more than 10^7 CFU g^{-1} LAB in all cases, but, on mMRS, this value was kept exclusively in trials added with the probiotic strain (Table 1). At day 12, LAB counts were still higher than 10^8 CFU g^{-1} in salami produced by the addition of probiotic cultures, and over 10^7 CFU g^{-1} in control salami, thus reflecting the levels recorded for total aerobic mesophilic microflora, only in the inoculated trials. Such outcomes perfectly match those reported for swine and venison nitrite-free dry-cured sausages.[6]

Counts on MRS agar added of bile salts reflect the optimal adaptation of the selected probiotic strain to the technological process

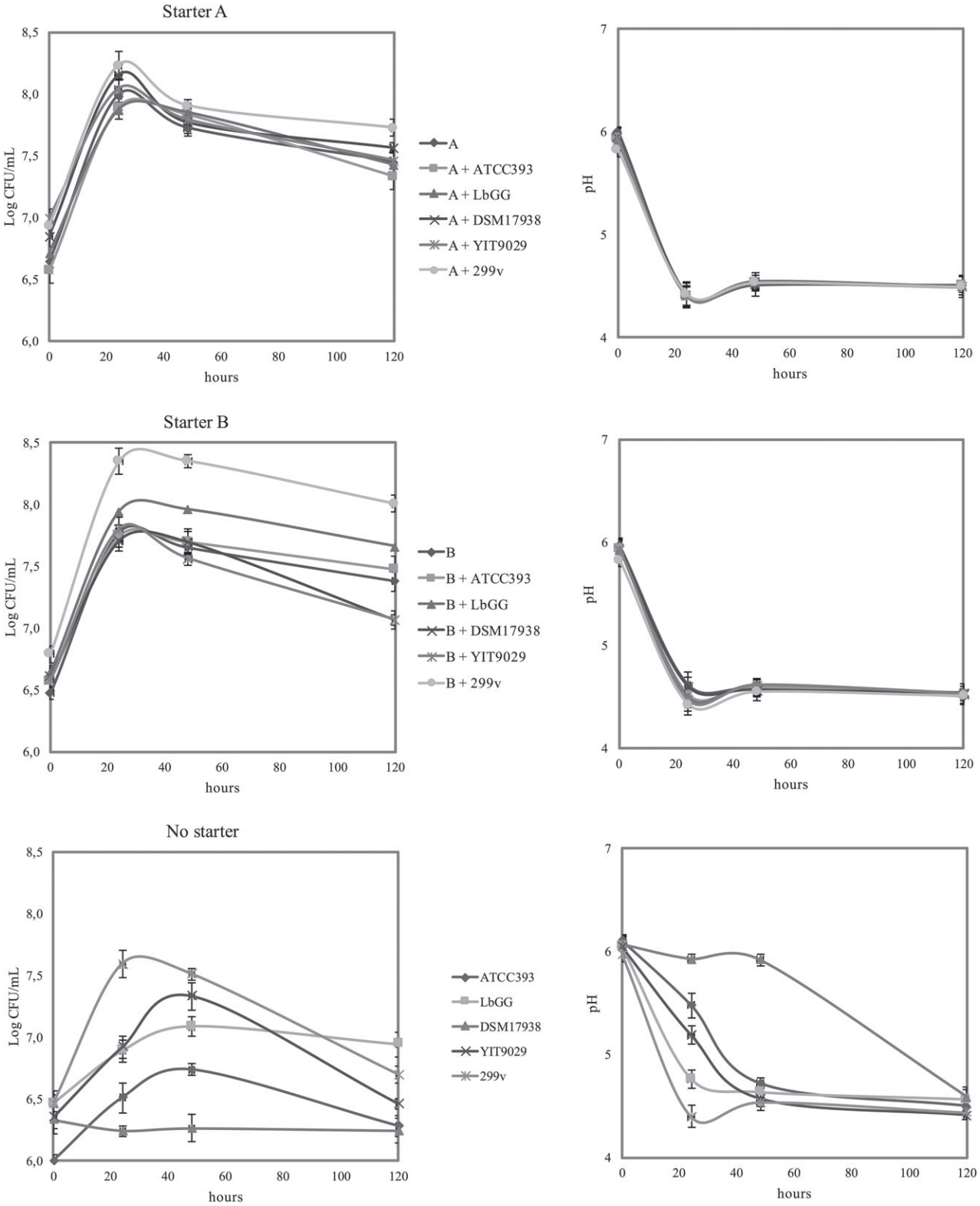


Figure 1. LAB counts (Log CFU mL^{-1}) and pH values in ECM-M medium at time 0 and after 24, 48 and 120 h of incubation at 22 °C. Probiotic strains were evaluated alone or in combination with the starter A and starter B.

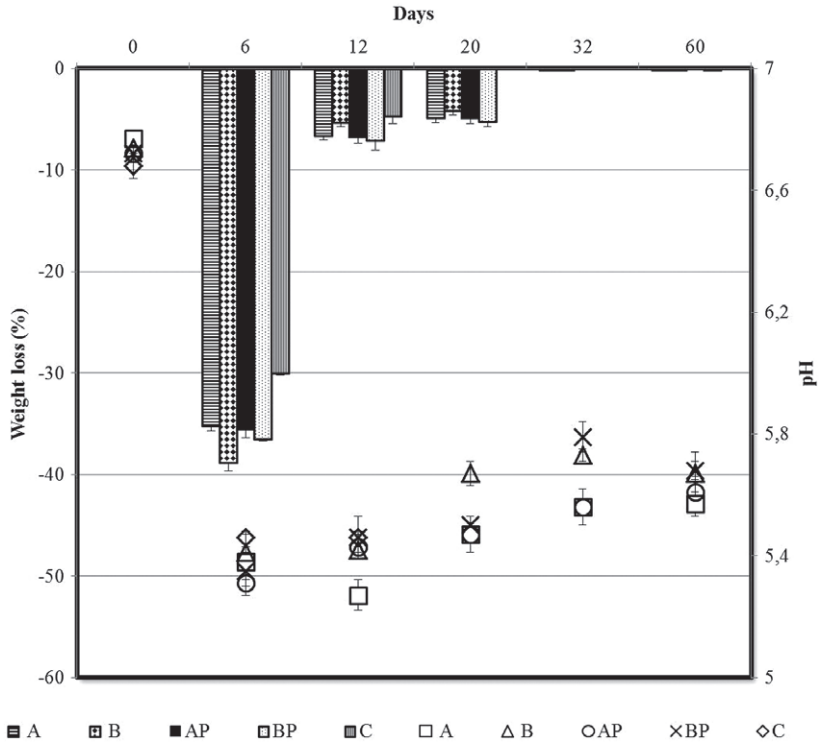


Figure 2. pH and weight loss values of the probiotic and control salami during 60 days of ripening and storage. A and AP, salami produced with starter A alone or in combination with the probiotic 299v. B and BP, salami produced with starter B alone or in combination with the probiotic 299v. C, salami produced by spontaneous fermentation.

conditions. In trials AP and BP, counts on MRS plus bile salts were always significantly higher. The occurrence of bile salt resistant LAB, even in salami not inoculated with probiotic strain 299v, confirms the potential existence of lactobacilli with functional traits in Italian salami, as recently pointed out.[27]

Enterococci increased of about 1 log in both trials added of the probiotic, as well as in salami achieved by using the sole starter culture B. A progressive drop could be observed starting by the 6th day of ripening. Up to the end of sampling, enterococci stayed always at lower levels in naturally fermented salami (Table 1).

Micrococcaceae were monitored on mannitol salt agar. On this medium, loads were almost the same, with the only exception of naturally fermented salami that showed, as expected, a lower level at the onset of the ripening (Table 2). Differences emerged with reference to coagulase-positive staphylococci, evaluated by counting on egg yolk tellurite emulsion-enriched Baird–Parker as selective substrate. Results reported in Table 2 indicate a control exerted by starter B against coagulase-positive staphylococci, which stayed below the 10^2 CFU g^{-1} (Reg. CE 2073/05) limit already after 12 days of ripening. After 32 days, presumed *St. aureus* colonies were not detectable (<100) for all trials (Table 2).

Pseudomonads initially decreased in all types of salami, but increased starting by the 20th day, which corresponds with the start of the salami storage at 4 °C (Table 2).

Yeasts and moulds populations, as monitored on WL agar, appeared nearly identical, except for naturally fermented salami, whose loads were higher. Values recorded for this parameter were significantly lower than those reported by Sidira *et al.*[28] during ripening of probiotic dry-fermented sausages produced by adding immobilised *Lb. casei* ATCC 393 cells.

Enterobacteriaceae and Coli-Aerogenes bacteria reached about 3 Log CFU g^{-1} at 6 days of ripening in salami produced with starter B, alone or in combination with the probiotic strain. In the next samplings, counts on both media and for all trials were below the detection threshold of the method (<100) (data not shown).

Lactobacilli monitoring by RAPD-PCR

One hundred and eight cultures, isolated from MRS and MRS added of bile salts agar plates at different ripening stages, could be presumptively told LAB after catalase test and Gram staining. Thirty-six strains could be reported to the genus *Pediococcus* spp. after observation at the microscope. As expected, all cultures isolated by salami obtained by using the only starter B were referable to pediococci. When starter B was combined with strain 299v, only some of the cultures could be ascribed to the *Pediococcus* genus (Table 3).

Seventy-two cultures, characterised by rod morphology, were biotyped by means of M13-R2 RAPD analysis. Except for one strain isolated by trial B after 20 days of ripening on MRS added of bile salts, all lactobacilli were characterised by pattern A1 (n. 32), namely the one of the *Lb. plantarum* strain used as starter A or by the profile of probiotic *Lb. plantarum* 299v (n. 39) (Table 4). Actually, *Lb. plantarum* and *Lb. pentosus* species are characterised by a moderate intra-specific polymorphism.[12]

All 23 lactobacilli isolated by trial A showed the RAPD profile of the starter (A1), independently by the isolation medium. When the starter was used in combination with probiotic lactobacilli, the dominating RAPD profile was that of the probiotic (299v). Surprisingly, within lactobacilli isolated from trial B, in which a *Pediococcus* strain was adopted as starter, six profiles out of seven were identical to that of the probiotic (299v), thus confirming the poor polymorphism occurring within members of the *Lb. plantarum* group.[12]

Table 1. Dynamics of total aerobic microflora, enterococci, lactobacilli and bile salts resistant lactobacilli in bovine salami during fermentation and ripening (Log CFU $g^{-1} \pm SD$)

		Days of ripening					
Medium	Trial	0	6	12	20	32	60
PCA	A	7.54 ± 0.02	8.60 ± 0.02	8.42 ± 0.17	8.26 ± 0.04	8.48 ± 0.05	7.86 ± 0.02
	B	7.79 ± 0.12	8.68 ± 0.02	8.15 ± 0.05	8.35 ± 0.03	6.93 ± 0.13	7.17 ± 0.05
	AP	7.73 ± 0.01	8.83 ± 0.11	8.79 ± 0.01	8.37 ± 0.02	8.06 ± 0.01	7.88 ± 0.01
	BP	7.52 ± 0.13	8.68 ± 0.05	8.45 ± 0.08	8.01 ± 0.09	7.62 ± 0.04	7.59 ± 0.02
	C	6.31 ± 0.09	8.15 ± 0.03	8.69 ± 0.07	–	–	–
MRS	A	7.38 ± 0.08	8.46 ± 0.06	8.10 ± 0.03	7.71 ± 0.04	7.97 ± 0.05	7.77 ± 0.03
	B	7.56 ± 0.04	8.44 ± 0.13	7.97 ± 0.02	8.11 ± 0.01	7.68 ± 0.04	7.04 ± 0.01
	AP	7.68 ± 0.01	8.77 ± 0.01	8.69 ± 0.03	8.50 ± 0.11	7.84 ± 0.01	7.70 ± 0.01
	BP	7.61 ± 0.12	8.76 ± 0.02	8.44 ± 0.15	7.96 ± 0.06	7.28 ± 0.06	7.38 ± 0.03
	C	7.32 ± 0.02	8.46 ± 0.01	6.71 ± 0.01	–	–	–
MRS + bile salts	A	4.29 ± 0.19	4.56 ± 0.07	4.39 ± 0.07	5.24 ± 0.06	4.16 ± 0.06	4.62 ± 0.04
	B	4.48 ± 0.02	5.56 ± 0.01	4.92 ± 0.04	4.79 ± 0.04	4.87 ± 0.01	5.31 ± 0.09
	AP	7.09 ± 0.01	8.60 ± 0.01	8.03 ± 0.02	7.26 ± 0.06	6.82 ± 0.01	6.53 ± 0.02
	BP	6.72 ± 0.02	8.49 ± 0.07	8.09 ± 0.06	7.09 ± 0.12	6.46 ± 0.05	8.27 ± 0.11
	C	2.88 ± 0.00	3.91 ± 0.09	4.71 ± 0.77	–	–	–
Slanetz and Bartley	A	7.31 ± 0.13	7.32 ± 0.07	4.62 ± 0.01	6.94 ± 0.17	5.59 ± 0.19	4.88 ± 0.24
	B	7.60 ± 0.02	8.50 ± 0.02	7.97 ± 0.03	7.93 ± 0.05	7.53 ± 0.02	6.66 ± 0.09
	AP	7.50 ± 0.02	8.42 ± 0.12	7.92 ± 0.07	7.40 ± 0.11	6.92 ± 0.04	6.71 ± 0.01
	BP	7.60 ± 0.04	8.68 ± 0.05	8.33 ± 0.17	7.64 ± 0.03	7.06 ± 0.08	6.72 ± 0.02
	C	7.32 ± 0.02	6.91 ± 0.09	6.76 ± 0.61	–	–	–

Table 2. Dynamics of micro-staphylococci, coagulase positive staphylococci, pseudomonads, yeasts and moulds in bovine salami during fermentation and ripening (Log CFU $g^{-1} \pm SD$)

		Days of ripening					
Medium	Trial	0	6	12	20	32	60
Mannitol salt agar	A	6.53 ± 0.10	7.75 ± 0.01	7.78 ± 0.05	7.78 ± 0.07	7.41 ± 0.01	7.30 ± 0.07
	B	6.70 ± 0.01	7.70 ± 0.01	7.59 ± 0.01	7.35 ± 0.02	7.28 ± 0.04	7.15 ± 0.04
	A + 299v	6.66 ± 0.01	7.73 ± 0.01	7.75 ± 0.02	7.46 ± 0.02	7.57 ± 0.03	6.66 ± 0.02
	B + 299v	6.64 ± 0.02	7.70 ± 0.02	7.60 ± 0.02	6.99 ± 0.11	7.48 ± 0.10	7.50 ± 0.05
	C	4.87 ± 0.05	7.41 ± 0.01	7.12 ± 0.03	–	–	–
Baird Parker	A	6.51 ± 0.11	4.58 ± 0.24	5.70 ± 0.02	3.42 ± 0.05	ND	ND
	B	4.32 ± 0.05	4.12 ± 0.08	2.06 ± 0.06	ND	ND	ND
	A + 299v	6.70 ± 0.01	6.68 ± 0.02	5.70 ± 0.01	3.40 ± 0.01	ND	ND
	B + 299v	4.12 ± 0.11	4.36 ± 0.06	2.06 ± 0.09	ND	ND	ND
	C	3.69 ± 0.07	6.45 ± 0.05	6.78 ± 0.01	–	–	–
Pseudomonas agar	A	6.74 ± 0.04	5.48 ± 0.12	4.87 ± 0.05	5.91 ± 0.13	4.75 ± 0.04	7.20 ± 0.01
	B	6.65 ± 0.02	5.62 ± 0.01	5.12 ± 0.05	4.44 ± 0.03	4.80 ± 0.02	7.38 ± 0.03
	A + 299v	6.73 ± 0.01	5.44 ± 0.03	4.69 ± 0.07	4.57 ± 0.01	5.17 ± 0.07	6.86 ± 0.02
	B + 299v	6.71 ± 0.02	5.58 ± 0.04	5.20 ± 0.07	4.59 ± 0.19	4.80 ± 0.06	7.47 ± 0.10
	C	6.75 ± 0.01	6.30 ± 0.07	5.85 ± 0.01	–	–	–
WL agar	A	6.03 ± 0.01	8.26 ± 0.10	8.03 ± 0.10	6.30 ± 0.04	4.75 ± 0.02	4.01 ± 0.18
	B	6.15 ± 0.03	7.87 ± 0.05	8.19 ± 0.03	5.39 ± 0.07	5.15 ± 0.05	4.03 ± 0.07
	A + 299v	6.03 ± 0.08	7.77 ± 0.03	7.22 ± 0.04	5.20 ± 0.07	5.43 ± 0.01	4.15 ± 0.08
	B + 299v	6.01 ± 0.12	7.70 ± 0.04	6.91 ± 0.09	5.18 ± 0.14	4.91 ± 0.13	5.00 ± 0.07
	C	6.12 ± 0.11	8.43 ± 0.02	6.40 ± 0.03	–	–	–

ND, not detectable.

Table 3. Number of cultures presumptively reported to the genus *Pediococcus* spp.

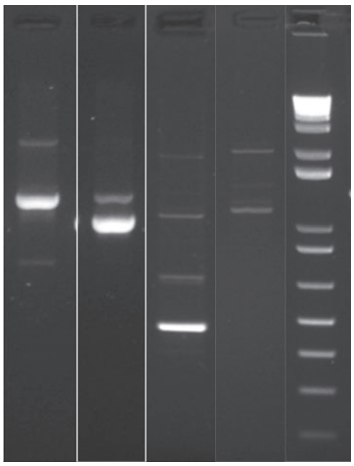
Trial	Isolation medium	Days of ripening 0	6	12	20	32	60
B	MRS	3 (−5)	3 (−6)	3 (−5)	3 (−5)	3 (−5)	3 (−4)
	MRS + BS	–	–	1 (−2)	–	1 (−2)	2 (−3)
BP	MRS	3 (−5)	2 (−6)	1 (−1)	2 (−5)	3 (−5)	1 (−5)
	MRS + BS	–	–	–	2 (−3)	–	–

The dilution of isolation is reported in parenthesis.
BS, bile salts.

Table 4. Distribution of RAPD profiles recorded for lactobacilli during monitoring

Trial	Isolation medium	Days of ripening 0	6	12	20	32	60
A	MRS	3 (−5) A_1	3 (−6) A_1	3 (−6) A_1	3 (−5) A_1	2 (−6) A_1	1 (−5) A_1
	MRS + BS	–	3 (−2) A_1	1 (−2) A_1	2 (−3) A_1	1 (−2) A_1	1 (−1) A_1
B	MRS	–	–	–	–	–	–
	MRS + BS	–	2 (−2) 299v	2 (−2) 299v	1 (−3) 299v	1 (−1) – 1 (−1) 299v – X_1	–
AP	MRS	3 (−5) A_1	1 (−6) – 1 (−6) A_1 – 299v	2 (−6) A_1	2 (−6) – 1 (−5) 299v – A_1	1 (−5) 299v	2 (−5; −6) A_1
	MRS + BS	–	3 (−6) 299v	3 (−6) 299v	2 (−5; −4) 299v	3 (−4) 299v	2 (−4) 299v
BP	MRS	–	1 (−6) 299v	2 (−5; −6) 299v	–	–	1 (−5) 299v
	MRS + BS	–	3 (−6) 299v	2 (−6) 299v	2 (−4) 299v	3 (−3, −4) 299v	2 (−4) 299v

RAPD profiles



Dilution of isolation is reported in parenthesis.
BS, bile salts.
[a] M13 R2 RAPD profiles of probiotic *Lb. plantarum* (299v), *Lb. plantarum* of starter A (A1), *Pd. pentosaceus* of starter B (B1) and of an unidentified *Lactobacillus* strain. M: 1 kb ladder DNA marker (Invitrogen).

Outcomes provided proof of the survival of *Lb. plantarum* 299v in salami up to 60 days of ripening, showing survival and co-existence ability of this strain at a high concentration in fermented meat products, as already reported by Rubio *et al.*[21]

Evaluation of microbial dynamics by PCR-DGGE

Total DNA was extracted by each type of salami during ripening. V3 inner hypervariable regions amplicons were separated by means of DGGE (Fig. 3). All samples were characterised by quite poor profiles with two, at most four, main bands up the end of ripening. Nine bands were in total purified and sequenced (Fig. 3). Two bands (8 and 5), corresponding to *Lb. plantarum,* were present in all types of salami up to 60 days of ripening, except for those inoculated with starter B or naturally fermented, even though the presence of lactobacilli had been evidenced by RAPD outcomes.

St. xylosus (bands 6 and 9) was retrieved in all types of salami up to the end of ripening, except in control fermentation, in which it seems to be replaced by *St. pasteuri*. This is an expected result since this species occurred in both starters.

Pd. pentosaceus (band 7) was recovered in all salami where starter B was added, either alone or combined with probiotic strain 299v.

Brochothrix thermosphacta (bands 2 and 4) was found in all salami samples at time 0. Cocolin *et al.*[29] reported that this species, which is a natural meat contaminant, disappear after the 3rd day of ripening in Italian salami. Equally, *Brochothrix thermosphacta* was identified in Italian salami 'Gentile' and 'Crespone' only at 0 and 2 days of ripening.[30]

Lb. ruminis was recovered in salami manufactured by natural fermentation. This formally recognised motile *Lactobacillus* species has been identified as part of the intestinal microbiota of several mammals, including cows and humans.[31]

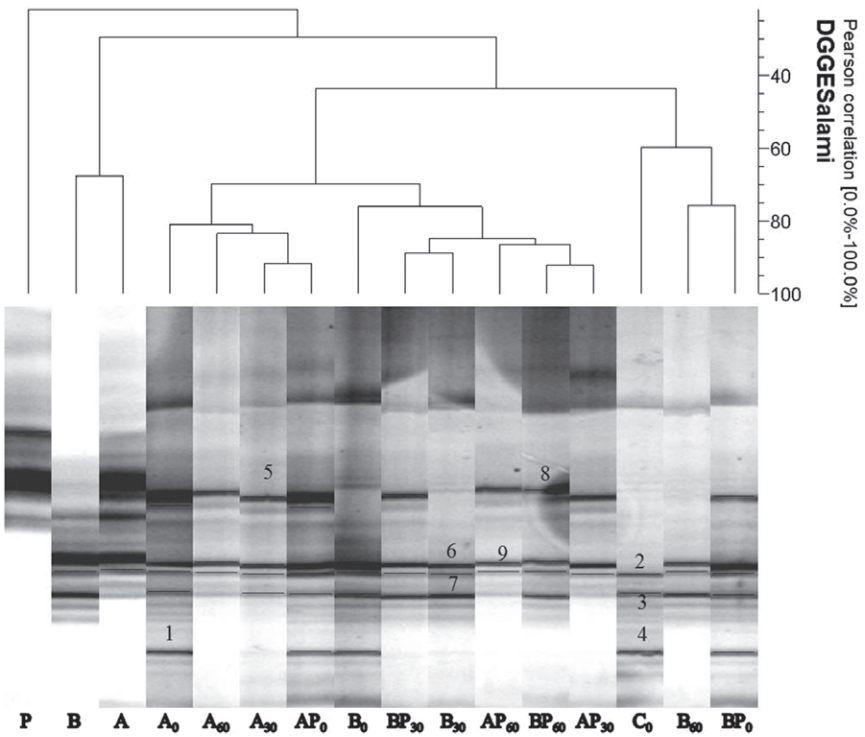
Sensory evaluation

Cultures different from traditional meat starters may have a negative impact on the sensory properties of salami. To determine whether the addition of probiotic cultures affected the sensory characteristics of the settled salami, a sensory analysis was carried out on salami after 20 days of ripening. Table 5 shows the mean scores of the sensory attributes selected by descriptive sensorial analysis to describe the samples and the overall acceptance of the experimental salami. Only three descriptors (ease of peeling, acid odour, and acid) significantly varied ($P < 0.05$) within salami

Table 5. Sensorial descriptors mean scores and standard deviations of bovine salami: starter A (A), starter B (B), starter A plus *Lb. pentosus* 299v (AP), and starter B plus *Lb. pentosus* 299v (BP)

	A		B		AP		BP	
Descriptor	Average	SD	Average	SD	Average	SD	Average	SD
Aspect								
Colour intensity	3.63[a]	1.40	3.88[a]	1.73	3.80[a]	1.10	3.88[a]	2.03
Ease of peeling	6.00[a]	0.93	6.75[a,b]	1.67	6.20[a]	0.43	7.25[b]	0.89
Flavour sensorial profile								
Odour intensity	6.88[a]	1.13	6.25[a]	1.28	6.88[a]	1.13	6.50[a]	0.93
Acid odour	4.50[b]	1.69	6.63[a]	0.93	5.63[b]	1.69	6.38[b]	1.41
Ripened odour	4.63[a]	1.92	3.50[a]	2.07	4.60[a]	1.92	3.88[a]	2.42
Butter odour	4.25[a]	2.05	4.16[a]	1.46	4.25[a]	2.05	4.23[a]	1.04
Gustative sensorial profile								
Salt	6.38[a]	1.19	6.13[a]	0.83	6.38[a]	1.19	6.24[a]	1.46
Sweet	4.50[a]	2.45	5.00[a]	2.00	4.50[a]	2.45	5.00[a]	2.20
Acid	6.88[a,b]	1.16	7.18[b]	0.83	6.38[a]	1.16	7.25[b]	0.52
Bitter	2.75[a]	1.39	3.38[a]	1.60	2.75[a]	1.39	3.75[a]	1.49
Ripened flavour	2.63[a]	1.19	2.38[a]	0.74	2.63[a]	1.19	3.00[a]	1.41
Tactile and quality profile								
Hard	5.75[a]	2.25	6.63[a]	1.19	5.75[a]	2.25	5.38[a]	2.39
Gummy	6.88[a]	1.13	6.13[a]	1.89	6.88[a]	1.13	5.88[a]	1.46
Overall impression	7.38[a]	1.51	6.88[a]	0.35	7.38[a]	1.51	7.13[a]	1.13

Values with different letters in the same row indicate statistical differences according to the Duncan's test at $P < 0.05$.



Band	Description	% similarity	Accession
1, 4	*Brochothrix thermosphacta* (FSL F6-1036)	99-100%	NR_113587.1
5, 8	*Lactobacillus plantarum* (WCFS1)	100%	NR_075041.1
3	*Lactobacillus ruminis* (ATCC 27782)	89%	NR_102839.1
7	*Pediococcus pentosaceus* (ATCC 25745)	100%	NR_075052.1
6, 9	*Staphylococcus xylosus* (JCM 2418)	99-100%	NR_113350.1
2	*Staphylococcus pasteuri* (psy-BHI19)	98%	JQ267517.1

Figure 3. Evolution of total microflora by means of PCR-DGGE. V3 amplicons obtained by DNA extracted by salami samples. For samples code see Fig. 2.

samples (Table 5). Acid descriptor was strongly perceived, as odour and taste, in line with recorded pH values (Fig. 2), in samples at 20 days of ripening. Actually, it is in a way expected that *Pd. pentosaceus*, alone and, above all, associated with probiotic lactobacilli, produces higher levels of lactic acid, causing higher acidification and higher scores in taste parameters.

With reference to samples aspect, only minor colour differences could be observed, while it was possible to discriminate samples for ease of peeling ($P < 0.05$). Focusing on odour and taste, all salami were characterised by adequate odour intensity (6.25–6.88 mean intensity ratings) and salt perception (6.13–6.38 mean intensity ratings). Lower, but detectable levels, of ripened (<4.63 ratings) and butter flavours (<4.25 ratings) were perceived. The overall quality of bovine salami (>6.88 ratings) was comparable to that reported by Rubio and co-workers for pork-fermented sausages (6.8)[21] and Spanish traditional *fuets* (6.1).[22] both produced by using the same probiotic strain.[21,22]

CONCLUSIONS

The addition of functional ingredients is widely suggested as a strategy to develop healthier meat and meat-derived products. Sausages could be a good vehicle to transfer probiotic cells into the intestine, because they are 'encapsulated' by a matrix consisting of meat and fat. In such food, probiotics might likely survive much better during the critical passage through the stomach and the small intestine, compared to their unprotected exposure to low pH and bile salts.[3] In spite of this, research reports related to inclusion of probiotics in fermented salami are still few in number. In this sense, the present survey, aimed to develop probiotic-enriched bovine meat-based salami, is certainly novel.

Data showed a remarkable viability of the probiotic strain even after 60 days of storage. *Lb. plantarum* 299v kept a concentration higher than 10^6 CFU g^{-1}, i.e. the level of probiotic bacteria recommended at the time of consumption to exert a healthy effect in humans.[28] Based on such criterion, the suggested dose of probiotics is largely overcome by consuming a 10–25 g daily amount of bovine salami, which is compatible with a nutritionally balanced diet.[21]

In conclusion, results here presented indicate the opportunity of using cattle meat for probiotic salami production, in particular using meat from cows on retirement and/or secondary or low quality beef cuts. Experimental salami proved to be hygienically safe, since coliforms and coagulase-positive staphylococci were not detectable in salami at the time of consumption as well as after more than 1 month of storage at chilling temperature. pH, weight loss and microbiological counts were within the ranges conventionally considered to be ideal for the formulation of fermented sausages. Moreover, probiotic-endowed salami exhibited the same level of acceptance and similar performances, if compared to control salami obtained by using only conventional meat starter cultures.

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