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TITOLO TESI/THESIS TITLE

Microbial ecology of fermented meat for the isolation of targeted strains as biopreservatives

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Abstract

Most of the pathogen contaminants in meat products, *Clostridium botulinum*, *Listeria monocytogenes* and *Salmonella* spp. included, are ubiquitous in the environment and may therefore be transmitted, from the raw material, along "farm to fork" chain. In fermented meat products, pathogens outgrowth is kept controlled by the addition of nitrite. Hitherto, nitrite is the most efficient agent against the *C. botulinum* germination and proliferation. The nitrites amount up to 150 mg/kg is allowed amount in the majority of the European Union countries, whereas a more stringent restriction is in force in Denmark (100 mg/kg) as a maximal ingoing amount in fermented meats. However, EFSA, in the last re-evaluation report on nitrites safety, concluded that the maximal amount of 150 mg/kg, added in the meat products, is within the safety limits. On the other hand, the conclusion drawn by the International Agency for Research on Cancer (IARC) on the toxicity of nitrosamine was that 'under conditions that result in endogenous nitrosation, ingested nitrate or nitrite is probably carcinogenic to humans (Group 2A)'. Therefore, there is a general concern about the nitrite addition in meat and is also supported by the continuous consumers demand for chemical free products. However, the raw meat used for fermented meat production is an extremely perishable matrix and its chemical-free conservation is a major nowadays challenge. Biopreservation is one of the most ancient microbial-based strategy to improve the product safety and extend its shelf-life, through the natural microflora and (or) their antibacterial products. Lactic acid bacteria have a major potential for use in biopreservation because they are safe to consume due to their QPS/GRAS status but also, they naturally dominate the microbiota of many fermented foods.

The present work explores the biopreservation as a potential alternative approach in a view of nitrites elimination or reduction in fermented meats. In particular, on one hand, it focuses on finding performant lactic acid bacteria strains, for their antimicrobial and techno-functional traits. On the other hand, the present work describes to what extent is safe to reduce the nitrites in the fermented meat products. Therefore, the antimicrobial activity of selected strains was tested through challenge tests, in nitrite-free and nitrite-reduced fermented salami, against *C. botulinum*, *L. monocytogenes* and *Salmonella* spp.

The observed outcomes demonstrated that the biopreservation is a promising approach for the pathogens outgrowth control in a nitrite-reduced fermented salami. In particular, one of the major outcomes is that the combination of 30mg/kg $NaNO_2$ and bioprotective/starter cultures is a safe approach for *Clostridium* spp. outgrowth control in fermented meat.

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LONGLIFE - Food Fermentations for Purpose: Health Promotion and Biopreservation, JPI Project

The present work was financed by the LongLife JPI Project from 2016 to 2019.

Fermentation is a traditional food processing method that allows the transformation of a raw primary material as milk, meat and cereals in a naturally fermented product. Nowadays, selection of food cultures is aimed in a techno-functional characteristics improvement as opposed to bio-functional capacity of the microbiota used in the fermentation process. Also, in an attempt to meet the consumers' demand for a clean label foods, the biopreservation features of the microbial cultures represent important characteristics that could guarantee the safety in traditional fermented foods.

LONGLIFE involved innovative processing of food substrates (milk, cereals and meat) into fermentates, using novel strains of lactic acid bacteria (LAB) and yeasts to produce value-added fermented products such as grain-derived foods and beverages, long-fermented sourdough bread and meat products with improved health benefits, organoleptic qualities and extended shelf-life.

Pre-screening of starter and protective cultures was performed for their bioactive metabolites as: exopolysaccharides, polyols and antimicrobial compounds. Strains with demonstrated *in-vitro* performances were used during fermentation of milk, cereals and meat together with the fermentation process improvement by manipulating processing conditions and to finally optimise food properties for health and economic benefit.

Additionally, new prebiotic ingredients developed based on novel superheated steam processing of bran substrates for enhancing bio-functionality in food/beverages. *Ex vivo* studies yield data on the validation of health effects (prebiotic and bioavailability/digestibility) of the generated ingredients and products.

Textural characteristics comparison with a bench-mark product, from same type of newly developed product within LongLife project, are going to be performed.

The specific LongLife goals are aimed at:

- Identification and characterization of food grade cultures with exopolysaccharides, polyol antimicrobial and antifungal-producing properties,
- Develop natural bio-engineered fibre-based ingredients and food products capable of releasing functional bioactive compounds,
- Develop a range of next generation foods with superior nutritional, health and techno-functional properties,
- Evaluate the digestibility, bioavailability, bio-accessibility and bioactivity of food compounds and ingredients within the food matrix,
- Demonstrate retention of health promoting activity following gastric transit, based on ex vivo studies to assess prebiotic activity and bioavailability/digestibility,
- Promote the scientific findings and technological solutions to all relevant stakeholders.

The consortium consists of 7 partners from 6 countries (Ireland, Italy, Netherlands, New Zealand, Poland and Romania). The partners involved are: Teagasc, University College Cork, University of Bologna, Netherlands Organisation for Applied Scientific Research, AgResearch Limited, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences and University of Agricultural Sciences and Veterinary Medicine Cluj Napoca. The LONGLIFE project management is under the responsibility of a coordination team: Catherine Stanton (IE) is the project coordinator.

The project will have a positive impact on both consumers, looking for healthier, nutritious and safer products, and on the food industry through the innovative processes development to guarantee products safety and quality.

1. Introduction

1. 1 Foodborne outbreaks

In the era where demand for ready to eat and preservative free products is constantly growing, the microbiological food safety has to be guaranteed, proportionally with this ongoing trend. The last report (2007-2015) of WHO (World Health Organization) estimates the global burden of foodborne disease to be caused by 31 hazards, with over 600,000,000 cases of illness, almost 420,000 deaths and 27,000,000 Years of Life Lost (YLL) (Havelaar et al., 2015).

Focusing on the European Union member countries, within the last EFSA foodborne outbreak (FBO) summary for the year 2017, were reported 5,079 outbreaks, corresponding to a 6.8% decrease compared to 2016. *Salmonella* (*S. enteritidis)* was the most common bacterial causative agent detected in foodborne and waterborne outbreaks in the EU (34.3% of all outbreaks), followed by bacterial toxins (16.1%), viruses (7.8%), other causative agents (3.6%) in particular histamine and parasites (0.6%), whereas for 37.6% of the outbreaks, the causative agent was not reported. In the same report, *Salmonella* caused the highest number of cases, hospitalisations and deaths, whereas *Listeria* and *C. botulinum* were associated with the highest case fatality, 5.1% and 7.7%, respectively, among the FBO cases.

Among 643 strong-evidence FBO (12.7% of total outbreaks), 60% were associated with food of animal origin; 'Meat and meat products' (i.e. including meat from poultry, pork, bovine, sheep and other unspecified red meats and their products) (EFSA, 2018).

1. 2 Fermented meat

Fermented meat products represent a fingerprint of traditions, culinary heritage and identity that is transmitted from generation to generation. Their origins date back from millennia with the first fermented sausages manufacturing by the Ancient Romans and Greeks, that spread throughout Europe in the Middle Age. The territorial environmental conditions led to

distinguish the processing conditions so that drying was applied in the Mediterranean area while smoking in Northern European countries (Flores and Toldrà, 2011, Ojha et al., 2015). A wide variety of semidry- and dry-fermented sausages is owed to the unique interactions between the primary material, microbiota composition and the processing conditions (Leroy et al., 2013; Tabanelli et al., 2012).

The meat matrix is a great nutrients source for microbial proliferation, i.e. glycogen, proteins, fats, amino acids, minerals and vitamins. Therefore, meat fermentation represents a complex physical, biochemical and microbial interaction. The initial enzymatic reactions are mostly guided by the endogenous enzymes, as cathepsins, and is further completed by the microbial ones (Flores and Toldrà, 2011). The microbial growth causes acidification of the meat batter that leads to textural changes, that include a solubilization and gelation of the muscular proteins, proteolysis and lipolysis. In addition, the mass and volume loss thorough the drying process contributes in a texture hardening and in aroma compounds development, typical for fermented salami (Toldrà, 2010). Lactic acid together with the proteolysis related products, as peptides, free amino acids, aldehydes, organic acids amines, volatile compounds, generated during the proteolysis and lipolysis, are the main contributors for the typical fermented meat taste. The safety of fermented salami is mainly guaranteed by the low pH and a_w. Lower the values, safer the hurdle barrier is, due to microbial pH sensitivity and their inability to cope when less water is available (Majou and Christieans, 2018, Toldrà, 2010).

However, these products are not exempted from the foodborne pathogen outgrowth, *L. monocytogenes*, *Salmonella* spp. and *C. botulinum* are some of the reported causative agents (EFSA, 2018).

1. 3 Foodborne pathogens

1. 3. 1 *Clostridium botulinum*

C. botulinum is a rod-shaped, Gram-positive, spore-forming, anaerobic bacteria. Botulinum endospores are widespread in the nature and could be found in the soil, dust, sediments, mammals intestine (i.e. pigs, birds, and fishes), therefore, eventually in the foods.

Is a producer of botulinum neurotoxins (BoNTs), the most lethal natural compound, among all bacterial, animal, plant and chemical poisonous compounds (Poulain and Popoff, 2019). Based on different phenotypic (i.e carbohydrate utilization, proteolysis and lypolysis) and genotypic (16S rRNA and Whole Genome Sequencing) markers comparison, neurotoxigenic *Clostridium* spp. belongs to at least six groups (I-VI). Their metabolic flexibility in adaptation to different substrates and/or to hosts defences, allowed to have a nowadays heterogeneity of *C. botulinum* types and subtypes. Botulinum toxins are produced as single-chain proteins, distinguished in a seven BoNT toxinotypes (A to G), based on the neutralization with corresponding specific antibodies, what list was recently added with another two, not completely described toxins, BoNT/H and BoNT/X (Poulain and Popoff, 2019). Considering meat products, a low prevalence of Group I and Group II *C. botulinum* spores in meats has been described, whereas A, B, E and rarely F types were described in a foodborne case (Keto-Timonen et al., 2012, Poulain and Popoff, 2019). However, regardless a botulinum toxins heterogeneity, their neurotropism results in an identical clinical disease, therefore the identification of the neurotoxin type is important for the antitoxin therapy. Foodborne botulism symptoms start 12–96 h after eating contaminated food with predominant nervous rather than gastrointestinal symptoms. Symptoms are characterized by the inhibition of cholinergic neurotransmission in the peripheral part of the nervous system, resulting in dysphagia, diplopia, ptosis, gland secretion inhibition (dysautonomia), muscle fatigue, flaccid and respiratory paralysis. Clinical signs usually develop within 6 days of ingestion and, in the absence of the antitoxin treatment,

death occurs within 8 days. Antitoxin therapy failure may also occur, therefore, treatments including administration of intravenous fluids and supplemental oxygen administration or mechanical ventilation are used, with a potential prolonged recovery up to 3 to 4 weeks (Mazzaferro and Ford, 2012). The intoxication severity depends on the amount of the ingested toxins. The infectious dose of 30-100 ng of preformed BoNT is sufficient to induce botulism in humans by the oral route. Little is known about the minimum infective dose of botulinum spores, but attention should be posed when conditions supports germination and vegetative cells growth, meaning supportive toxin production environment. The drawbacks in the cell detection, prior their lysis in the direct plate counting, avoided to confirm the reliable toxins detection, that was in one case reported to be detected when the initial inoculum was 2-log CFU/g followed by 100- or 1000-fold microbial increase, whereas others reported BoNT detection when 1-log or prior to any colony increase in the plate counts (Glass and Marshall, 2013).

Nowadays, this pathogen is reported in a canned food, artisanal food products with critical physio-chemical characteristics, as well as in a ready to use foods, both, from animal and vegetable origin, where temperature abuse mostly occurs. However, data collected about *C. botulinum* cases are mostly from high-income European countries, that also in certain cases is misdiagnosed (Havelaar et al., 2015, EFSA, 2018).

1. 3. 2 *Listeria monocytogenes*

L. monocytogenes is a rod-shaped, Gram positive, mobile, non-sporeforming, facultative anaerobic microorganism. Is another rare foodborne pathogen, but highly fatal with 2,480 infections estimated in 2017 in Europe, with a mortality of 24%, prevalently in elderly patients. The target group includes also neonates, pregnant women and immunocompromised patients, listeriosis is mostly attributable to severe central nervous system infections, septicaemia,

spontaneous abortions and neonatal listeriosis (EFSA, 2018). *L. monocytogenes* is ubiquitous in the nature as well as in a food-processing plants, due to its ability to form biofilms that, in certain cases, possess tolerance against commonly used disinfectants. Currently 13 serotypes exist, classified by the somatic (O) and flagellar (H) antigens, but only 1/2a, 1/2b, 4b serotypes are more frequently associated to human listeriosis (Borucki and Call, 2003). The microorganism is highly resistant to acid and salt stress, it is able to grow at temperatures between 0 and 45°C, and requires minimum water activity of 0.92. These characteristics allow the bacterium to contaminate different ready to eat foods, fish and meats products, soft and semi soft cheese, fruits and vegetables, but *Listeria* could also be sampled in all sampling stages and units in the production plants (Stoller et al., 2019, EFSA, 2018). Therefore, this pathogen is nowadays the biggest health-related problem and represent a challenge for the food industries (D'Ostini et al., 2016).

1. 3. 3 *Salmonella* spp.

Salmonella spp. is a zoonotic pathogen, with 91,662 confirmed human cases reported in the European countries in 2017, with a low mortality rate (EFSA, 2018). Is a Gram-negative, rod-shaped, facultatively anaerobic enteric bacteria. Is commonly found in the intestinal tract of a wide range of domestic and wild animals. Most commonly reported serovars in *S. enteritidis, S. typhimurium*, *S. infantis* and *S. newport*. The salmonellosis more often causes gastroenteritis, but in a rare, severe cases enteric fever, septicaemia and death. From farm to fork is the main route of transmission, that include, infecting the carcass during the slaughtering, handling infected raw material or when foods are contaminated by infected individuals with scarce hygienic practice (EFSA, 2018). Fermented foods as sausages are not exempted from *Salmonella* spp. outgrowth where *Salmonella typhimurium* was found in pork carcasses (Christieans et al., 2018). The optimal growth range is at 35–37°C and at pH 6.5–7.5, even

though its wide range of potential growth goes from 5.2–46.2°C and the pH from 3.8–9.5 (Wirtanen and Salo, 2016).

1. 4 Nitrites in fermented meats

Curing with nitrites is an ancient preservation method that is still used in the salami production. Its addition to a raw meat matrix enabled to strongly reduce the risk of *C. botulinum* outgrowth and their spore germination inhibition, as well as of other foodborne pathogens outgrowth in meat products (Hospital et al., 2016). It was reported that the anti-clostridial activity increases with the increase of the added nitrite rather than with the residual amounts (Sindelar and Milkowski, 2012). Nitrites addition is regulated due to the health-related safety concern (IARC, 2010). The European Commission has recently approved a Danish national provision in setting more stringent restrictions, allowing 100 mg/kg of nitrites as a maximal ingoing amount in fermented meats, whereas the amount of 150 mg/kg remains unchanged in the rest of the EU countries (European Commission, 2018; EFSA, 2017a). Additionally, with the EC Regulation Nº 889/2008 for organic meat products, almost 50% reduction of the maximum amount was established: 80 mg/kg for added nitrite and 50 mg/kg for residual nitrite (European Commission 2008). However, EFSA in the last re-evaluation of nitrites safety, concluded that its addition and consumption is within the safety limits (EFSA, 2017a). The nitrites healthy related concern, relies on their interaction with a secondary amine in the human gastrointestinal tract that leads to formation of a potentially carcinogenic N-nitroso compounds (EFSA, 2017a). The genotoxic nature of nitrosamines is due to the *in vivo* enzymatic degradation into reactive diazo compounds that can bind to DNA (de La Pomelie, 2018). In particular, IARC concluded that 'under conditions that result in endogenous nitrosation, ingested nitrate or nitrite is probably carcinogenic to humans (Group 2A)' (IARC, 2010). This multifunctional additive is known for its outstanding technological properties, as for its contribution for the typical

"cured" flavour, the red meat colour development and as an antioxidant inhibits the lipids rancidity (Majou and Christieans, 2018, Alahakoon et al, 2015). However, the current consumers demand is for additive-free products, challenges the industries in finding bio-based alternatives, while preserving products safety and quality.

1. 5 Biopreservation in fermented foods

Food preservation has been a historical and an ongoing challenge. The ancient preservation methods as drying, salting and fermentation are still commonly used preservation methods. The earliest foods fermentation was based on the spontaneous fermentation of the raw materials, naturally colonised with different microbial communities. A natural contamination of the raw material, or during the fermentation process, with a spoilage microorganism or with microbial pathogens, lead to look for new approaches for the fermented foods preservation improvement. Therefore, "backslopping" was introduced and is referred to a raw materials inoculation with a fermented one, where a successful fermentation has previously occurred. The inoculation of the new matrix with already adapted dominant strains to the same matrix, was an "unconscious" introduction of a nowadays widely used *starter cultures*, aimed at reducing the process failure and to its shortening (Flores and Toldra, 2011).

However, with the gradual shift from a small scale to a large-scale industrial foods production, an intense use of a chemical preservatives, i.e. nitrites, were introduce to guaranty the foods safety and quality. In parallel, with an increase consciousness of the foods composition, the consumers have proportionally increased the demand for healthier and chemical-free products (Hung et al., 2016b).

Therefore, biopreservation approach has started to be intensively studied over the last decades, to fulfil the consumers and industrial need for a healthier and safer product. "Biopreservation refers to extended storage life and enhanced safety of foods using the natural microflora and

(or) their antibacterial products. Lactic acid bacteria have a great potential for use in biopreservation because they are safe for the consumer and during storage they naturally dominate the microflora of many foods. In milk, brined vegetables, many cereal products and meats with added carbohydrate, the growth of lactic acid bacteria produces a new food product. In raw meats and fish that are chill stored under vacuum or in an environment with elevated carbon dioxide concentration, the lactic acid bacteria become the dominant population and preserve the meat with a 'hidden' fermentation." (Stiles, 1996).

By definition, lactic acid bacteria (LAB) are mostly employed organisms in foods fermentation. LAB in foods can be defined as starters or as protective cultures, depending on their application goal. In particular, starters are characterized by their outstanding metabolic performances, being of a great technological and quality insuring importance, whereas protective cultures have a main aim to guarantee the products safety by the growth and survival inhibition of pathogenic microorganisms. The ideal strain candidate would fulfil both, the metabolic and antimicrobial characteristics. In addition, bacteriocins, bacteriophages and bacteriophage-encoded enzymes are also taking part of the "green" biopreservation approach (Garcia et al., 2010).

1. 6 Microbial food cultures

Food cultures as lactic acid bacteria are classified as safe by EFSA and are within Qualified Presumption of Safety (QPS) list; whereas in the USA the same are approved by FDA and classified as Generally Recognised as Safe (GRAS). In both cases their safe use is due to the history of safe consumption and a body of knowledge. The General EU Food Law relies on the food cultures suppliers' obligations for a careful safety assessment on the new released products (Laulund et al., 2017). Therefore, beside the QPS/GRAS status, the safety of starter

and protective cultures should be guaranteed, and strains that have antibiotic resistance traits and that produce biogenic amines should be excluded (Herman et al., 2019).

Fermented salami is restricted ecological niche, harbouring as main genera: *Lactobacillus*, *Staphylococcus, Debaryomyces* and *Penicillium* (Franciosa et al.., 2018). Microbial cultures isolated from traditional, spontaneously fermented salami, represent a good food cultures candidate, due to their previous adaptation to the meat environment, that could lead to a further microbiota control in the product where will be applied to (Cocconcelli and Fontana, 2008). Lactic acid bacteria followed by coagulase-negative staphylococci (CNS), yeasts or moulds, alone or in a combination are commonly used cultures in meats fermentation (Galvez et al., 2010).

In a view of nitrites reduction or elimination from fermented meat products, protective cultures were studied over the last years as a promising "green approach" in fermented meat products (Oliveira et al., 2018). Among lactic acid bacteria, the mostly studied strains are belonging to the genus: *Lactobacillus*, *Lactoccocus* and *Pediococcus* were most commonly explored namely, *L. sakei*, and *L. curvatus* as the mostly studied species due to their dominance in the meat matrix (Jansen et al., 2018), followed by *L. plantarum*, *L. fermentum*, *Lc. lactis*, and *P. acidilactici* (Albano et al., 2007, Benkerroum et al., 2005, Nikodinoska et al., 2019, Marcos et al., 2007, Nieto-Lozano et al., 2010).

2. Scope of the thesis

Raw meat used for fermented sausage production is an extremely perishable matrix and its chemical-free conservation is a major challenge nowadays. In this view, the scientific community is challenged in finding alternative for nitrites, the main chemical agents used for pathogen control in the meat, that can give rise to toxic and harmful compounds. Bioprotective strains, with GRAS/QPS status, may have the potential of effectively replace these chemical additives.

With the final aim of finding alternatives for nitrites use or reducing their amount in fermented meat products, the present work proposes the combined use of low levels of sodium nitrite in combination with the use of bioprotective microbial cultures. Therefore, the main focus of the present PhD study is the isolation, characterization and use of microbial strains to be used as protective cultures in the meat and, as the same time, as starters for the fermentation process. These strain should possess antimicrobial activity against meat pathogens along with techno-functional properties in meat fermentation. A particular focus is given to understand the effectiveness of the selected cultures, alone or in combination with low nitrites concentration, as biopreservation method in a fermented meat product (salami). Moreover, since the fermented meat salami are an ecological niche that harbours a variety of native microorganisms, the characterization of microbial ecology and further isolation of dominant strains was also performed.

Considering this, the specific aims of this study are to:

1. study the possibility of using available LAB strains as bioprotective cultures against meat pathogens (Paper 1 and 2)

1.study the microbial ecology of artisan fermented meat-salami prepared without the addition of starter bacteria cultures and additives (Paper 3);

2. isolate and screen LAB strains for their antimicrobial and techno-functional characteristics (paper 3);

3. study the strain stability and dominance in the food matrix as well as the antimicrobial activity in "chemical-free" *in carnis* model (Paper 4);

4. develop potential product prototype, fermented with selected bioprotective/starter cultures (Paper 4).

CHAPTER 1

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

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Abstract

In meat fermented foods, *Clostridium* spp. growth is kept under control by the addition of nitrite. The growing request of consumers for safer products have led to consider alternative bio-based approaches, the use of protective cultures being one of them. This work is aimed at checking the possibility of using two *Lactobacillus* spp. strains as protective cultures against *Clostridium* spp. in pork ground meat for fermented salami preparation. Both *Lactobacillus* strains displayed anti-clostridia activity *in vitro* using the spot agar test and after co-culturing them in liquid medium with each *Clostridium* strain. Only one of them, however, namely *L. plantarum* PCS 20, was capable of effectively surviving in ground meat and of performing anti-microbial activity *in carnis* in a challenge test where meat was inoculated with the *Clostridium* strain. Therefore, this work pointed out that protective cultures can be a feasible approach for nitrite reduction in fermented meat products.

1. Introduction

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

Fermented meat products, such as salami, are partially dehydrated to favor their preservation at ambient temperature. The acidic pH (in the range 5.1-6.0) and water activities lower than 0.94 make microbial growth difficult to occur. Among foodborne pathogens in meat products, a major concern is represented by *Clostridium* species, in particular *Clostridium perfringens* and *Clostridium botulinum* (Akhtar et al., 2009; Golden et al., 2009; Linton et al., 2014). Both can be isolated from raw meat as well as from cooked or uncooked cured meat products (Akhtar et al., 2009; Linton et al., 2014). Regarding *C. perfringens*, almost all outbreaks are the result of temperature abuse, allowing multiplication of clostridia, reaching 6-7 Log CFU/g (Huang, 2003). Spores and, to a certain extent, vegetative cells ingested through contaminated food can survive the acidic conditions of the stomach. In the large intestine, during sporulation and/or germination process, production and release of enterotoxins occur, respectively. Occasionally death may occur, particularly in elderly patients (Songer, 2010). On the other hand, botulism occurs after ingestion of a neurotoxin formed in food when spores germinate and vegetative cells multiply. The toxins of *C. botulinum* are relatively sensitive to heat and are inactivated by heating at 80°C for 10 min (Mataragas et al., 2008). In dry-processed meat products, nitrites and nitrates are commonly used as curing agents. Their addition in raw meat processing enabled to strongly reduce the risk of clostridia and other foodborne pathogen growth in meat products (Parthasarathy and Bryan, 2012). In particular, the use of these preservatives still

remains the most efficient strategy to inhibit the spore germination of *C. botulinum* (Linton et al., 2014). The first half of the 20th century brought a gradual shift from nitrate to nitrite and research studies allowed then to outline that nitrite could result in formation of carcinogenic N-nitrosamines (De Mey et al., 2014; Honikel, 2008). Finally, the IARC (International Agency for Research on Cancer) in 2006 concluded that ‘‘Ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans’’ (IARC, 2010).

Nevertheless, the adverse effects of nitrite on health as well as the increasing number of consumer demands for natural, chemical free products (Akhtar et al., 2009; Ojha et al., 2015), has stimulated the research of new strategies to substitute or to reduce the nitrites and other additives, with alternative natural friendly and possibly bio-based methods. In this context, the approach of using protective cultures, in particular lactic acid bacteria (LAB) against food pathogens appears particularly interesting, as shown by the large number of research works and reviews on the use of protective cultures in food (Chaillou et al., 2014; Comi et al., 2015; Gaggia et al., 2011; Galvez et al., 2010). A large number of LAB, including those used as protective cultures, are considered safe for human consumption as they possess the GRAS (Generally Recognised As Safe) and/or QPS (Qualified Presumption of Safety) status from the US FDA and the EFSA, respectively. Certain protective LAB strains can produce anti-microbial peptides such as bacteriocins, as well as organic and metabolic compounds formed along the fermentation process, which can inhibit foodborne pathogens growth (Varsha et al., 2015). In addition, a direct competition of protective cultures with potential pathogens is another important mechanism to restrict the growth of undesired organisms. Several works have focused on the use of LAB against foodborne pathogens in meat such as *Listeria* spp., *Salmonella* spp., *Campylobacter jejuni*, *Escherichia coli* (Baffoni et al., 2012; Maragkoudakis et al., 2010; Melero et al., 2012; Osés et al., 2015; Raimondi et al., 2014; Santini et al., 2010), but only a few studies have been specifically focused on *Clostridium* spp. and the possibility

of combating this pathogen with the use of protective cultures, as reviewed by Allaart et al. (2013). One of the most relevant application *in carnis* of LAB against clostridia has been described by Nieto-Lozano et al. (2010). This study showed that, during the storage period, *Pediococcus acidilactici* MCH14 pediocin-producing strain was able to reduce *C. perfringens* in Spanish dry-fermented sausages.

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

2. Material and Methods

2.1 Microorganisms and culture conditions

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

C. perfringens DSM 756 and *Clostridium sp.* DSM 1985 were used as target microorganisms. The latter is a surrogate strain, which, based on partial 16S rDNA sequence and as reported on the DSMZ website (https://www.dsmz.de/catalogues/details/culture/DSM-1985.html), is closely related to *Clostridium botulinum* type E, strain ATCC 23387; it does not produce a

neurotoxin but a bacteriocin-like substance. Both *Clostridium* strains were grown in TPY medium supplemented with 20% (v/v) Chopped Meat broth (CM, Oxoid) under anaerobic conditions at 37 °C for 24h or in Reinforced Clostridial Medium (RCM, Oxoid). For evaluation of viable cell population Reinforced Clostridial Agar (RCA, Oxoid) was used, using the same incubation conditions described above.

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

2.2.1 Spot agar test

The procedure described in Savino et al. (2011) was used with some modifications as follows. Each *Lactobacillus* strain was grown in MRS broth for 24 h, the culture was neutralized to pH 7 with 1N NaOH, and centrifuged at 7.000 *g* for 10 minutes. Cells were washed and suspended in saline at the concentration of 6 Log CFU/ml. TPY-CM agar plates were inoculated with 50 µl of each *Clostridium* culture at 7 Log CFU/ml. Two sterile paper blank disks (diameter 6 mm) were placed on the agar plate and imbibed with 50 µl of washed *Lactobacillus* cells. After incubation for 24 h at 37°C in anaerobic conditions, the presence of inhibition zones was evaluated. Each assay was performed in duplicate.

2.2.2 Antimicrobial activity in liquid co-cultures

The capability of the two LAB strains of interfering with the growth of *Clostridium* spp. was evaluated by co-culturing both strains in the same tube and checking survival of both LAB and clostridium strains in above mentioned selective agar plates. After verification that LAB growth was not altered in TPY-CM medium with respect to TPY (data not shown), both the LAB and the *Clostridium* strains were pre-grown on TPY-CM medium under anaerobic conditions at 37 °C up to early stationary phase, corresponding to about 8 Log CFU/ml. Tubes containing 10 ml of TPY-CM medium (pH was set to 6.5 with NaON 1N) were inoculated with: i) 1 ml of each *Lactobacillus* strain (*L. plantarum* PCS20 or *L. delbrueckii* DSM 20074),

ii) 1 ml of each *Clostridium* strain (*C. perfringens* DSM 756 and *Clostridium sp.* DSM 1985), iii) 0.5 ml of each *Lactobacillus* strain and 0.5 ml of each *Clostridium* strain (LAB-*Clostridium* co-culture). Subsequently, tubes were incubated in anaerobic conditions at 37 °C. At 4 h intervals, tubes containing cultures were centrifuged for 15 min at 5.000 g and pellets were resuspended in new sterile TPY-CM medium, in order to exclude growth inhibition due to pH variation or nutrient limitation. After this washing step, tubes were incubated in anaerobic conditions as described above. Determination of LAB and *Clostridium* spp. growth was followed after 24 h, 48 h and 72 h of incubation period. TPY agar plates incubated in aerobic conditions at 37 °C was used to allow growth of only LAB strains and in RCA plus mupirocin (100 μg/ml) in anaerobic conditions at 37 °C to allow growth of only *Clostridium* spp. Each experimental condition was set up in triplicate.

2.3 Study design in ground meat

This study was designed to evaluate the potential inhibitory activity of *L. plantarum* PCS20 and *L. delbrueckii* DSM 2074 against a *C. perfringens* strain and a *Clostridium* sp. strain related to *C. botulinum* type E in pork ground meat used for fermented sausage production (salami). The pork ground meat was provided by a local butcher in Bologna (Italy), and contained: ground pork meat containing 30% pork fat, NaCl 2.5% (w/w), dextrose 0.5% (w/w) and no other preservatives. The study was performed in the following sequence:

a) evaluation the survival of the two protective cultures in pork ground meat upon inoculation of the strains: with quantitative methods for total lactobacilli evaluation, both in the inoculated and non-inoculated meat at different incubation times, by traditional plate counts and real-time PCR; randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) analysis on MRS grown picked colonies were also performed with the aim to evaluate the percentage of each protective cultures present in the meat, in comparison with native lactic acid

bacteria; b) challenge test aimed at studying, *in carnis,* the antimicrobial activity of the protective cultures against *Clostridium* spp., inoculated in pork ground meat in the presence and in the absence of $NaNO_2$ 150 mg/kg in meat.

2.4 Survival of the two protective cultures in pork ground meat

The inocula for the *Lactobacillus* strains were prepared as follows: glycerinated frozen cultures were pre-grown in MRS broth as previously described, 10 ml MRS tubes were then inoculated (2% v/v) and incubated overnight at 37°C under anaerobic conditions. The concentration of overnight culture growth was determined through plate counts after serial dilutions and prior their inoculation in pork meat batter. Subsequently, the cultures were centrifuged and cells resuspended in Ringer's solution (Oxoid) in order to achieve 8 Log CFU/mL. Aliquots of this suspension were used to inoculate 30 g of meat, at the theoretical initial concentration of protective culture of about 6 Log CFU/g. The real concentration after inoculation in the meat was evaluated with plate counts.

Three different conditions were set up, each one in triplicate: 1) meat inoculated with *L. plantarum* PCS20, 2) meat inoculated with *L. delbrueckii* DSM 2074, 3) uninoculated control. Each meat portion was placed in a synthetic casing (supplied by Morgan, Florence, Italy). The meat fermentation was developed in 3 days in microaerophilic conditions generaterd by CampyGen™ Compact (Oxoid) at 22°C. Meat was sampled after inoculation and after 1, 2 and 3 days of incubation for plate counts and DNA extraction for real-time PCR.

2.5 Challenge test on ground meat

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

Table 1. Experimental study design of the Challenge test

Control Batches	Protective culture	*Clostridium* strain	$NaNO_2$ 150 ppm
1	-	*C. perfringens*	+
2	-	*C. perfringens*	-
3	-	*Clostridium* sp.	+
4	-	*Clostridium* sp.	-

Challenge Test Batches	Protective culture	*Clostridium* strain	$NaNO_2$ 150 ppm
5	*L. plantarum*	*C. perfringens*	+
6	*L. plantarum*	*C. perfringens*	-
7	*L. plantarum*	*Clostridium* sp.	+
8	*L. plantarum*	*Clostridium* sp.	-

2.6 Plate counts on meat samples

Meat samples of 2 g were removed aseptically from each tray and homogenized in 18 ml of Buffer Peptone Water (BPW, AESlaboratoire, Bruz, France) for 2 min in 50 mL sterile tubes.

Serial dilutions (1:10 in BPW) of the meat homogenate were performed. 1.0 mL aliquot from different dilutions were transferred to Petri dishes and subsequently MRS agar medium was poured. Plates were incubated in jars, for 48 h at 37°C under anaerobic conditions.

2.7 Quantification of *Lactobacillus* spp. on meat samples with real-time PCR

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

using 16S rRNA PCR product of *L. brevis* DSM 20054. PCR products were purified with a commercial kit DNA purification system (NucleoSpin® Extract II kit, MACHEREY-NAGEL GmbH & Co. KG, Germany) and the concentration measured at 260 nm. Serial dilutions of PCR product were performed and 10^2, 10^3, 10^4, 10^5, 10^6 copies of the gene were used for calibration.

2.8 RAPD PCR

RAPD-PCR was carried out on DNA extracted from randomly selected colonies obtained on MRS agar plates used for counting the total LAB in meat samples. This technique relies on DNA amplification with an arbitrary primer followed by separation on agarose gel. For each experimental conditions (meat inoculated with *L. plantarum* PCS20 and meat inoculated with *L. delbrueckii* DSM 20074), about 100 colonies were picked. DNA extraction from pure colonies was carried out by suspending one colony in 50 µl sterile Tris-EDTA in a single well of a 96 multiwell plate. The plate was subjected to heat treatment at 95°C for 10 min, cooled at 4°C, centrifuged at 5,000 *g* for 5 min to pellet cell debris. DNA containing supernatant was subjected to RAPD-PCR amplification, using primer M13 (5′- GAG GGT GGC GGT TCT) as previously reported in different studies (Cocolin et al., 2009; Rossetti and Giraffa, 2005). Reactions were carried out in a final volume of 20 µl containing 1.2 µl $MgCl_2$ (1.5 mM) (Sigma, Madrid, Spain), 10 µl HotStarTaq Master Mix (Qiagen) 2 µM primer M13 (2 µM), 3.8 µl RNase-free water (Qiagen) and 3 µl template DNA. The amplification cycle was as follows: 45 repetitions at 94°C for 1 min, 34°C for 1 min, ramp to 72°C for 2 min. An initial denaturation at 94°C for 3 min, and a final extension at 72°C for 7 min, was also carried out, and subsequently the samples were held at 4°C in a Mastercycler gradient (Eppendorf, Madrid, Spain). RAPD-PCR products were analyzed by electrophoresis on 2% (w/v) agarose gels supplied with EtBr, in 1X Tris-Borate-EDTA buffer (TBE, Thermo-Fisher Scientific, Waltham, MA, *USA*) at 120 V for 4 h.

2.9 Statistical analysis

A one-way ANOVA was performed to determine significant differences among samples. All the analyses obtained from the Challenge tests were performed using the Statistica 8.0 StatSoft Inc.USA. Differences among means were tested by Duncan's multiple range test (significance $P<0.05$).

3. Results

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

The results obtained from the spot agar test evidenced that washed cells of both *Lactobacillus* spp. strains were capable of inhibiting the growth of both *Clostridium* strains. The radius of the halos obtained were in the range 2.5-3.5 cm for all the combinations LAB-*Clostridium* assayed. As an example, the inhibitory activity of the two *Lactobacillus* strains assayed against *C. perfringens* DSM756 strains is shown in Fig. 1 (inhibition halo of 3.0 cm).

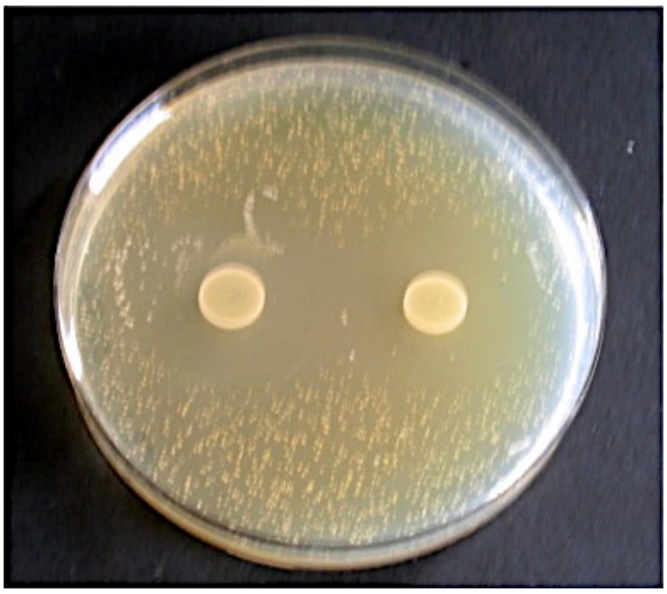

Fig. 1 Spot agar text showing growth inhibition of *C. perfringens* DSM 756 by *L. delbrueckii* DSM 20074 (left) and *L. plantarum* PCS20 (right) strains.

The results of the anti-microbial activity studies in liquid co-cultures are shown in Fig. 2 and 3, which also report the growth of the *Lactobacillus* and the *Clostridium* strains as single

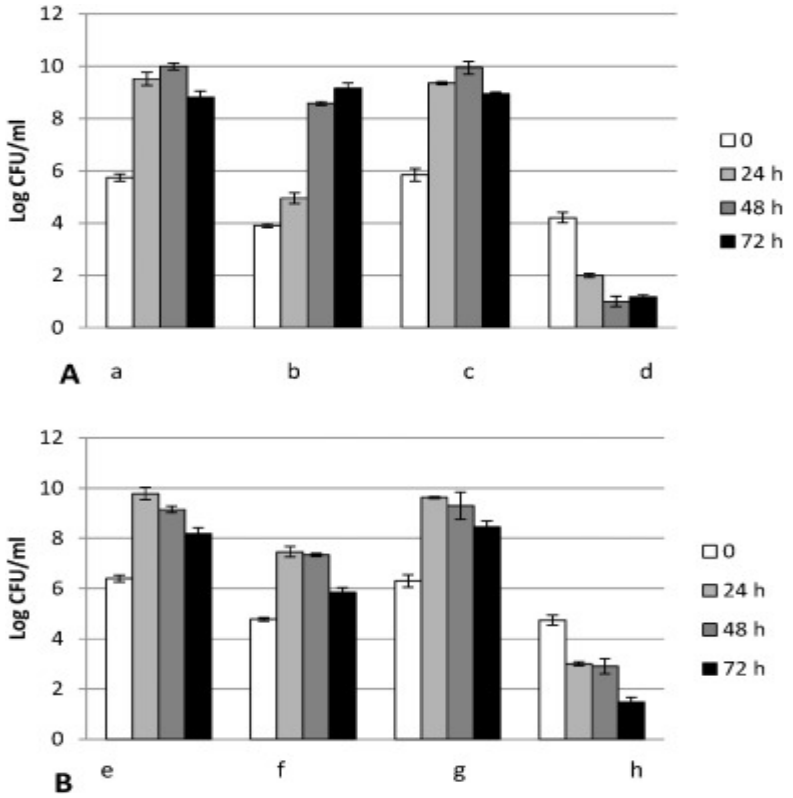

Fig. 2 Antagonistic activity of *L. plantarum* PCS20 in liquid cultures against *Clostridium* sp. DSM 1985 (Fig. 2A): a) *L. plantarum* PCS20 single culture, b) Clostridium sp. single culture, c) *L. plantarum* PCS20 co-culture, d) *Clostridium* sp. in co-culture; and *C. perfringens* DSM 756 (Fig. 2B):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

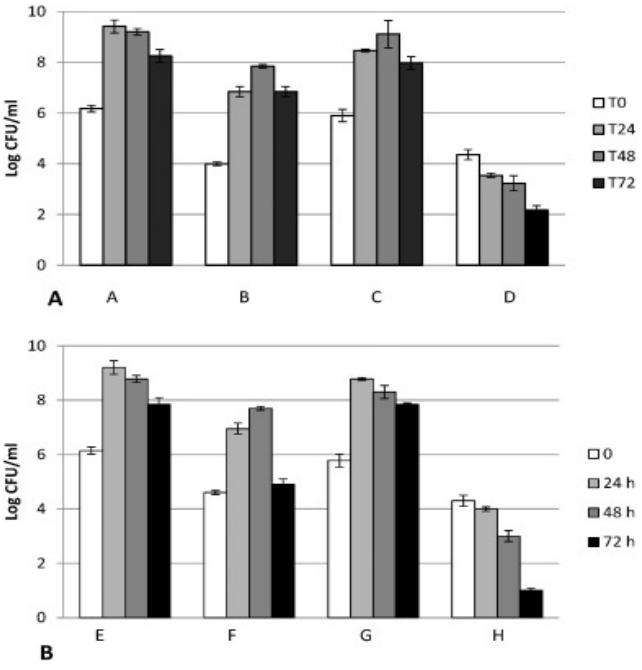

Fig. 3 Antagonistic activity of *L. delbrueckii* DSM 20074 in liquid cultures against *Clostridium* sp. DSM 1985 (Fig. 3A): A) *L. delbrueckii* DSM 20074 single culture, B) *Clostridium* sp. single culture, C) *L. delbrueckii* DSM 20074 co-culture, D)

Clostridium sp. in co-culture; and *C. perfringens* DSM 756 (Fig. 3B): E) *L. delbrueckii* DSM 20074 single culture, F) *C. perfringens* single culture; G) *L. delbrueckii* DSM 20074 co-culture, H) *C. perfringens* in co-culture.

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

3.2 Survival of the two protective cultures in pork meat batter

The survival of protective cultures in pork meat batter was evaluated in two batters obtained by the same butcher at two different times. Meat fermentation, developed in laboratory conditions, was performed in microaerophilic conditions at 22°C, chosen as the closest scenario compared to the realistic one. Plate counts on MRS-agar of meat samples not inoculated and inoculated with PCS20 or DSM 20074 are shown in Table 1.

Table 2. Microbial counts on MRS agar plates of the two meat batters considered in the work inoculated with *L. plantarum* PCS20 or with *L. delbrueckii* DSM 20074 and not inoculated with any culture (Control). Counts on meat batter 2 have also been performed with real-time PCR using primers targeted to the *Lactobacillus* genus

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

Real-time PCR was also performed for the lactobacilli quantification in one of the two meat batters. The initial counts of native bacterial strains capable of growing on MRS was very different in the two meat samples, being 4 Log CFU/g of meat in one of them and 7.15 Log CFU/g in the other one. After two days of incubation, the total count increased in all batches (inoculated with the exogenous *Lactobacillus* spp. and not inoculated) in the range 1.5-2.0 Log CFU/g. At the end of the study period (day 6), MRS agar counts reached the same value of about 9 Log CFU/g in all batches, with and without protective culture inoculation. The morphology of the colonies on MRS plates was variable both in inoculated and not inoculated plates. Therefore, molecular fingerprinting analyses were performed to better understand the survival of each protective culture strain in pork ground meat. As an example, the RAPD pattern profiles obtained from 20 out of the 100 colonies isolated from meat inoculated with *L.*

plantarum PCS20, at time zero and after 6 days of incubation, are shown in Fig. 4A and 4B, respectively.

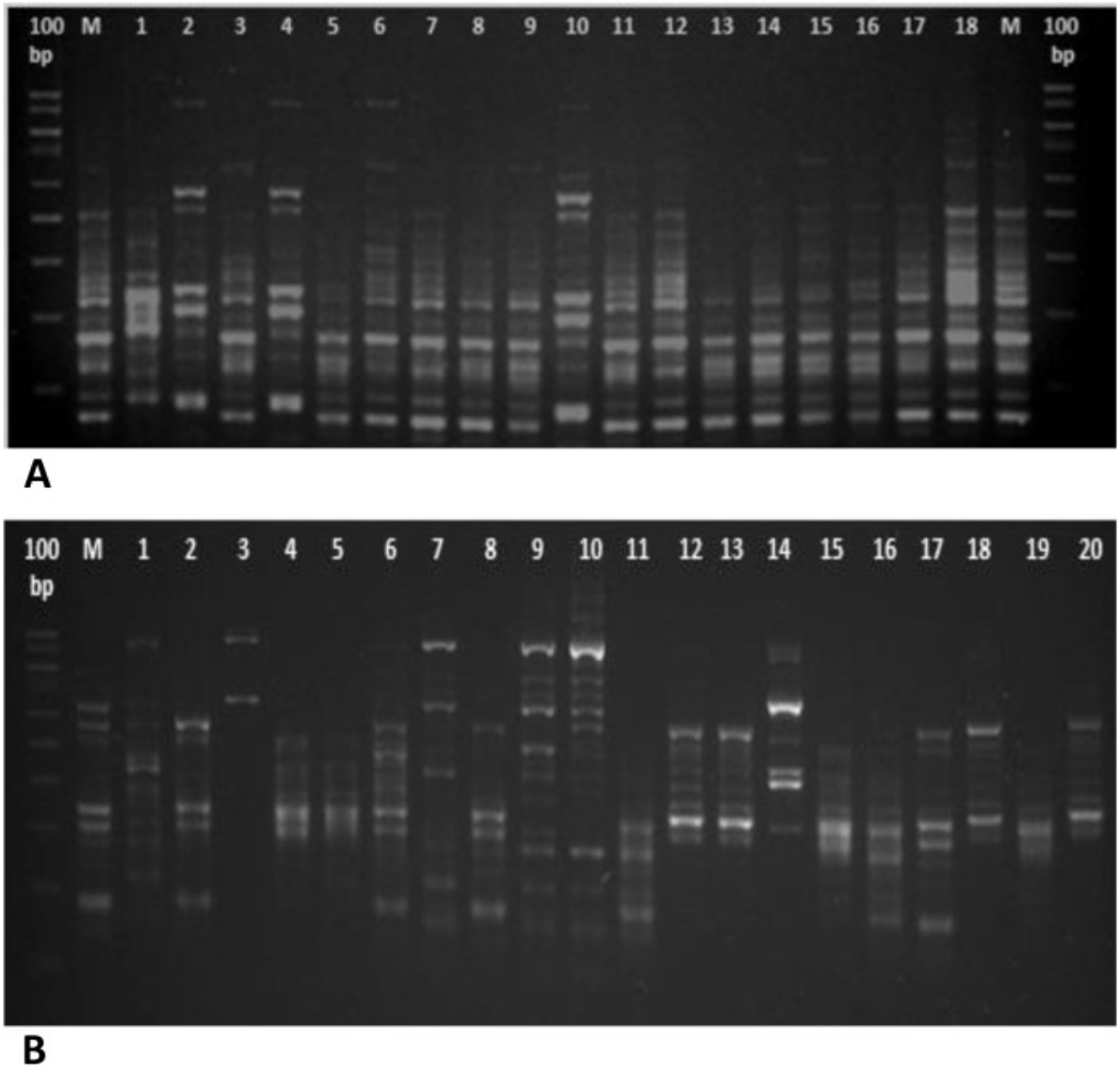

Fig. 4 RAPD profiles of the DNA obtained from 18 and 20 picked colonies. Colonies were obtained from plates resulting from microbial counts of the meat inoculated with protective cultures *L. plantarum* PCS 20 (Fig. 4A) and *L. delbrueckii* DSM 20074 (Fig. 4B). Molecular weight ladder (100 bp); protective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

On the whole, the results obtained showed that about 70% of the colonies isolated at time zero had a RAPD profile which could be ascribed to the inoculated strain, whereas 47% had the same profile of the PCS20 strain after 6 days. Conversely, 60% and 11% of the picked colonies

could be ascribed to *L. delbrueckii* DSM 20074 at time zero and 6 days of incubation, respectively (data not shown). RAPD analyses of the control sample did not show any colonies with a profile typical of both *L. plantarum* PCS 20 and *L. delbrueckii* DSM 20074 strains (data not shown)

3.3 Challenge tests on meat batter

The meat batter used for the challenge tests was checked for absence of detectable clostridia before starting the experiments (data not shown). The effect of *L. plantarum* PCS20 on the growth and survival of *C. perfrigens* DSM 756, both inoculated in pork meat batter in the presence or absence of 150 mg/kg $NaNO_2$, is shown in Fig. 5A.

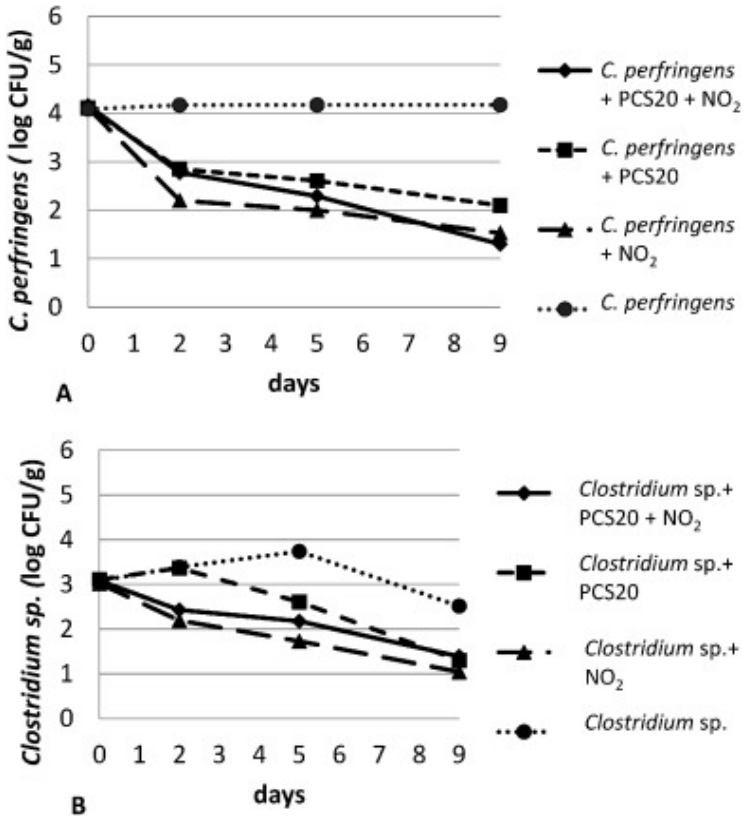

Fig. 5 *Clostridium* counts of pork ground meat challenged with *C. perfringens* DSM 756 (Fig. 5A) and with *Clostridium* sp. DSM 1985 (Fig. 5B) in the presence and absence of *L. plantarum* PCS20 and 150 mg/kg nitrite.

The count of inoculated *C. perfrigens* DSM756 in the meat was 4.2 Log CFU/g at the beginning of the incubation. The inoculated *C. perfrigens* strain was found to survive well in the batter, whereas a significant ($p < 0.05$) reduction of its concentration was observed when $NaNO_2$ was added. When *L. plantarum* PCS20 (initial concentration 9 Log CFU/g, as determined by real time PCR counts) was added to the batter without nitrite treatment, a significant reduction ($p<0.05$) of *C. perfrigens* was observed between day 2 and 5 in comparison with the batch containing only the pathogen. The results from the last sampling day showed that, after 9 days of the fermentation process, *C. perfrigens* growth was inhibited to 1.5 Log CFU/g in the batches inoculated with the protective culture.

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

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

4. Discussion

Clostridium spp. are a serious risk of disease in meat products (Akhtar et al., 2009; Golden et al., 2009; Linton et al., 2014). However, they are kept under control by the addition of nitrite or nitrate as antimicrobial agents, which at present make the risks of *Clostridium* diseases close to zero (Toldrà, 2010). The growing request of consumers for safer products and the concern

due to nitrosamine formation have led to the consideration by the food industry of chemical-free alternative approaches (Akhtar et al., 2009; Ergonul, 2013; Ojha et al., 2015), the use of protective cultures being one of them. However, although biopreservation studies have led to reports of efficient protective cultures, some bottlenecks actually limit their application in meat products. In particular, *in vitro* observed antagonistic effects have often been described as abolished *in carnis*, where the adaptation and metabolic activity of inoculated protective cultures can be impaired (Chaillou et al., 2014; Jones et al., 2008, 2009; Linton et al., 2014).

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

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

Meat for salami production is a harsh environment, as it contains a high amount of fat and an initial amount of 2.5% NaCl which increases during the ripening process due to water evaporation. PCS20 is a well-studied *L. plantarum* strain (Cho et al., 2010; Maragkoudakis et

al., 2010; Nissen et al., 2009; Dimitrovski et al., 2014), known to possess the *pnl* gene for plantaricin production and capable of surviving *in vitro* to low water activity conditions and starvation stresses (Santini et al., 2010). Moreover, it was also found to possess gut health promoting action (Nissen et al., 2009). As Gaggia et al. (2011) pointed out, foods inoculated with LAB protective cultures may have additional functional and beneficial properties for the consumers; thus, the concept of "protective cultures" is a broad one and it is not strictly related to food safety concept.

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

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

This paper also pointed out that real time PCR is an effective and time-saving method for *Lactobacillus* counts in fermented meat products, allowing to obtain results comparable to the officially used plate count procedures. There are several ongoing debates regarding the accuracy of qPCR based approach versus traditional count methods in food matrixes (Castillo

et al., 2006; Rantsiou and Cocolin, 2006). However, the study here described confirms that qPCR is a reliable method for this purpose.

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

CHAPTER 2

Protective cultures against foodborne pathogens in a nitrite reduced fermented meat product

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LWT- Food Science and Technology Journal, 101(2019) 293-299

Abstract

In the present work, a combined hurdle approach for fermented meat preservation was investigated. *In situ* challenge tests were performed in *Chorizo* sausage model using the maximum allowed $NaNO_2$ amount (150mg/kg), a reduced amount (75 mg/kg) and no nitrite, with and without protective cultures inoculation. Cocktail strains of *L. monocytogenes* and *Salmonella* spp. were used as indicator strains. The reduced amount of nitrites did not change the *L. monocytogenes* growing trend in the sausage model when compared with those containing 150 mg/kg $NaNO_2$. The combined approach, 75mg/kg $NaNO_2$ and *L. plantarum* PSC20, was capable of lowering the *L. monocytogenes* counts up to 2.62 log cfu/g when compared with sausages without protective cultures. None of the protective cultures demonstrated *in situ* antagonistic activity against *Salmonella* spp.

This work pointed out that the reduction of nitrites with the combined use of a protective culture could be a feasible approach to control *L. monocytogenes* growth in fermented meat foods.

1. Introduction

In the era where demand for ready to eat and preservative free products is constantly growing, the microbiological food safety has to be guaranteed, proportionally with this ongoing trend. In the recently published European Food Safety Authority (EFSA) foodborne outbreak report, referred to 2016, *Salmonella* spp. human infections had the same high level of the previous year (94.530 confirmed cases), whereas human listeriosis, caused mainly by *Listeria monocytogenes, showed* 9.3% increase (2.536 confirmed cases) (EFSA 2017). Despite the relatively low incidence of listeriosis, compared with the number of campylobacteriosis and salmonellosis cases, its importance is due to the severity of the disease and the higher case-fatality rate (Baffoni et al., 2017; D'Ostuni et al., 2016; EFSA, 2017).

Curing with nitrite is the most used approach to control foodborne pathogens in the meat (Honikel, 2008). Nitrites have additional functions in the meat, as they help to prevent lipid oxidation and rancidity, guarantee a bright red color and a typical "cured" flavor (Sebranek and Bacus, 2007). Although nitrites are widely used in the meat industry, they are classified by International Agency for Cancer Research as potentially carcinogenic agents (IARC, 2010), due to their ability to react with amines in the gastrointestinal tract, resulting in N-nitrosamines formation. Nitrites, hitherto, are the most effective solution against *C. botulinum* growth in meat products (EFSA, 2003; Hospital et al., 2014; Hospital et al., 2016). Therefore, 150 mg/kg $NaNO_2$ and 300 mg/kg $NaNO_3$ are authorized as maximum added levels in meat and other food products (EFSA, 2003; European Commission, 2011). The Danish authorities proposed more stringent national regulations related to nitrites (100 mg/kg of nitrite as maximum allowed amount in fermented sausages), which were approved by the European Commission (European Commission, 2015). Specifically, Denmark will collect data regarding nitrite added/residual level to correlate them with acceptable health risks and benefits for 3 years. Additionally, with the EC Regulation Nº 889/2008 for organic meat products, almost 50% reduction of the

maximum amount was established: 80 mg/kg for added nitrite and 50 mg/kg for residual nitrite (European Commission, 2008).

Therefore, meat industries are challenged to employ healthier and safer approaches for meat preservation. In the attempt of finding alternatives to nitrites for fermented food preservation, several authors suggested the use of lower nitrite levels in combination with other compounds or processing technologies, in a way that antimicrobial properties against the common foodborne pathogens could be guaranteed without alteration of sensory qualities (Alahakoon et al., 2015; Cavalheiro et al., 2015). Lactic acid bacteria (LAB) with demonstrated *in vitro* antimicrobial activity against a wide spectrum of foodborne pathogens (Leroy et al., 2013) as well as the addition of natural extracts or phytochemicals are the mostly studied approaches for the development of innovative processed meat products (Alahakoon et al., 2015; Gaggia et al., 2011; Oliveira et al., 2018). However, several natural extracts may contain even more than the allowed nitrate amount, thus the nitrosamine formation is questioned (Bedale et al., 2016). LAB strains with demonstrated sensorial or health promoting properties are approved by FDA as Generally Recognized as Safe (GRAS) and by EFSA with the Qualified Presumption of Safety (QPS) status.

In the present work, we studied the effectiveness of a combined hurdle approach, *i.e.* a 50% reduction of nitrites plus the addition of previously characterized *Lactobacillus* strains (*Lactobacillus plantarum* PCS20 or *Lactobacillus delbrueckii* DSM 20074), against common foodborne pathogens in *Chorizo*, a dry fermented sausage produced in Spain.

2. Material and methods

2.1 Bacterial strains

L. plantarum PCS20 (MSCL P977) and *L. delbrueckii* DSM 20074 were used as protective cultures for their demonstrated anti-microbial activity against several pathogens (Di Gioia et al., 2016; Savino et al., 2011). They were grown in de Man Rogosa Sharpe medium (MRS, Oxoid Ltd., Basingstoke, England) in anaerobic conditions (Anaerogen, AN0025A, Oxoid), at 37 °C for 48 h.

A cocktail of *Listeria monocytogenes* strains (serovar 4a and 4b, CECT 5366, CECT 934, CECT 4032 and LTA0020) and a cocktail of *Salmonella* strains (isolated from different sources at the Department of Biotechnology and Food Science, Burgos, Spain) were used in the challenge tests (Melero et al., 2012; Melero et al., 2013). The strains were grown at 37°C in Brain Heart Infusion Broth (BHI, Oxoid).

For viable *L. monocytogenes* counts, Chromogenic *Listeria* agar (Oxoid) supplemented with OCLA (ISO) Selective Supplement (SR 0226E, Oxoid) and Brillance *Listeria* Differential Supplement (SR 0228E, Oxoid) was used. Brilliance *Salmonella* agar (Oxoid) supplemented with *Salmonella* Selective Supplement (SR 0194, Oxoid) was used for *Salmonella* spp. viable cell counts.

2.2 Study design

Two Challenge tests in sausage prototypes were designed, referred to as 1 and 2. Challenge test 1 aimed at studying the effect of *L. plantarum* PCS20 against *L. monocytogenes* and *Salmonella* spp. in fermented sausages, both without nitrite addition and with 150 mg/kg of nitrite. Challenge test 2 was focused on the effects of two protective cultures, *L. plantarum* PCS20 and *L. delbrueckii* DSM 20074, against *L. monocytogenes* strains in pork meat batters treated with 75 mg/kg and 150 mg/kg of nitrite. Challenge test protocols are detailed below (2.3 and 2.4).

2.3 Inocula preparation

2.3.1 Pathogen strains

Each *L. monocytogenes* and *Salmonella* spp. strain was grown at 37°C overnight in BHI broth up to 9 log cfu/ml. Cells were washed and suspended in sterile Ringer solution (Oxoid). For Challenge test 1, dilutions were performed in order to obtain a final concentration of 4.5 log cfu/g in the meat batter (Figure 1), whereas for Challenge test 2, meat batter was inoculated with *L. monocytogenes* cocktail strains in order to obtain the final concentration of 3 log cfu/g (Figure 2).

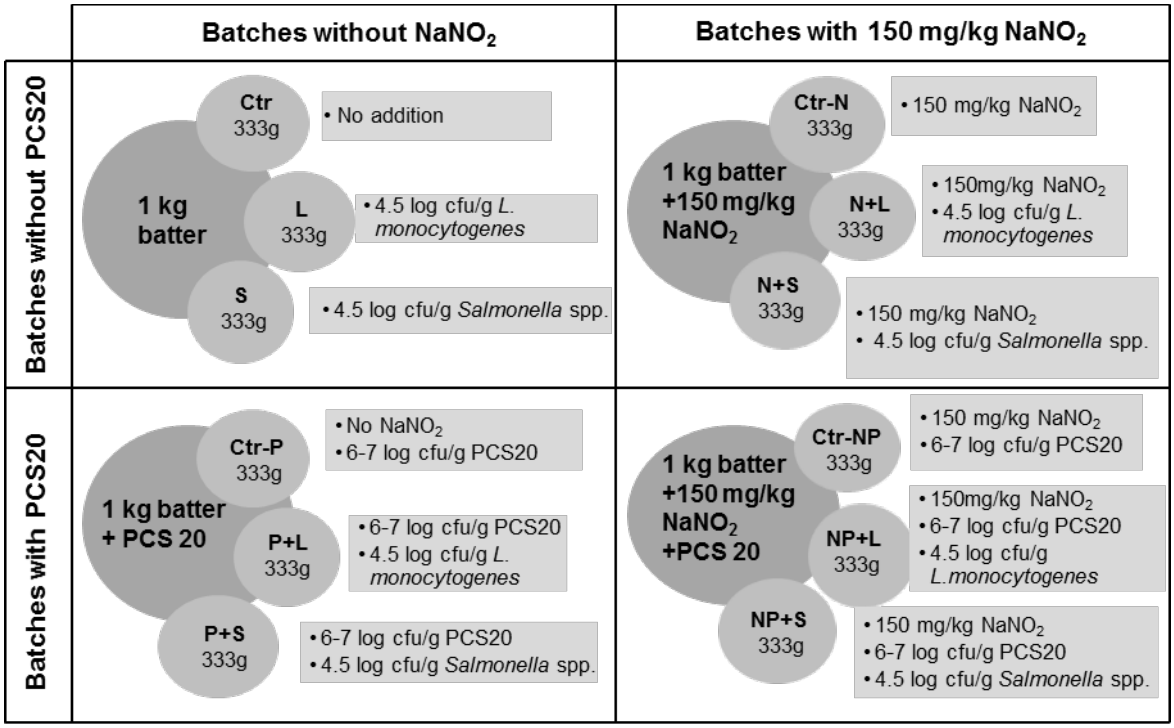


Figure 1. Challenge test 1 - Batch: Ctr, control; N, 150mg/kg NaNO2; P, L. plantarum PCS20; L, L. monocytogenes; S, Salmonella spp.

2.3.2 Protective cultures

L. plantarum PCS20 and *L. delbrueckii* DSM 20074 were grown at 37°C overnight in MRS broth up to 9.5-10 log cfu/ml. Cells were washed and suspended to a final concentration of 6-7 log cfu/g (Figure 2).

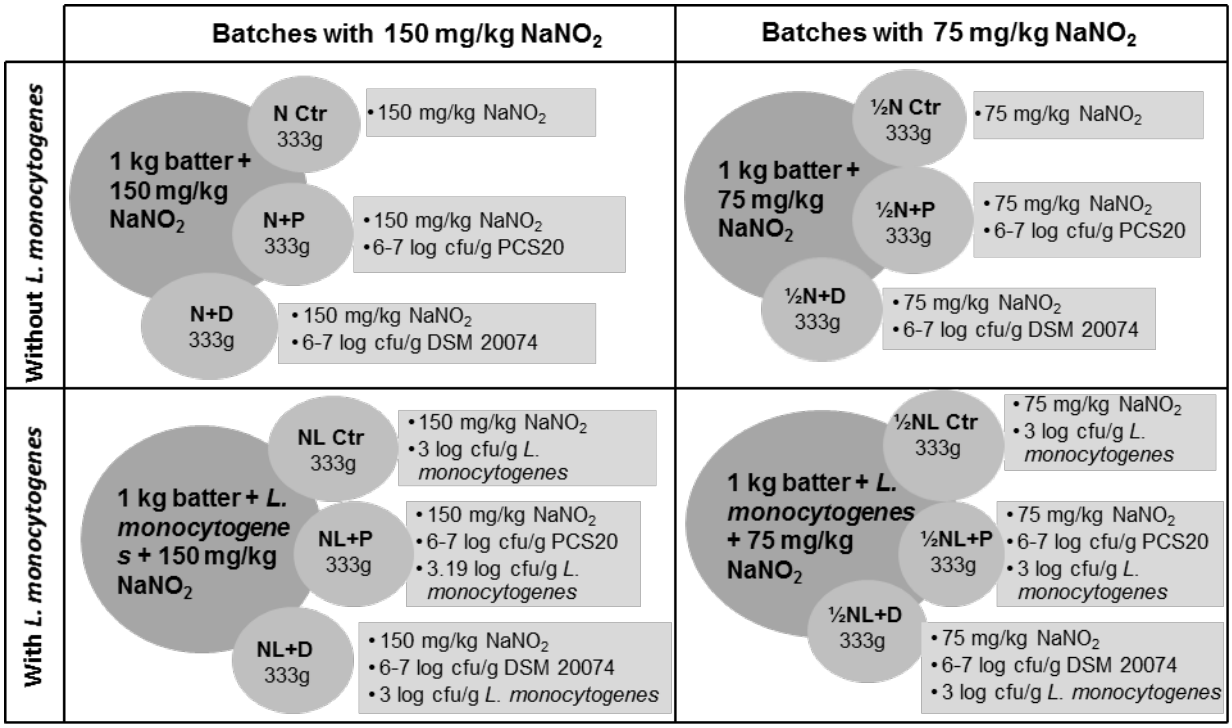


Figure 2. Challenge test 2 - Batch: Ctr, control; ½N, 75 mg/kg NaNO2; N, 150mg/kg NaNO2; P, *L. plantarum* PCS20; L, L. *monocytogenes;* D, *L. delbrueckii* DSM20074

2.4 Challenge tests

The batter was composed of grounded pork meat and fat (70% and 30%, respectively) supplied by a meat processing company in Burgos (Spain). Spices were not used not to interfere with the results obtained.

For Challenge test 1, the ground meat (4 kg) was divided in 2 trays of 2 kg meat each. In one tray 2% NaCl was added whereas, in the other tray, meat was supplemented with 2% NaCl plus 150 mg/kg $NaNO_2$ (Figure 1). After homogenization in a vacuum mixer, each 2 kg portion of batter was divided in two parts. 1 kg was inoculated with *L. plantarum* PCS20 and aseptically mixed by hand. The other kg was not inoculated. Subsequently, each kg was divided in 3 batches (333 g), one inoculated with the cocktail of *Salmonella* strains, the second one with the *L. monocytogenes* strains and the last one was not inoculated with any pathogen (control). The 12 conditions and the relative acronyms are shown in Fig. 1.

For Challenge test 2, the ground meat (4 kg) was divided in 2 trays of 2 kg meat each. 2 kg were treated with 2% of NaCl, 0.5% dextrose and 75 mg/kg $NaNO_2$ and 2 kg with 2% of NaCl,

0.5% dextrose and 150 mg/kg $NaNO_2$. Each tray was divided in two (1 kg each one): one inoculated with *L. monocytogenes* and aseptically mixed by hand and the other not inoculated. Then each kg of meat was divided in three batches (333 g each) and submitted to different treatments: inoculated with PCS 20, with DSM 20074 and not inoculated. The 12 conditions and the relative acronyms are shown in Fig. 2.

Each batch containing 333 g of meat batter was used to produce two sausages, each one containing 166.5 g of batter, which were stuffed in collagen casings (45 mm diameter) (Viscofan, Navarra, Spain). For Challenge test 1, the fermentation was performed for 2 days at 23°C, 95% humidity and followed by a short ripening of 6 days at 15°C and lower humidity (80-75%). pH evaluation and microbiological analyses were performed at the following days: D0, D1, D2, D4, D6 and D8. For Challenge test 2, the fermentation was studied for 2 days followed by 5 days of short ripening in the same conditions as for the Challenge test 1. pH evaluation and microbiological analyses were performed at the following days: D0, D3, D5 and D7.

For each Challenge test, the meat was also analyzed for the presence of *L. monocytogenes* and *Salmonella* before inoculation at D0.

2.5 pH analysis

pH was measured with a pin electrode of a pHmeter (micropH2001, Crison, Barcelona, Spain) inserted directly 3 times into the sample.

2.6 Microbiological Analysis

Meat samples (10 g) were aseptically removed from each *Chorizo* and homogenized in 90 ml of *Buffered Peptone Water* (BPW; AES Laboratoire, Combourg, France) for 2 min in a sterile plastic bag using a Smasher (AES Laboratoire). For cell counts, decimal dilutions (1:10 in BPW) of the meat homogenate were prepared and aliquot of 100 µl were inoculated onto solid agar plates. The counts were performed in duplicate. *Lactobacillus* was counted on MRS agar

plates, incubated anaerobically for 48 h at 37°C. *L. monocytogenes* was enumerated according to ISO 11290–2:1998 (ISO, 1998) for artificially contaminated samples, on Chromogenic *Listeria* agar plates supplemented as previously described. The presence or absence of viable *Salmonella* cells in the initial dilution was investigated by enrichment according to ISO 6579:2002 (ISO, 2002).

ISO protocols were used for the detection of natural contamination in not artificially inoculated batches: ISO 11290–1:1996 (ISO, 1996) and ISO 6579:2002 (ISO, 2002) for *L. monocytogenes* and *Salmonella* spp., respectively.

2.7 Statistical analysis

The results of microbiological analysis were obtained from two chorizo per treatment, for each sampling point and subjected to one-way ANOVA analysis. Differences among means were tested by Duncan's multiple range test (significance $P<0.05$). All the analyses obtained from the Challenge tests were performed using the Statistica 8.0 (StatSoftInc., USA). Results of statistical analysis are presented as mean value ± standard deviation.

3. Results

3.1 Challenge test 1

3.1.1 pH analysis

No differences in pH were observed during the fermentation and short ripening process (data not shown). Considering the slight decrease of pH observed, 0.5% of dextrose was added in pork meat batter in Challenge test 2 with the aim of stimulating the *Lactobacillus* growth and acidification.

3.1.2 Microbiological analysis

The growing trend of *L. monocytogenes* and *Salmonella* spp. in Challenge test 1 is shown in Figure 3. Both pathogens demonstrated ability *to* survive and colonize the pork meat in the sausage model.

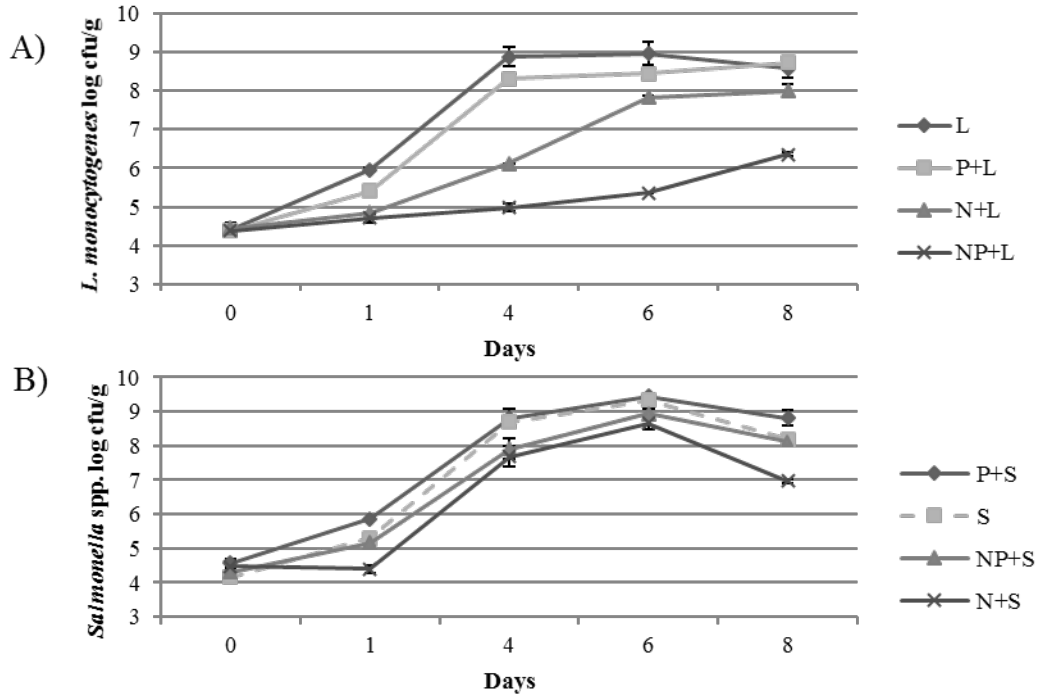


Figure 3. Antimicrobial activity of L. plantarum PCS20 against L. monocytogenes and *Salmonella* spp. in dry fermented sausage with and without 150 mg/kg NaNO2. A) *L. monocytogenes* counts; B) *Salmonella* spp. counts within the ripening period. N=150 mg/kg NaNO2.; L= *L. monocytogenes* P= *L. plantarum* PCS20; S= *Salmonella* spp.

Regarding *L. monocytogenes* growth, a significant reduction ($p<0.05$) of 0.95 and 2.78 log cfu/g, was observed at day 4 and 6, respectively, in the batch with 150 mg/kg $NaNO_2$ and PCS20 (NLP) with respect to the batch with nitrite but without PCS20 (NL) (Figure 3A). Moreover, in batch NL, *L. monocytogenes* counts increased 3.55 log cfu/g at the last sampling time (D8) compared with the initial inoculum, whereas this increase was of 1.96 log cfu/g in batch NL+P (Figure 3A). Lower, but significant reduction ($p<0.05$) of 0.60 and 0.52 log cfu/g in *L. monocytogenes* counts was observed at day 4 and 6, respectively, in batches P+L compared to L that are without nitrite addition, whereas no significant differences were observed at D8.

Lower *Listeria* counts were obtained in batches where $NaNO_2$ was added (NL/NL+P) in comparison with batches without additives (L/P+L). A significant reduction ($p<0.05$) of 2.37 log cfu/g was obtained comparing NL+P and P+L batches and a significant reduction ($p<0.05$) of 0.58 log cfu/g was observed at the last sampling day (D8) comparing NL Ctr and L.

Salmonella spp. counts within the study period are shown in Figure 3B. *L. plantarum* PCS20 did not show antimicrobial activity against *Salmonella* spp. growth. However, nitrites demonstrated a significant reduction ($p<0.05$) of *Salmonella* spp. growth (1.23 log cfu/g) in batch N+S in comparison with batch S at D8.

Initial counts of LAB in the meat without protective culture were between 3-4.5 log cfu/g. The level of PCS20 inoculum was 5.6-5.9 log cfu/g. After 3 days, when the fermentation conditions were settled, LAB counts increased in all batches of 2.5-3.5 log cfu/g, reaching values in the range 7-9 log cfu/g in batches with protective culture and 7-8 log cfu/g in uninoculated batches, at the end of the study (data not shown).

3.2 Challenge test 2

3.2.1 pH analysis

pH trend in the meat subjected to different treatments is shown in Table 1.

Table 1. Challenge test 2 - The trend of pH during the fermentation and ripening period

Batches**	**Days ***			
	0	**3**	**5**	**7**
N Ctr	5.96 ± 0.03^{B}	5.78 ± 0.03^{C}	6.12 ± 0.06^{A}	6.03 ± 0.06^{B}
½N Ctr	5.90 ± 0.09^{B}	5.99 ± 0.03^{B}	6.11 ± 0.04^{A}	5.90 ± 0.01^{B}
½NL Ctr	5.77 ± 0.08^{B}	5.89 ± 0.02^{A}	5.92 ± 0.02^{A}	5.91 ± 0.02^{A}
NL Ctr	5.85 ± 0.03^{B}	5.99 ± 0.01^{A}	6.10 ± 0.04^{A}	5.85 ± 0.02^{B}
N+P	5.86 ± 0.06^{A}	5.44 ± 0.02^{B}	5.23 ± 0.02^{C}	5.02 ± 0.04^{D}
½N+P	5.85 ± 0.07^{A}	5.28 ± 0.06^{B}	5.21 ± 0.02^{B}	5.05 ± 0.02^{C}
NL+P	5.77 ± 0.06^{A}	5.31 ± 0.01^{B}	5.14 ± 0.04^{C}	5.09 ± 0.04^{C}
½NL+P	5.83 ± 0.01^{A}	5.30 ± 0.01^{B}	5.16 ± 0.01^{C}	5.02 ± 0.01^{D}
N+D	5.87 ± 0.03^{A}	5.93 ± 0.04^{A}	5.93 ± 0.05^{A}	5.89 ± 0.04^{A}
½N+D	5.80 ± 0.04^{B}	5.93 ± 0.02^{A}	5.93 ± 0.02^{A}	5.89 ± 0.05^{A}
NL+D	6.05 ± 0.01^{A}	5.94 ± 0.03^{B}	6.04 ± 0.05^{A}	5.97 ± 0.03^{B}
½NL+D	6.17 ± 0.04^{A}	5.91 ± 0.01^{B}	5.94 ± 0.04^{B}	5.80 ± 0.04^{C}

* Data are expressed as mean of n=3 measurements.

Batch : **N Ctr=meat batter added with 150mg/kg $NaNO_2$; **½N Ctr**=75 mg/kg $NaNO_2$ added; **½NL Ctr**=75mg/kg $NaNO_2$+*L.monocytogenes* added; **NL Ctr**=150mg/kg $NaNO_2$+*L.monocytogenes* added; **N+P**=150mg/kg $NaNO_2$+PCS20 added; **½N+P**=75mg/kg $NaNO_2$+PCS20 added; **NL+P**=150mg/kg $NaNO_2$+*L.monocytogenes*+PCS20 added;

½NL+P=75mg/kg $NaNO_2$+*L.monocytogenes*+ PCS20 added; **N+D**=150mg/kg $NaNO_2$+DSM 20074 added; **½N+D**=75mg/kg $NaNO_2$+DSM 20074 added; **NL+D**=150mg/kg $NaNO_2$+*L.monocytogenes*+DSM 20074 added; **½NL+D**=75mg/kg $NaNO_2$+DSM 20074 added.
***A,B,C,D: Mean values in the same row (corresponding to the same batch) differ significantly ($p < 0.05$).

As expected, the addition of 0.5% dextrose caused a significant pH reduction at D7 (from 5.80 to 5.05; $p<0.05$), in all batches where *L. plantarum* PCS20 was inoculated. Differently, the addition of *L. delbrueckii* DSM 20074 did not lead to a significant pH reduction ($p>0.05$).

3.2.2 Microbiological analysis

Figure 4 shows the trend of *L. monocytogenes* inoculated at 3 log cfu/g in all batches.

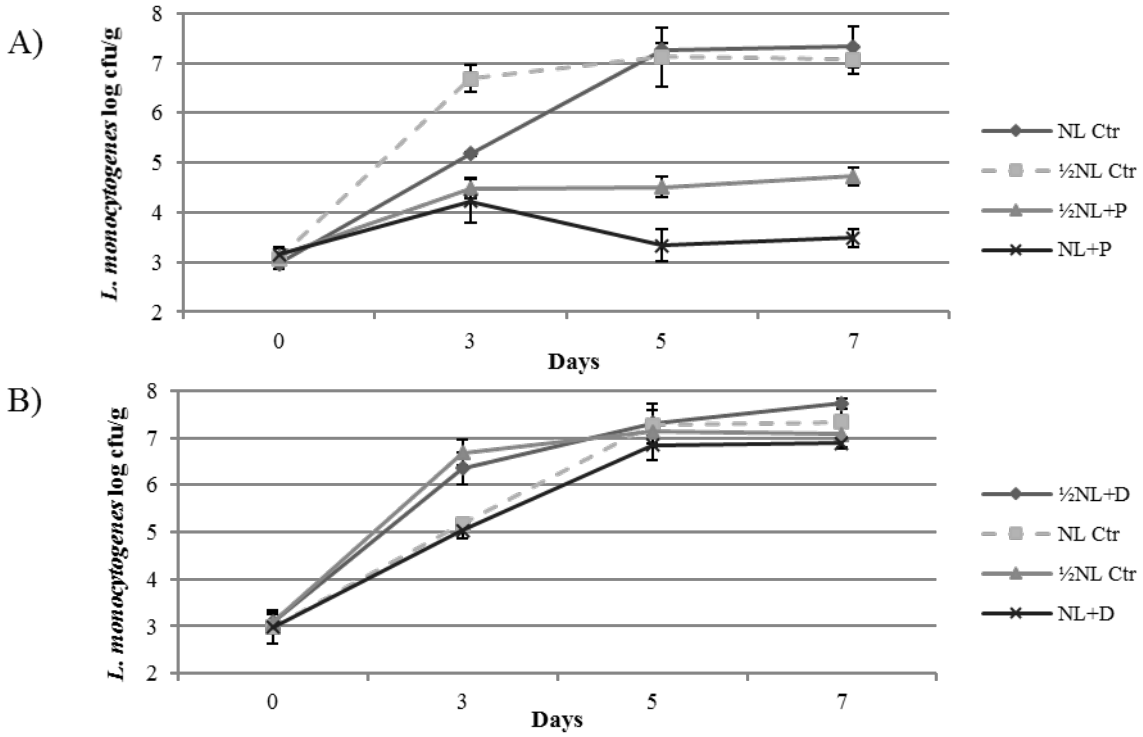


Figure 4. Antimicrobial activity of L. plantarum PCS20 (A) and L. delbrueckii DSM20074 (B) against L. monocytogenes in dry fermented sausage added with 75 or 150 mg/kg NaNO2. A) L. monocytogenes counts within the ripening period. ½N=75 mg/kg NaNO2; N=150 mg/kg NaNO2.; L=L. monocytogenes; P=L. plantarum PCS20; B) L. monocytogenes counts within the ripening period. ½N=75 mg/kg NaNO2; N=150 mg/kg NaNO2.; L=L. monocytogenes; D=L. delbrueckii DSM20074

Comparing batches containing 75 mg/kg $NaNO_2$, with and without PSC20 (batches ½NL+P and ½NL Ctr, respectively, Fig. 4A), a significant reduction ($p<0.05$) of 2.20 and 2.62 log cfu/g of the inoculated *L. monocytogenes* was observed at day 3 and 5, respectively, in the batch where PCS20 was inoculated (½NL+P); this reduction was maintained until D7.

Interestingly, considering the initial inoculum, the pathogen counts increased of only 1.61 log cfu/g in the batch ½NL+P compared with a 3.99 log cfu/g increase in the batch ½NL Ctr, at D7. On the other hand, in batches with higher nitrites concentration a significant reduction of *L. monocytogenes* ($p<0.05$) of 3.93 log cfu/g was observed at D5, in batch containing PCS20 as protective culture (NL+P) in comparison with batch without PCS20 (NL Ctr), with a final reduction of 3.84 log cfu/g at D7. In summary, it is possible to observe that the pathogen growing trend, without PCS20, was the same when the meat was treated with 75 or 150 mg/kg of nitrites (½NL Ctr and NL Ctr batches), while with PCS20,

L. monocytogenes counts were higher in ½NL+P compared with NL+P (difference of 1.49 log cfu/g).

Figure 4B shows the *L. monocytogenes* growth in pork meat batter with 150 mg/kg or 75 mg/kg $NaNO_2$ with or without *L. delbrueckii* DSM 20074 inoculum. At the end of the study, no significant differences in *L. monocytogenes* growth were observed among batches.

Counts of LAB growth were under the detection limit (<2 log cfu/g) in the control batches without protective culture inoculum at D0; whereas LAB counts were in the range 6-7 log cfu/g in the batches inoculated with PCS20 at D0 (Table 2).

Table 2. Challenge test 2 - LAB counts (log cfu/g) within the 7 days of fermentation and ripening period

Batches**	Days *			
	0	**3**	**5**	**7**
N Ctr	<2 ±0.00^{D}	6.63 ±0.25^{C}	7.82 ±0.12^{B}	7.89 ±0.12^{A}
½N Ctr	<2 ±0.00^{B}	7.18 ±0.46^{A}	7.60 ±0.17^{A}	7.09 ±0.34^{A}
NL Ctr	<2 ±0.00^{D}	6.59 ±0.28^{C}	8.15 ±0.07^{A}	7.38 ±0.29^{B}
½NL Ctr	<2 ±0.00^{B}	6.89 ±0.27^{A}	7.15 ±0.51^{A}	7.43 ±0.28^{A}
N+P	6.34 ±0.15^{C}	8.87 ±0.20^{B}	9.09 ±0.12^{AB}	9.13 ±0.10^{A}
½N+P	6.34 ±0.13^{D}	9.06 ±0.13^{B}	9.26 ±0.06^{A}	8.03 ±0.13^{C}
NL+P	6.59 ±0.17^{C}	8.86 ±0.12^{B}	9.10 ±0.07^{A}	9.14 ±0.09^{A}
½NL+P	6.61 ±0.13^{B}	9,04 ±0.07^{A}	9.04 ±0.08^{A}	9.17 ±0.08^{A}
N+D	5.71 ±0.26^{C}	6.49 ±0.22^{B}	7.29 ±0.38^{A}	5.89 ±0.37^{C}
½N+D	5.84 ±0.11^{D}	6.98 ±0.19^{B}	7.44 ±0.15^{A}	6.36 ±0.13^{C}
NL+D	5.98 ±0.11^{D}	6.52 ±0.12^{C}	7.69 ±0.28^{A}	7.19 ±0.08^{B}
½NL+D	6.09 ±0.15^{C}	6.35 ±0.04^{B}	7.44 ±0.15^{A}	7.58 ±0.13^{A}

* Data are expressed as mean of n=3 measurements.
Batch **N Ctr=meat batter added with 150mg/kg $NaNO_2$; **½N Ctr**=75 mg/kg $NaNO_2$ added; **N+P**=150mg/kg $NaNO_2$+PCS20 added; **½N+P**=75mg/kg $NaNO_2$+PCS20 added; **N+D**=150mg/kg $NaNO_2$+DSM 20074 added; **½N+D**=75mg/kg $NaNO_2$+DSM 20074 added; **NL Ctr**=150mg/kg $NaNO_2$+*L.monocytogenes* added; **½NL Ctr**=75mg/kg $NaNO_2$+*L.monocytogenes* added; **NL+P**=150mg/kg $NaNO_2$+*L.monocytogenes*+PCS20 added; **NL+P**=75mg/kg $NaNO_2$+*L.monocytogenes*+ PCS20 added; **NL+D**=150mg/kg $NaNO_2$+*L.monocytogenes*+DSM 20074 added; **½NL+D**=75mg/kg $NaNO_2$+DSM 20074 added.
***^{A,B,C,D}: Mean values in the same row (corresponding to the same batch) differ significantly ($p < 0.05$).

At the end of the study, LAB counts reached 7-8 log cfu/g in batches without PCS20, and 8-9.2 log cfu/g in batches with PCS20. Batches inoculated with DSM 20074 did not reach the same LAB count level as PCS20. In particular, 5.89 log cfu/g were obtained in the control batch with 150 mg/kg $NaNO_2$ and 6.36 log cfu/g in that with 75mg/kg $NaNO_2$, at D7. These counts are almost 3 log lower than those obtained for PCS20.

Similarly to the previous experiment, significant differences ($p<0.05$) were observed between D1 and D3, i.e. in the final part of the fermentation period (3rd day). At the end of the short ripening period, LAB reached counts in the range 7-9 log cfu/g.

4. Discussion

The aim of the present work was to evaluate the possibility of using protective cultures to eliminate or reduce nitrite amount in fermented meat products. For this purpose, the biopreservative activity of previously characterized LAB strains, *L. plantarum* PCS20 and *L.*

delbrueckii DSM 20074, was studied against *L. monocytogenes* and *Salmonella* spp. in a dry fermented sausage model without nitrite, with half (75 mg/kg) and maximum (150 mg/kg) allowed nitrite amount.

The results showed that the addition of *L. plantarum* PCS20 as protective culture in nitrite-free sausages inoculated with the pathogen is capable of significantly reducing the pathogen load after 4 and 6 days from the beginning of the fermentation, although the same effect was not observed at D8. On the contrary, the antimicrobial activity of PCS20 was not observed against the cocktail of *Salmonella* strains, whereas their growth was significantly ($p<0.05$) reduced in the presence of 150 mg/kg nitrites. Interestingly, Hospital et al. (2014) obtained complete *Salmonella* inactivation using a halved nitrite amount (75 mg/kg or) in fermented sausages at the end of the storage period. Other works showed the ineffectiveness of commercial protective cultures, as well as of meat-isolated *Lactobacillus* strains, against *Salmonella* spp., when inoculated in different meat models (Dias et al., 2013; Kotzekidou and Bloukas, 1998). The outcomes of this study support the Hugas (1998) consideration on the hurdle effect strategy.

Our study also shows that it is possible to reduce *Listeria* counts by inoculating the meat with *L. plantarum* PCS20 and a halved amount of nitrite (75 mg/kg). This result is particularly important considering the EC decision of adopting more stringent criteria for potential carcinogenic additives. Therefore, the combination of a protective culture with a reduced nitrite amount is an effective hurdle approach in fermented sausage production that may allow both to reduce pathogen load and to have the known positive effects of nitrites, such as the bright color.

The anti-*Listeria* activity observed is in agreement with a recent work (Giello et al., 2018) that showed the effectiveness of the bacteriocin-producing *Lactobacillus curvatus* 54M16 strain in fermented sausages. Several authors pointed out that bacteriocin action can be hindered *in carnis* by bacteriocin binding to food matrixes or degradation by proteases or their production

can be prevented by nitrites (Galvez et al., 2007; Kouakou et al., 2009). Therefore, non-bacteriocin producing strains showing anti-listerial activity can be of great importance in fermented meat production, in particular in the presence of nitrites. This is the case of *L. plantarum* PCS20 strain, that does not produce bacteriocins (Cho et al., 2010) and exerts anti-microbial activity in the presence of a reduced amount of nitrites. Its anti-microbial activity against *L. monocytogenes* can be attributed to cell-to-cell contact mechanisms or the production of organic acidic metabolites. An additional strength of our study is the use of four different *L. monocytogenes* strains, belonging to different serovars (Lianou and Koutsoumanis, 2013; Scott et al., 2005).

Moreover, our work confirmed that dextrose is an important pH lowering agent, allowing to reach pH values between 4.5 and 5.5, a range in which nitrite is mainly in the undissociated state, possessing the greatest antibacterial activity. Moreover, a rapid pH drop below 5.1 is considered as a desirable acidification rate for protective cultures in fermented meat products (Ammor and Mayo, 2007). On the other hand, the inability of *L. delbrueckii* DSM 20074 strain to demonstrate a significant pH lowering, resulted in an antagonistic failure against *L. monocytogenes* at the end of the study.

Our study supports the outcomes of a recent survey (Hung et al., 2016), in which meat industry stakeholders expressed interest in the development of innovative and healthier processed meat products but asked the scientific community to provide additional evidences of the microbiological safety of developed approaches. Consumers are important players in industrial innovation shaping, thus the taste and the microbiological safety are the most important criteria for the novel food formulations (Bedale et al., 2016; Hung et al., 2016).

5. Conclusions

This work pointed out that a combined approach based on half of the allowed nitrite amount and of protective culture may be effective in a dry-fermented meat product (*chorizo*) to reduce the growth of *L. monocytogenes*, a pathogen with high case fatality incidence and causing severe diseases. This study has also shown that the effectiveness of nitrites against this pathogen is not related to their amount; the inoculation with lactic acid bacteria contributing to pH lowering and to reach the effective dissociation state of nitrite is probably a crucial factor for their effectiveness. However, further studies aimed at better elucidating the anti-microbial mechanisms against pathogens in food matrix need to be pursued.

Additionally, this study can provide additional scientific evidence that the combined hurdle approach is promising for an innovative fermented meat development.

CHAPTER 3

Characterization of lactic acid bacteria isolated from artisanal salami for their bioprotective and techno-functional properties

Ivana Nikodinoska,..

Manuscript to be submitted

1. Introduction

Fermented meat products, such as salami, represent a cultural fingerprint dating back from millennia. Traditional salami production in Italy is often run in a small-scale family-based environment, with an in-house production of all primary material (Roccato et al., 2017). The artisanal products bring the "taste of tradition" where indigenous microbiota, raw materials and production conditions are the main protagonists (Tabanelli et al., 2012). Spontaneous fermentation in artisanal small-scale salami production is led by environmental and meat indigenous biota whereas the industrial-scale fermentation is standardized with starter cultures employment (Franciosa et al., 2018). Fermented salami is a restricted ecological niche, harbouring *Lactobacillus*, *Staphylococcus, Debaryomyces* and *Penicillium* as main bacterial and fungi genera. Starter cultures for fermented meat are 'preparations which contain living or resting micro-organisms that develop the desired metabolic activity in the meat' (Hammes, 1996). *Lactobacillus* is the most employed bacterial genus as a starter for several matrixes including meat, due to its techno-functional and safety performances (Franciosa et al., 2018). The high genetic diversity with the over 241 *Lactobacillus* species reported in the *List of Prokaryotic names with Standing in Nomenclature* "LPSN" (September 2019, www.bacterio.net) can explain the adaptation in different environments. The limited carbon availability and the rich amino acid and lipid content in the meat matrix, are the shaping

parameters of the *Lactobacillus* metabolic behaviour during the fermentation and late ripening process. The initial lactic acid production followed by amino acid catabolism have a significant impact on the hygienic and sensory quality of the long-ripened fermented meats (Gänzle, 2015).

Fast microbial growth together with a rapid pH drop are the most desirable techno-functional and hygienic characteristic of meat starter cultures. The fast acidification in salami manufacturing mainly leads to the inhibition of spoilage pathogens, product shelf life stability, textural stability of the final product, due to the coagulation of muscle proteins and new sensory properties of the final product (Lücke, 2000).

Nitrites are widespread multifunctional additives used in the fermented dry-cured salami. Beside their remarkable chemical properties that contributes to the red colour stability, lipid oxidation prevention and a typical cured flavour, nitrites represent the most efficient anticlostridial agent. However, the use of nitrite in the meat industry is being questioned due to their involvement in nitrosamine formation (IARC, 2010). There is a general tendency in the EU to reduce nitrite amount in the meat with respect to the current allowed amount (150 mg/kg of meat) (European Commission, 2018) and meat industries are challenged in finding alternatives for nitrites, also considering the additional increased consumers demand for "chemical-free" foods.

Biopreservation is one of the most ancient microbial-based strategy to improve the product safety and extend its shelf-life (Stiles, 1995). The production of antimicrobial compounds such as organic acids, hydrogen peroxide and lytic agents are some of the antagonistic mechanisms employed by these bioprotective bacteria (Galvez et al., 2010). Protective cultures used in fermented foods, might also be starters aimed in controlling both the native microbiota and the pathogens outgrowth (Zagorec and Champomier, 2017). The mostly studied antagonistic mechanisms is through the production of bacteriocins, peptides of few aminoacids, known for

their bactericidal effects at a certain concentration, prevalently against Gram-positive bacteria (Chikindas et al., 2018). Antagonistic activity of released bacteriocins generally considers pore formation or cell wall synthesis inhibition in a target cell (Collins et al., 2017; Inglis et al., 2013). Recently, the antagonistic potential of a sub-concentrations of bacteriocins have been investigated as a novel quorum quenching mechanism (Melian et al., 2019; Mathur, 2018) that might lead to a re-evaluation of bacteriocins employment.

The safety assessment of a food grade cultures in Europe is regulated by EFSA that defines the species having the Quality Presumption of Stafety staus. The General EU Food Law relies on the food cultures suppliers' obligations for a careful safety assessment on the new released products (Laulund et al., 2017). Therefore, the safety of the new microbial candidate strains should be guaranteed by the study of antibiotic resistance traits and biogenic amines production (Herman et al., 2019). However, antibiotic resistant strains and biogenic amines producers isolated from foods have been reported (Jaimee and Halami, 2016; Coton et al., 2018). Considering this, despite the low healthy-related risks associated with food-grade microbial strains, a more efficient safety characterization is required.

In this work we used comparative *in-vitro* phenotypic and *in-silico* genomic analysis to characterize newly isolated meat-derived lactic acid bacteria (LAB) for their bioprotective function, safety assessment and techno-functional performances. The final objective is to obtain a number of LAB strains that could be used as starters for salami production and have at the same time bioprotective activity.

2. Materials and methods

2. 1. Isolation of new lactic acid bacteria (LAB) strains and DNA extraction

Three artisanal, homemade, starter-free salami produced in different Italian regions (*Salame Romagnolo*, from the Emilia Romagna Region; *Salame Bresciano*, from the Lombardia region; *Salame Basilicata*, from the Basilicata Region) were the source of new LAB strains isolation.

25g of fresh meat from each salami were homogenised with 225 ml BPW (Oxoid, UK), followed by 10-fold dilutions and plating on MRS (WVR, USA) agar. Plates were incubated at 30°C for 48h. Colonies were randomly picked with sterile loop and single colony lines were streaked onto new MRS agar plates, obtaining enumerated bacteria stock libraries for the further steps. Each selected colony was then inoculated in 10ml MRS broth and incubated at 30°C for 24h. Therefore, 1 ml overnight culture was submitted to DNA extraction with the Wizard® Genomic DNA Purification Kit (Promega Corporation). The remaining bacterial culture was centrifuged and from the obtained pellet single strain skim milk stocks were prepared and kept at -80°C. Manufacturer's instructions for Isolating Genomic DNA from Gram Positive Bacteria were followed, except for the use of an additional lysis step (100mg/ml of lysozyme were added followed by an overnight incubation at 37°C).

2. 2 Fingerprinting based clustering and 16S rRNA identification of isolated LAB strains

Clustering of 209 isolated LAB strains was performed by Random Amplification of Polymorphic DNA (RAPD)-PCR as described by Di Gioia et al. (2016). Cluster analysis of obtained RAPD profiles was carried out with GelCompar II, 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) using Dice's Coefficient of similarity with the un-weighted pair group method arithmetic averages clustering algorithm (UPGMA). Based on the genotypic clustering, amplification of 16S rRNA gene region of representative isolates was performed, according to Gaggia et al. (2018) and then sequenced (MWG, Eurofins genomics). The obtained forward and reverse sequences were edited, and consensus sequences were built using the BioEdit sofware package. Sequences assignment to species or genera was achieved with the genomic data available on NCBI by BLASTn procedure (http://www-ncbi-nlm-nih-gov.ezproxy.unibo.it/genbank/).

2. 3 Whole Genome Sequencing (WGS)

DNA from 5 meat-isolated LAB strains was extracted again as described before except for the final elution in 10mM Tric-HCl solution. Sequencing of extracted genomes was outsourced at MicrobeNG with Illumina platform, using 2x250bp paired-end reads, obtaining draft genome sequences annotated with PROKKA. The obtained genomes were further annotated using the web-based software RAST (Rapid Annotations using Subsystems Technology) (RAST) (http://rast.nmpdr.org/), in particular RASTtk annotation scheme and default settings for automatically fix errors (Aziz et al., 2008). Putative bacteriocin genes were searched with BAGEL4, whereas antibiotic resistance related-genes were analysed with CARD and manually by using BLASTp and Artemis as a genome viewer for tracking their position and the flanking genes. The output from this software and sequences of interest were double checked in NCBI Protein BLAST and in UniProt blast (https://www.uniprot.org/). Multiple Sequence Alignment of genes of interest was performed with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Whole genome sequenced strains were aligned with progressive MAUVE software that allows the visualization of the conserved genomic regions and large-scale rearrangements (Darling et al., 2010).

2. 4 Determination of antibiotic susceptibility

Antibiotic susceptibility profiles of 42 LAB strains were phenotypically determined, using Lact-1 and Lact-2 VetMIC microplates, purchased from the National Veterinary Institute (SVA, Sweden). Briefly, individual colonies were suspended in a sterile glass tube containing 4 ml Maximum Recovery Diluent (Biolofe, Italy) to a turbidity of 1 in the McFarland scale (~$1x10^8$ CFU/ml). 20µl from the bacterial suspension were diluted in 10ml ISO-MRS broth (90% Iso-sensitest IST broth, Oxoid + 10% MRS broth) to obtain a final inoculum of ~$5x10^5$ cfu/ml. After filling with 100µl of the final suspension ($5x10^5$cfu/ml), VetMIC plates were

sealed with provided clear film and incubated at 30 °C for 24h-48h, depending on the growth of the strain in the control wells.

Inverted light microscope was used for results interpretation. MIC was considered as the lowest concentration completely inhibiting visible growth. The resistance was screened against the following antibiotics: Gentamicin, Kanamycin, Streptomycin, Neomycin, Tetracycline, Erythromycin, Clindamycin, Chloramphenicol, Ampicillin, Penicillin, Vancomycin, Quinupristin-dalfopristin, Linezolid, Trimethoprim, Ciprofloxacin, Rifampicin. Strain susceptibility and resistance were interpreted according the FEEDAP Panel and adopted by EFSA (EFSA-FEEDAP, 2012)

2. 5 Antimicrobial activity assay

The potential antagonistic activity of isolated LAB strains was evaluated using different indicator strains (listed in Table 1). The activity of the whole cells was tested with spot on the lawn assay, whereas the potential production of antimicrobial compounds present in the cell-free supernatant was tested with the Well-Diffusion Assay (WDA). For the direct antagonistic activity, 10 µL of fresh cell pellet, from previously overnight grown LAB strains, were spotted onto MRS agar plates and incubated for 24h at 30 °C. Then, plates were overlaid with 10 mL of 0.8% BHI or RCM soft agar containing 10^5 CFU/mL *Listeria* spp. or *Clostridium* spp., respectively, and incubated as indicated in Table 1. Supernatants from meat-borne LAB strains, grown in MRS broth for 24h at 30°C, were obtained by centrifugation at 5000g at 4 °C for 10 min. Unmodified and neutralized supernatants at pH 6.8-7.2 with 0.1-1M NaOH were used for the WDA. After solidifying of the MRS-agar, BHA (BHI+1.5% agar) and RCA (RCM+1.5% agar) plates inoculated with 10^5 CFU/mL of the indicator strain, 50 µL of supernatant were inoculated into preformed 6-mm diameter wells. The plates were initially placed for 2h at 4°C and further incubated as indicated in the Table 1. Both assays were performed in duplicate and the presence of inhibition zones around the spotted cells or around the wells were analysed.

Table 1. Indicator strains used for the in-vitro antimicrobial activity

Indicator strains used in the assays	Cultivation conditions	Strains used in a Well-diffusion assay (WDA)	Strains used in a Spot test
Clostridium sporogenes	RCM, 37°C, 24-48h, anaerobic	+	+
Listeria innocua DPC 3572	BHI, 37°C, 24h	+	+
L. monocytogenes DPC 1768	BHI, 37°C, 24h	+	+
E. coli DPC 6054	BHI, 37°C, 24h	+	+
S. enterica ssp. *typhimurium* DPC 6046	BHI, 37°C, 24h	+	+
L. curvatus DSM 20019T	MRS, 30°C, 24-48h	+	-
L. sakei ssp. *sakei* LMG 13558t	MRS, 30°C, 24-48h	+	-
L. delbrueckii ssp. *bulgaricus* LMG 6901	MRS, 37°C, 24-48h, anaerobic	+	-
L. plantarum DPC6124	MRS, 37°C, 24-48h,	+	-
L. paracasei ssp. *paracasei* DPC 6130	MRS, 30°C, 24-48h	+	-
L. brevis LMG 6906	MRS, 37°C, 24-48h, anaerobic	+	-
L. fermentum DPC 6193	MRS, 37°C, 24-48h, anaerobic	+	-

2. 6 Purification of lactocin 705 variant

Lactobacillus sakei E23B was grown aerobically at 30°C in 1L MRS. The culture was centrifuged at 8280g for 20 minutes. The cell pellet and supernatant were retained. The cells were resuspended in 250 ml of 70% 2-propanol 0.1% TFA, stirred at room temperature for 3-4 hours and the suspension was centrifuged as described above. Culture supernatant was applied to an Econo column containing 30 g Amberlite XAD16 beads prewashed with Milli-Q water. The column was washed with 400 ml of 30% ethanol and the antimicrobial peptides were eluted in 400 ml of Isopropanol (IPA) (Sigma-Aldrich, Wicklow, Ireland). IPA was removed by rotary evaporator (Buchi) and the sample was applied to a 60-ml 10-g Strata-E C18 SPE column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The column was washed with 60 ml of 30% ethanol and the inhibitory activity was eluted in 60 ml of IPA. The C18 SPE IPA eluent was concentrated using rotary evaporation before separation of the peptides using RP-HPLC. Aliquots of approximately 4 ml were concentrated to 2 ml through the removal of IPA by rotary evaporation. The concentrated aliquots were applied to

a Phenomenex (Phenomenex, Cheshire, UK) Proteo Jupiter (RP)-HPLC column (250 × 10.0 mm, 4μ, 90Å,) previously equilibrated with 27.5% acetonitrile, 0.1% trifluoroacetric acid (TFA). Peptides were eluted in a gradient of 27.5% acetonitrile 0.1% TFA to 75% acetonitrile 0.1% TFA over 35 minutes at a flow rate of 2.5 ml/min. The peptides of interest were collected, subjected to rotary-evaporation to remove the acetonitrile and freeze-dried. The purity of the peptide was analysed by MALDI-TOF Mass Spectrometry.

2. 7 Biogenic amine production (Bover-Cid and HPLC)

The identification of biogenic amines production was performed with the use of the medium Bover-Cid-Holzapfel and further confirmed by HPLC analysis of the culture media used to study the competition as described by Tabanelli et al. (2014). Briefly, for the first assay, strains were streaked onto MRS agar added with 0.1% biogenic amines precursors, namely tyrosine, histidine, ornithine, lysine and phenylalanine, and the colour modification was evaluated after incubation for 48h at 30°C. For the HPLC analysis, MRS broth containing the same precursors was used, inoculated with an overnight LAB strains suspension and incubated as previously described.

2. 8 Kinetic modelling of microbial growth and acidification

Growth and acidification kinetics of isolated LAB strains were tested in MRS broth at 30 °C. Frozen stock isolates were streaked on MRS agar plates, incubated for 48h and colonies propagated overnight in MRS broth at 30 °C. Then, 1% of strain suspension, corresponding to final concentration of 10^5 CFU/mL, was inoculated in 10mL of MRS containing 0%, 2%, 4%, 6% and 8% (w/v) of NaCl. Cell turbidity was recorded after 0, 4, 6, 8, 12, 14, 17, 19, 21, 24, 30, 48, 52, 72h with DEN-1B McFarland densitometer (Biosan, Latvia) and pH was monitor for the same time points with. Furthermore, 10 strains were studied for their growth performance at 23 °C for 24h, recording the microbial turbidity after 0, 4, 6, 8, 10, 12, 14, 17, 20, 21, 24, whereas pH was measured at the beginning and end of the experiment. Experiments

were performed twice. McFarland values obtained from microbial growth at different % of NaCl and different temperatures were fitted with the Gompertz equation as modified by Zwietering et al. (1990).

$$y=A\exp(-\exp((\mu max e/A)(\lambda-t)+1))$$

For microbial growth kinetics, y is the McFarland values at time (t), A represents max growth (log reached after 72h), μmax is the maximal increase of the growth rate (generations/h) in the exponential phase and λ is the lag time (h). Differently, acidification kinetic considers the pH decrease with respect to the initial value; therefore, A represents maximum acidification at time (t), μmax is the maximum velocity of pH decrease in exponential phase (pH unit/h) and λ is the lag time duration (h).

3. Results and discussion

3. 1 Clustering of isolated LAB strains and 16S rRNA sequencing

97 colonies were picked from plates deriving from Salame Romagnolo, 50 from Salame Bresciano, and 62 from Salame Basilicata. After purification, DNA was extracted from each obtained strain. RAPD fingerprinting and a clustering analysis were performed. 12 clusters and 8 single strains were obtained from Salame Romagnolo, 6 clusters and 5 single strains from Salame Bresciano and 5 clusters and 5 single strain from Salame Romagnolo (results are shown in the Supplementary material 1S).

One or few representative strains for each group were subjected to 16S rRNA sequencing and results are shown in Table 2.

Table 2. Identification of LAB isolated from three artisanal salami

Isolation source	Strain	Closest match	% identification NCBI	Accession Number
Salame Bresciano	C3B	*L. sakei*	99.857	MN120894
	C10B	*L. sakei*	99.786	MN173305
	C14B	*L sakei*	99.929	MN173306
	C16B	*L. sakei*	99.929	MN173307
	C17B	*L. sakei*	99.857	MN173308
	C21B	*L. sakei*	99.930	MN173309
	C22B	*L. sakei*	99.859	MN173310
	E3B	*L. sakei*	99.860	MN173311
	E7B	*L. curvatus*	99.786	MN173312
	E15B	*L. sakei*	99.930	MN173313
	E23B	*L. sakei*	99.616	MN215967
Salame Romagnolo	C10G	*L. sakei*	99.858	MN173314
	C12G	*L. sakei*	99.929	MN173315
	C16G	*L. sakei*	99.721	MN173316
	C22G	*L. sakei*	99.857	MN173317
	C26G	*L. curvatus*	99.785	MN173318
	C27G	*L. sakei*	99.929	MN173319
	C45G	*L. sakei*	99.786	MN173320
	C48G	*L. sakei*	100.000	MN173321
	E1G	*L. sakei*	99.786	MN173322
	E3G	*L. sakei*	99.929	MN173323
	E8G	*L. curvatus*	99.645	MN173324
	E13G	*L. sakei*	99.857	MN173325
	E15G	*L. sakei*	99.929	MN173326
	E17G	*L. sakei*	99.786	MN173327
	E18G	*L. curvatus*	99.856	MN173328
	E19G	*L. sakei*	100.000	MN173329
	E26G	*L. sakei*	99.929	MN173330
	E28G	*L. sakei*	99.929	MN173331
Salame Basilicata	BC1	*Leuconostoc mesenteroides*	99.930	MN173332
	BC6	*L. sakei*	99.930	MN173333
	BC20	*L. sakei*	99.930	MN173334
	BC33	*L. sakei*	99.930	MN173335
	BC35	*L. sakei*	100.000	MN173336
	BC50	*L. sakei*	99.929	MN173337
	BE2	*L. curvatus*	99.788	MN173338
	BE16	*L. sakei*	100.000	MN173339
	BE23	*Leuconostoc mesenteroides*	100.000	MN173340
	BE28	*L. curvatus*	99.861	MN173341

The results showed that *L. sakei* was the dominant species with great strain specific variability (Table 2). *L. curvatus* was the second most abundant species among the isolates, with an abundance of 11.3 % in Salame Romagnolo (R), 11.4 % in Salame Bresciano (B) and 14.5 % in Salame Basilicata (BAS), whereas, *Lactococcus mesenteroides* was detected only in Salame BAS, representing 4.5% of the isolates. On the whole, 85% of isolates from 3 different artisanal salami belongs to *L. sakei*, confirming the strong adaptation of strains of this species to meat

matrix and their wide use as starter cultures in a small and industrial-scale salami production (Tremonte et al., 2017).

The characterization of the microbial composition of spontaneous fermentation in artisanal salami is a valuable approach for gaining insights for new isolates history and performances. In a recent study, microbial assertiveness and competition was monitored in a meat fermentation process guided by a cocktail of different starter cultures (Janßen et al., 2018). Two main scenarios were observed: i) a single strain specific competitiveness, resulting in strain dominance that outgrows the autochthonous microbiota and ii) co-dominance or cooperation between different strains where more than one strain make up the major part of the microbiota.

From the RAPD clustering (Fig.1S), different scenarios in the spontaneously fermented salami were observed: i) dominance of a specific strain in Salame Basilicata, represented in the cluster containing more than 80% of all isolates; ii) about 50% single strain dominance in Salame Brescia; iii) a co-dominance of two clusters that comprises more than 50% of the isolates in Salame Romagnolo.

3. 2 Whole Genome Sequencing

Five *L. sakei* strains, namely E23B, C21B, C22G, E13G and E28G, were chosen for WGS analysis in order to study in depth or compare some of the studied phenotypic features. In particular, E23B and C21B were chosen due to their anti-LAB *in-vitro* activity, C22G due to its phenotypic antibiotic multi-resistance, E13G and E28G due to their absence of antibiotic resistances.

General genome features of sequenced strains are shown in the Table 3. The G+C content was quite similar among the strains, being ~41% and with a coding sequences ranging from 1.896 to 2.054. *Lactobacillus sakei* E13G possess the smallest genome of 1.908.827 bp, whereas E28G genome showed the greatest one (2.012.405 bp).

Table 3. Whole genome sequences and genomic features of meat-borne isolates used in the genomic comparisons

Strain	Genome coverage (X)	Size (bp)	GC%	CDS	Source	Accession Number
Lactobacillus sakei C21B	34.80	2.011.454	40.9	2.029	Salame Bresciano	CP043730
Lactobacillus sakei E23B	40.25	1.973.255	41.0	1.932	Salame Bresciano	CP043731
Lactobacillus sakei E13G	63.39	1.908.827	41.0	1.896	Salame Romagnolo	VSTE00000000
Lactobacillus sakei C22G	54.77	1.976.826	41.0	1.934	Salame Romagnolo	CP043729
Lactobacillus sakei E28G	77.44	2.012.405	40.9	2.054	Salame Romagnolo	CP043728

With the genome evolution, different magnitude of adaptive process modifications is printed on sequences. Locally collinear blocks (LCB) identified upon the whole chromosome alignments from the five *Lactobacillus* genomes are shown in Figure 1. C21B was chosen as a reference strain for the present alignment. LCBs number of a contiguously coloured matching regions, represents a high homology among the strains genomes. However, a large number of prevalently short blocks, shown below a genome's centre line, indicate a frequent chromosomal rearrangement. In particular, strains E23B and C21B showed the highest homologues, followed by a mosaic rearrangement in E28G genome. Additionally, C22G and E13G showed a larger sequence blocks rearrangement, possibly because of the presence of specific mobile genetic elements. Interestingly, regardless the smallest genome size and lowest CDS number, E13G genome has a high CG% content that suggests a loss of the genes due to the evolutive or environmental adaptation (Huang et al., 2019).

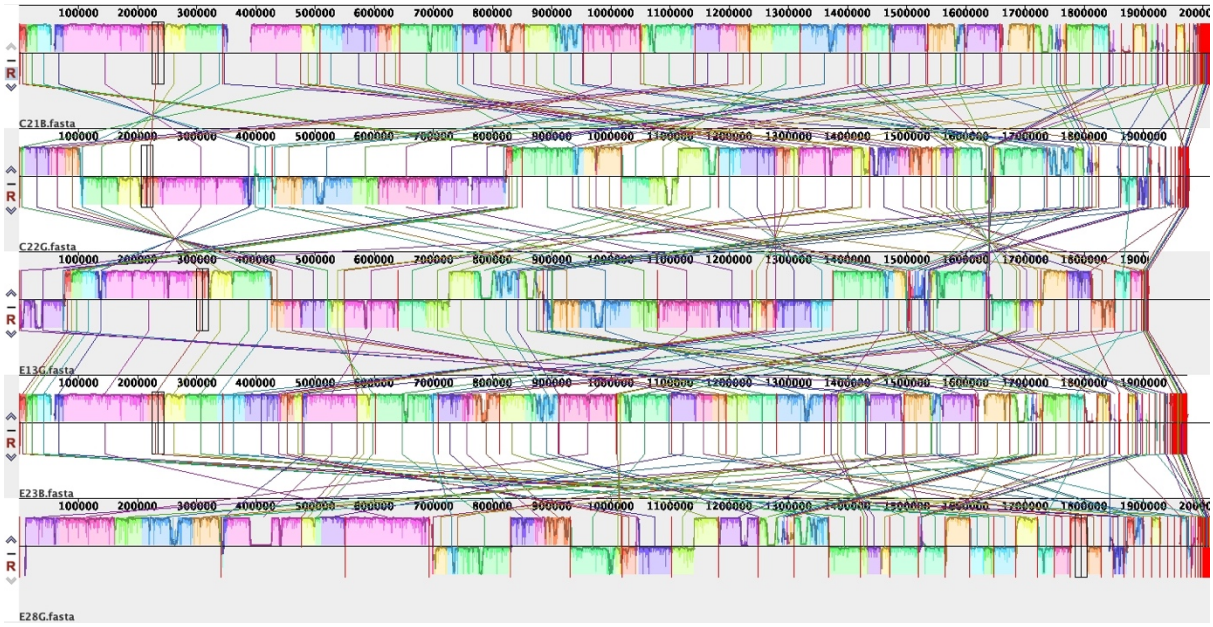


Figure 1. MAUVE alignments of five whole genome sequenced *Lactobacillus sakei* strains

To date, 50 *L. sakei* genome sequences are available (NCBI Microbial genomes, 2019), with size ranging from 1.88466 to 2.10357 Mb for the complete genomes. Intraspecies chromosome size variation from ~1.8 to ~2.3 Mb is reported, that explains its wide phenotypic diversity and adaptation to different environment (Zagorec and Champomier, 2017).

3. 3 Determination of antibiotic susceptibility (MIC) and detection of genes for antibiotic resistance in selected genomes

The WHO reports the alarming increase of the antibiotic resistance (AMR) including a wide range of infective agents (WHO, 2014). AMR is responsible for an estimated 33,000 deaths per year in the EU and AMR spread leads to a reduced antibiotics effectiveness toward diseases transmitted from animals to humans, such as salmonellosis and campylobacteriosis (EFSA, 2019). In addition, the farm-to-fork chain is reported as one of the routes that contributes to their dissemination. Resistant strains from the animal intestinal tract are found in the unprocessed meat used for the salami production, even when proper manufacturing conditions are applied (Fraqueza et al., 2015). Although LAB are known for some intrinsic, non-transmissible resistances, such as for vancomycin, multi-resistant strains with a transferable

resistance were also found in spontaneously fermented salami (Federici et al, 2014). Being a complex, multidisciplinary approach, food microbiologists are also challenged in providing data for observed antibiotic traits in food-borne bacteria, but also in filling the gap in knowledge about microbial resistant genetic traits.

Antibiotic resistance mechanisms are complex and involve drug expulsion by efflux mechanism, presence of immunity proteins and target bypass, target modification, production of enzymes that inactivates drugs. Genes coding for these complex mechanisms could also be transmitted with mobile elements like plasmids or transposons (Alekshun and Levy, 2007).

In the present study, minimal inhibition concentration of 16 antibiotics was examined in all 42 LAB strains and results of strains with a phenotypical resistance, are shown in Figure 2. Table 1S (Supplementary Material) reports detailed MIC value for each studied strain.

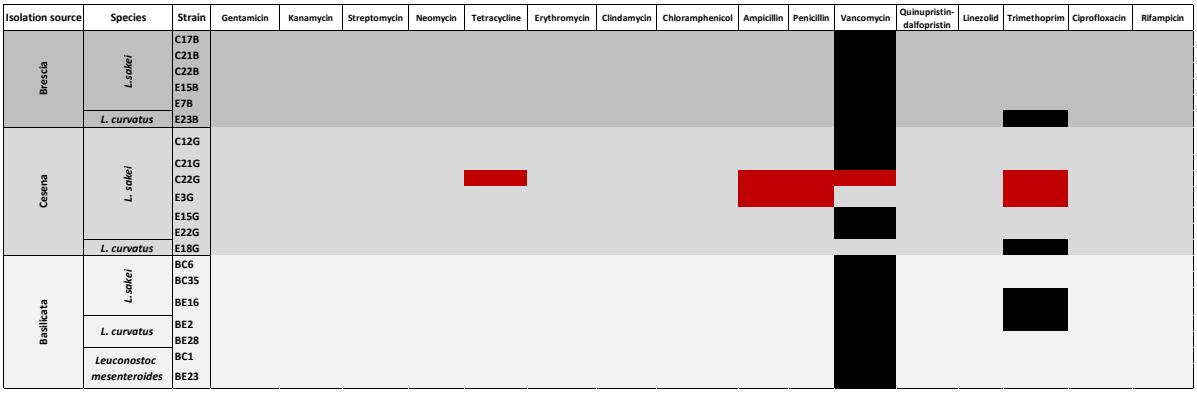


Figure 2. Phenotypic characterization of the antibiotic resistance in LAB meat borne isolates. Black colour indicates a single antibiotic resistance whereas the red one stays for a multiple resistance.

Antibiotic resistant strains were isolated from all artisanal salami. 20 out of 42 isolates showed phenotypic resistance; vancomycin, followed by trimethoprim resistance were found as the most distributed phenotypes. Trimethoprim resistance, in most of the cases, was coupled with vancomycin resistance. Multi-resistant strains were only observed in Salame Romagnolo. Penicillin and ampicillin resistance were found in 2 strains (C22G and E3G), whereas additional resistance to tetracycline was observed in one of the multi-resistant strain (C22G).

In order to study in depth these phenotypic features, five strains, listed in the Table 2. were further analysed for phenotypic-genotypic comparison. Phenotypical characterization of the five selected isolates, is reported in Table 4.

Table 4. Antibiotic resistance phenotypes of five whole genome sequenced strains

Strains	Antibiotic with the MIC (µg/mL)														
	Gm	Km	Sm	Nm	Tc	Em	Cl	Cm	Am	Pc	Va	Qda	Lz	Tm	Ci	Ri
C22G	1 (S)	16 (S)	32 (S)	4 (S)	>64 (R)	0.12 (S)	0.25 (S)	4 (S)	>16 (R)	>16 (R)	128 (S)	4 (S)	4 (S)	>64 (R)	16 (S)	2 (S)
E13G	1 (S)	8 (S)	64 (S)	1 (S)	8 (S)	0.12 (S)	0.06 (S)	8 (S)	2 (S)	1 (S)	64 (S)	1 (S)	4 (S)	2 (S)	4 (S)	0.25 (S)
E28G	0.5 (S)	<2 (S)	4 (S)	0.5 (S)	8 (S)	0.12 (S)	0.03 (S)	4 (S)	1 (S)	0.5 (S)	32 (S)	1 (S)	2 (S)	1 (S)	2 (S)	0.25 (S)
C21B	<0.5 (S)	4 (S)	16 (S)	8 (S)	4 (S)	0.12 (S)	0.12 (S)	2 (S)	4 (S)	1 (S)	>128 (R)	1 (S)	2 (S)	32 (S)	8 (S)	0.5 (S)
E23B	0.5 (S)	2 (S)	8 (S)	0.5 (S)	8 (S)	0.12 (S)	0.06 (S)	4 (S)	2 (S)	1 (S)	>128 (R)	2 (S)	4 (S)	>64 (R)	8 (S)	1 (S)

All isolates, except E28G and E13G, showed phenotypic antibiotic resistance for one or more antibiotics. Vancomycin resistance, with MIC higher than 128 µg/mL, was found in C21B and E23B strains, whereas C22G was sensitive with a MIC 128 of µg/mL. Trimethoprim resistance, with MIC higher than 64 µg/mL, was observed in strains E23B and C22B.

Literature reports that vancomycin resistance is due to D-Ala-D-Ala ligase (*ddl*)- substrate aspecificity. Vancomycin is a glycopeptide that has high affinity for D-Ala-D-Ala C-terminus of the growing peptidoglycan and binding leads to a sterical occlusion of the nascent bacterial cell wall. The generation of a modified dipeptide precursor, where D-Ala in a C-terminus is replaced by D-Lac, is a well described resistance mechanism in *Lactobacillus* spp. (Kleerebezem et al., 2010). Vancomycin resistant (Van^r) and sensitive (Van^s) *Lactobacillus* strains are generally distinguished based on the presence of Phe^{261} (F-type, resistant) or Tyr^{261} (Y-type, sensitive), in the active site of D-Ala-D-Ala ligase *ddl* (Campedelli et al., 2018).

The multiple alignment analysis of *ddl* amino acid sequences, from the five whole genome sequenced isolates are shown in Figure 3.

Figure 3. *ddl* amino acid sequences alignment from five *L. sakei* strains

The F-type was observed in all sequences, regardless their different phenotypic behaviour. Similar divergence in phenotypic and genotypic antimicrobial resistance traits was recently reported by Campedelli et al. (2018) where "Y-type" was observed in a phenotypically resistant strains and "F-type" in vancomycin sensitive *Lactobacillus* type strains. A susceptible phenotype with a resistant genotype might be explained by the presence of a silent and potentially down-regulated gene, whereas, an unknown resistant gene might account for the resistant phenotype and susceptible genotype (Fraqueza et al., 2015). However, a "silent" genotype could still be a potential hazard in AMR spreading.

Interestingly, D'Costa et al. (2011) investigated the modern phenomenon of antibiotic resistance through metagenomic studies on ancient DNA from 30,000-year-old Beringian permafrost sediments, concluding that is a natural mechanism that predates the clinical antibiotic use.

Findings in our study showed that the spontaneously fermented salami are harboured with a vancomycin and trimethoprim resistant and sensitive LAB strains.

Trimethoprim is a widely used antibiotic for veterinary and clinic purpose. It belongs to diaminopyrimidines category and inhibits dihydrofolate reductase (*dhfr*) that catalyses the last step of bacterial folic acid synthesis. Trimethoprim resistance in lactobacilli could be explained by their limited folic acid biosynthetic ability (Katla et al., 2001; Campedelli et al., 2018). However, sensitivity to this antibiotic was observed in the present study. An attempt for explanation of this bias was given by Danielsen et al., 2004 who questioned the adequateness

of the MRS medium for trimethoprim susceptibility studies. In particular, thymidine or thymine presence in the MRS media, due to beef extract and peptones, stimulates alternative metabolic pathway in thymine requiring bacteria, leading to trimethoprim insensitivity due to the absence of the drugs target.

Beta-lactam resistance is commonly referred to the production of ß-lactamase enzymes and/or in a modification in Penicillin Binding Proteins (PBP). PBP are group of transglycosylase and transpeptidases that are involved in peptidoglycan cross-linking of microbial cell walls, thus having a vital function in the cell growth, division and rest (King et al., 2016). Also, they are target proteins for ß-lactam drugs, therefore their sequence modification prevents the drug from binding. Among PBP proteins, PBP2 and PBP1A are mostly studied in pathogenic bacteria whereas limited information is available about LAB foodborne strains (Behmard et al., 2019; King et al., 2016). In the present study, *in silico* elaboration of the sequenced *L. sakei* genomes showed modification in PBP1A protein sequence, whereas modifications in PBP2 sequence were not observed. Alignment of PBP1A obtained from five genomes showed a substitution of lysine in a position 53 with arginine only in the penicillin resistant strain C22G (Figure 4).

Figure 4. PBP 1A gene alignment from five *L. sakei* strains

The present substitution might not fully explain the resistance, since the mutations in a C-terminal in PBP1A are the most studied critical resistance spots (Behmard et al., 2019). However, in a majority of beta-lactams resistances, a mosaic PBPs genes were found, which ones evolved by the accumulation of point mutations in genes of a sensitive commensal strains,

thus encodes for proteins with decreased antibiotics affinity. The same mosaic PBP genes were described in a streptococci penicillin resistant isolate (Sibold et al., 1994).

Multi-resistance is attributed to strains that are showing more than three phenotypical resistance to antibiotics, which is mostly observed in sensitive strains upon horizontal acquisition of resistance islands (Devirgiliis et al., 2011).

The obtained output from CARD, UniProt and BLASTp database showed the presence of the TetM gene in the phenotypically resistant C22G strain. The manual genome browsing, viewed by Artemis, showed genes for plasmid encoding cassette in the up and downstream of the TetM gene in the C22G genome, suggesting the potential horizontal transferability of this resistance. Most of the genes correlated with the plasmid genome organization belonged to the conjunction proteins and repA, in line with a previous observation (Oliveira et al., 2017). The same resistance was not observed in the rest of sequenced strains.

3. 4 Determination of biogenic amines

All identified isolates were tested for the amino acid decarboxylation potential using the Bover-Cid medium. In particular, the ability of decarboxylating tyrosine, histidine, ornithine and lysine was investigated. *Lactobacillus curvatus* C26G was the only strain biogenic amine producer. Purple-colored culture Bover-Cid was the indicator of putrescine production (data not shown). The HPLC analysis of C26G supernatant for biogenic amine production confirmed the production of putrescine, being 600 mg/l out of 1000 mg/l of the precursor. The production of putrescine by *L. curvatus* isolated from cheese, dairy products, meat and sausage, has been observed in the range of 10-10000 mg/L in different studies (Wunderlichova et al., 2014).

Genome analysis of the sequenced strains confirmed the lack of decarboxylase related genes: *tdc/tyrdc* (tyrosine decarboxylase), *hdc* (histidine decarboxylase), *agdi/aguA* (agmatine deiminase) and *odc* (ornithine decarboxylase). The present genotypic approach for biogenic amines production in the sequenced LAB strains confirmed the strain phenotypic traits.

3. 5 Antimicrobial activity assay and bacteriocin genes prediction

Cell to cell interactions, interference of molecular actors, potential quorum sensing signalling or quorum-quenching are some of the mechanism that describes the complexity of the direct antagonism (Melian et al., 2019). In the present study, the direct antagonistic activity of the LAB isolates against foodborne pathogens showed that all strains were able to inhibit at least one strain, with a different magnitude. Some strains, namely C10B, C12G, E13G and E15G, were shown as the most performant antagonistic strains against the four screened pathogens (Figure 5). Conversely, the same effect against pathogens was not observed when the antimicrobial activity of non-neutralized and neutralized LAB supernatants were tested.

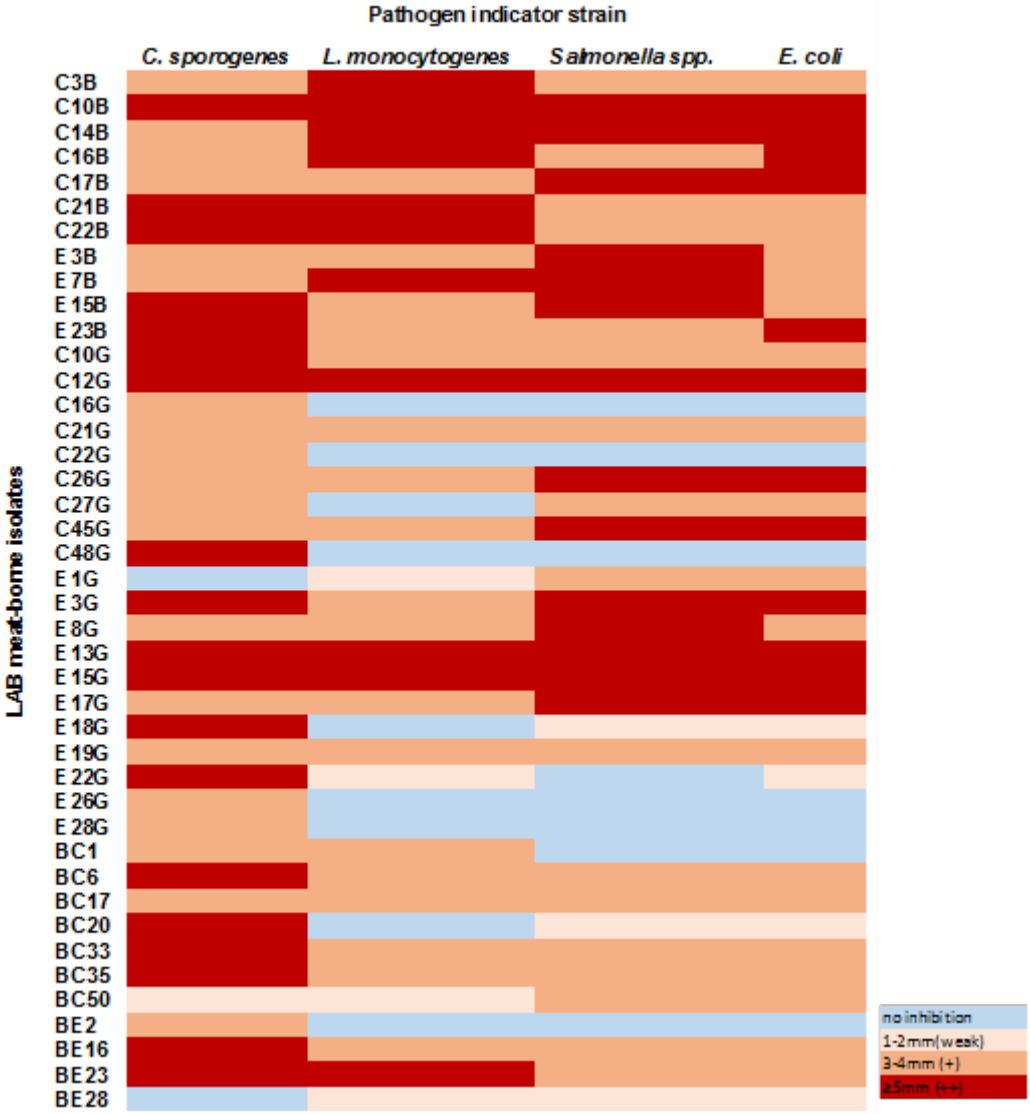


Figure 5. Direct antagonistic activity of meat-borne LAB against foodborne pathogens, based on spot test assay

Despite these results, WGS analysis showed that three out of the sequenced five strains were found to possess the genes for bacteriocin production. However, an anti-LAB activity was observed in some strains. In particular, each of the strains E23B and C21B, isolated from the same source but belonging to different RAPD-PCR cluster, possessed the genes for two bacteriocins: alfa and beta lactocin_705 variant and a putative bacteriocin belonging to the class IIc (Figure 6.). The BAGEL4 outputs related to bacteriocin related genes found in the sequenced genomes are listed in the Table 5 and Figure 6.

Table 5. Bacteriocins encoded in the *Lactobacillus* genome sequences

Strain	**Core proteins (BAGEL4)**	**Class**	**Similarity with protein with accession number**	**% of identity protein with accession number**	**Flanking mobile element**
E23B	Lactocin_705_alfa Lactocin_705_beta	IIb	*L. curvatus* CRL705 (GCA_000235705) (NP_542216.1) alfa (NP_542217.1) beta	100.000% (54/54) - FX989_09480 98.214% (55/56) - FX989_09475	RepA, Rep_3, integrase,
	Putative bacteriocin inducing peptide	IIc	*L. sakei* 23K (WP_011374266.1)	100.00% (44/44) - FX989_04935	-
C21B	Lactocin_705_alfa Lactocin_705_beta	IIb	*L. curvatus* CRL705 (GCA_000235705) (NP_542216.1) alfa (NP_542217.1) beta	100.000% (54/54) - FXV74_09250 98.214% (55/56) - FXV74_09245	RepA, integrase, Rep_3
	Putative bacteriocin inducing peptide	IIc	*L. sakei* 23K (WP_011374266.1)	100.00% (44/44) - FXV74_05195	-
C22G	-	-	-	-	-
E28G	Putative bacteriocin inducing peptide	IIc	*L. sakei* 23K (WP_011374266.1)	100.00% (44/44) - FX990_05045	-
E13G	-	-	-	-	-

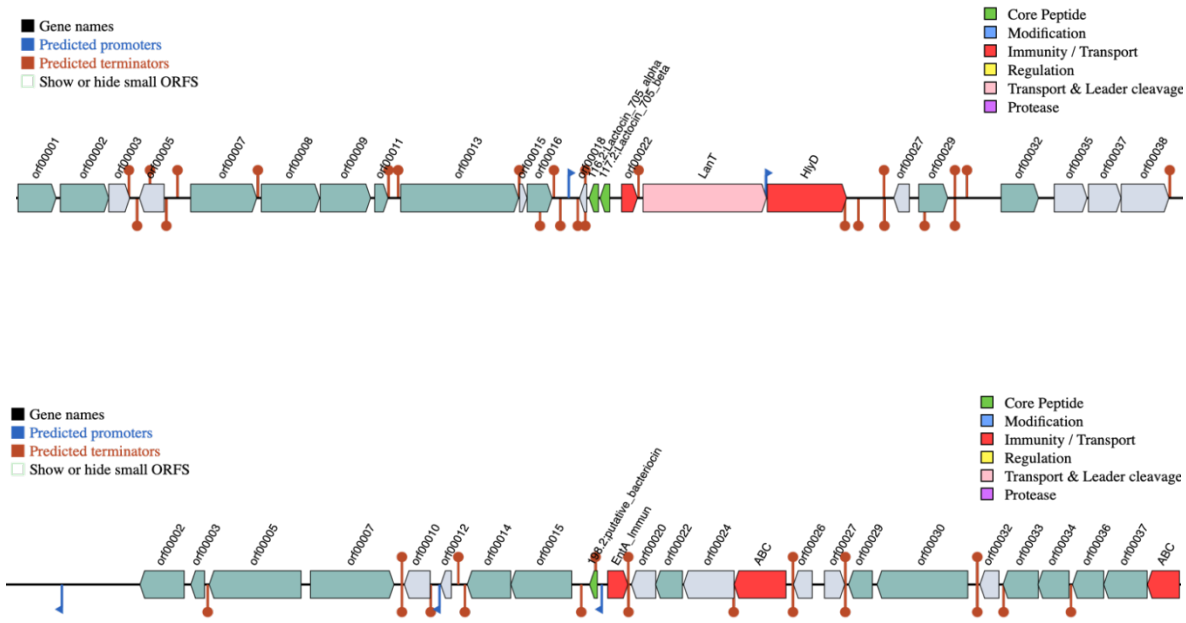


Figure 6. Schematic representation of lactocin 705 variant bacteriocin and putative bacteriocin clusters in E23B and C21B

When the supernatant activity of E15B and lactocin_705 variant strains (E23B and C21B) was tested against the indicator type strains *L. curvatus* DSM 20019^{T} and *L. sakei* ssp. *sakei* LMG 13558^{T}, an inhibitory activity was observed (Figure 7). The first step of salami fermentation is held at about 22°C, whereas 30°C is the optimal *in vitro* temperature for LAB growth. In the attempt to understand whether the production of the antimicrobial compounds is temperature dependent, E15B, E23B and C21B strains were grown at 22°C and 30°C. Figure 7 shows that the inhibitory activity of the three isolates is not affected by growth at both temperatures.

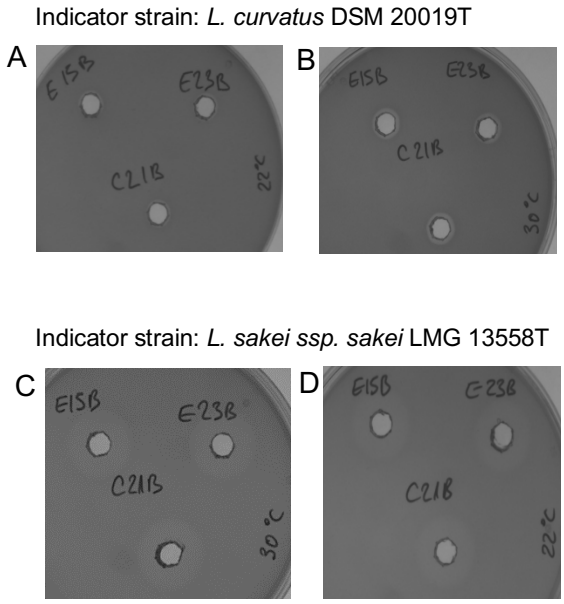


Figure 7. Anti-LAB activity of lactocin 705 variant producers

When bacteriocin sequences of *L. curvatus* CRL705, E23B and C21B strains were aligned, one amino acid substitution in W26G subunit alpha was observed also in E23B and C21B. In addition, flanking mobile elements next to the bacteriocin genes, as reported in Table 5, suggests that lactocin_705 variant is potentially encoded by a plasmid. The mass of the purified lactocin_705 variant components was also confirmed with MALDI TOF MS:

Peptide 1: GFGGGLGYIAGRVGAAYGHAQASANNHHSPING MW = 3179.38 Da

Peptide 2: GMSGYIQGIPDFLKGYLHGISAANKHKKGRLGY MW = 3578.11 Da

L. curvatus CRL705 is widely studied as meat bioprotective agent and recently as an antimicrobial compound in an active food packaging coating (Melian et al., 2019). The two-component plasmid encoded lactocin_705 is one of the two bacteriocins produced by this strain with demonstrated antagonistic activity against closely related species such as *L. casei, L. plantarum* and *Leuconostc* spp. The lactocin_705 variant found in E23B and C21B strains, shares 98.214% identity with the one isolated from the *L. curvatus* CRL705 strain. When anti-LAB activity was tested, the purified peptides are inactive when used alone, meaning that both are required for activity (Figure 8). This observation agrees with Cuozzo et al. (2000), who

described the two component lactocin_705 activity when both subunits are present at equimolar concentration, as commonly find in a class IIb bacteriocins.

Figure 8. Purified lactocin 705 variant

Therefore, despite the amino acid substitution, the bacteriocin variant described in this work is still able to inhibit the growth of the closely related species (Figure 8).

Interestingly, Janßen et al. (2018) described an anti-LAB producer strain, namely *L. curvatus* TMW 1.624, as a dominant strain in the meat microbiota, with a total of 81.6% of the microbiota. This suggests that the anti-LAB bacteriocins, produced by starters/bioprotective cultures, might be a strain specific strategy in the occupation of the meats ecological niche (Janßen et al., 2018). The positive outcome from this strategy, might be also the control of the aminobiogenic LAB, as reported by Tabanelli et al. (2014).

For comparison purposes, when the genome of *L. curvatus* CRL705 with the accession number GCA_000235705 was screened for bacteriocins production with BAGEL4, three bacteriocins with high homology with SakacinQ, SakacinP and Enterocin_NKR-5-3D were found. The production of these three bacteriocins might also explain the anti-listerial activity observed in a food model by Castellano et al. (2006), highlighting the importance of the additional information that can be obtained from the whole genome sequences, that cannot be revealed only with the phenotypic and a gene targeted analysis.

Interestingly, among the sequenced strains in the present study, E13G, a strain without bacteriocin genes detected, was the most performant strain in a direct inhibition antimicrobial assay. Moreover, this strain belongs to a cluster that counts 21.6% from all isolates in Salame Romagnolo, being one of the two co-dominant clusters that suggests its competitive performances in such a complex environment. In addition, this strain showed a short lag phase as well as one of the highest acidification performances among all the tested strains (data not shown). These observations are in line with bioprotective cultures characteristics, that include performant starter strains possessing antimicrobial activity without bacteriocin producing genes (Orihuel et al., 2018).

Recently, an alternative approach of protective cultures applications was reported. In particular, when suboptimal concentrations of Lactocin AL705 was used, a successful inhibition of *L. monocytogenes* biofilm formation was observed, hypothesizing due to the signal molecule inactivation, different than a known autoinducer, AI-2 quorum sensing molecule (Melian et al., 2019). Differently, when *Lactobacillus* strains were co-cultured with class IIb plantaricin A, an increased biofilm formation by *L. plantarum* DC400 was observed, giving us an opportunity to re-evaluate the bacteriocins applications in an undesired microbial outgrowth control (Calasso et al., 2013; Mathur, 2018). Also, molecular fundaments in microbial interactions is nowadays reconsidered when the antagonistic activity is shown in the absence of bacteriocins production (Orihuel et al., 2018).

3. 6 Growth and acidification kinetics

The growth and acidification performances of new starter cultures are important parameters for safety and quality ensuring in fermented meats. Lag phase is an indicator of the duration of the strain's adaptability in a new environment. The shorter the lag phase, the faster is the starter colonization of the food matrix, resulting in a potential inhibition of pathogens or undesirable microbial outgrowth (Swinnen et al., 2004). In addition, high acidification performances,

expressed as a high delta pH in a certain amount of time as well as the pH units lowered per hour, represent another hurdle barrier for a pathogens outgrowth as well as an improvement of the product quality (Ammor et al., 2005).

In the present study, the growth and acidification behaviour of isolated LAB strains was monitored through the strain turbidity increase and pH variation; the kinetic data were modelled with the Gompertz equation (Zwietering et al., 1990). The growth of new isolates at different % of NaCl, over 72h, was evaluated by comparison of the parameters for their a) growth kinetics: growth rate (μmax, MF unit/h), lag phase (λ, h) and max growth (A, MF units after 72h) and b) acidification kinetics: acidification rate (μmax, pH unit decrease/h), lag phase (λ, h), max acidification (A, pH units after 72h).

Table 6. Acidification and growth performances of LAB strains isolated from three artisanal salami

Isolation source	% NaCl	λ	μmax	ΔpH
Salame Romagnolo (R)	0	6.88 (±1.36)^{a}	0.73 (±0.12)^{a}	1.83(±0.31)^{a}
	2	8.26 (±2.14)^{a}	0.60 (±0.07)^{ab}	1.77(±0.28)^{a}
	4	12.00 (±2.97)^{b}	0.52 (±0.06)^{b}	1.56(±0.29)^{b}
	6	19.55 (±3.08)^{c}	0.37 (±0.07)^{c}	1.36(±0.26)^{c}
	8	32.85 (±9.68)^{d}	0.15 (±0.06)^{d}	
Salame Bresciano (B)	0	7.01 (±0.43)^{a}	0.97 (±0.22)^{e}	1.92(±0.08)^{a}
	2	7.58 (±0.54)^{a}	0.99 (±0.20)^{e}	1.95(±0.05)^{a}
	4	10.07 (±2.54)^{ab}	0.60 (±0.03)^{b}	1.72(±0.13)^{ab}
	6	14.90 (±3.35)^{b}	0.33 (±0.09)^{c}	1.48(±0.16)^{b}
	8	24.72 (±6.06)^{d}	0.13 (±0.03)^{d}	
Salame Basilicata (BAS)	0	5.98 (±0.72)^{a}	0.96 (±0.12)^{e}	1.73(±0.06)^{a}
	2	6.41 (±0.22)^{a}	0.85 (±0.27)^{e}	1.72(±0.08)^{a}
	4	10.28 (±3.39)^{ab}	0.65 (±0.06)^{a}	1.54(±0.27)^{b}
	6	14.88 (±6.02)^{b}	0.51 (±0.05)^{b}	1.54(±0.14)^{b}
	8	23.73 (±7.36)^{c}	0.23 (±0.11)^{cd}	

Growth kinetic of selected Lactobacillus strains in 5 different NaCl %: 0%,2%,4%,6%,8%, 72h. Statistical analysis: potential interactions between factors (%NaCl and Isolation source) were explored by the general linear model (GLM) procedure using two-way ANOVA of Statistica (StatSoft Italy srl, Vigonza, Italy) on growth parameters of Gompertz equation. Fisher's Least Significant Difference (LSD) test was used to identify significant differences ($p< 0.05$). Means with the same letter in the column are not statistically different ($p>0.05$).

Table 6 summarises growth and acidification properties of LAB strains, based on the source of isolation. The values of standard deviation highlight a great variability in the strain performances, especially at the highest NaCl %, both, within the same species and among different isolation source. Best growth performances for λ and the greatest pH decrease was observed between 0 and 2 % NaCl without significant differences ($p>0.05$), with λ comprised between 6-8h and pH drop being between 1.72-1.95 units. When considered μmax, strains isolated from Salame Bresciano (B) showed overall significantly lower growth rates. Strains isolated from Salame Romagnolo (R) and Salame Basilicata (BAS) showed significantly ($p > 0.05$) higher growth rates at 0 and 2 % NaCl with respect to those isolated from B, being between 0.85-0.99 gen/h, whereas BAS strains showed significantly better ($p <0.05$) growth performances at higher NaCl concentration (6 and 8%) The growth and acidification parameters in 4% of NaCl showed no significant difference among the isolates, except for BAS strains that showed significantly higher growth rates. Significant differences in acidification performances were observed between 0-2% group and 4-6% group, with the exception for strains from R that had a lower performance.

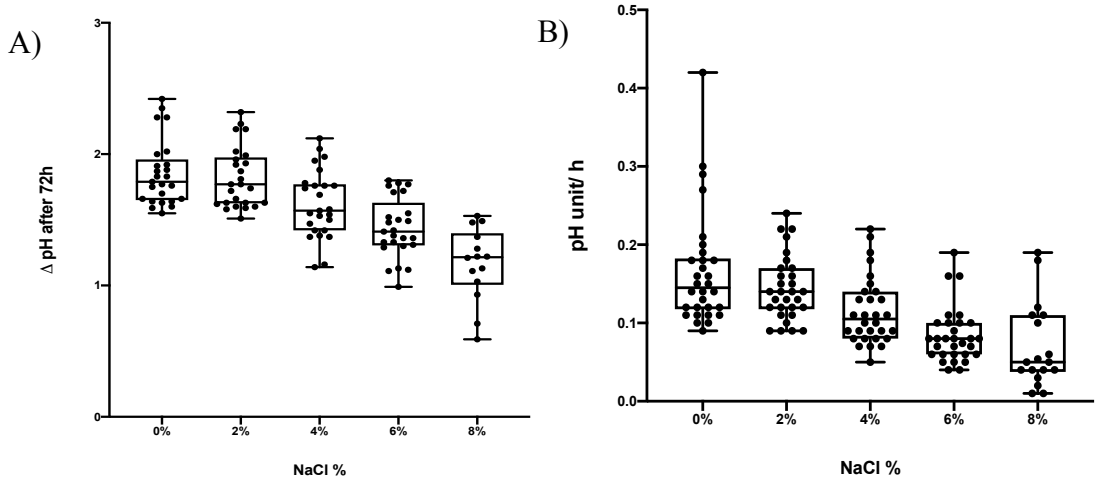


Figure 9. *In-vitro* acidification performances of LAB isolates. A) variation of pH after 72h, B) acidification rates of LAB strains (μmax)

The two plots in Figure 9 show the acidification performances of the isolated bacteria under different osmotic stress (NaCl%). With the increase of the osmotic stress (6% and 8% of NaCl) the strains showed a decreased acidification capability compared to a lower osmotic stress. However, some of the strains under higher osmotic stress (8% NaCl) are more performant as shown in the upper extreme of the same boxplot. Strains under lower salt stress are able to decrease pH with different magnitude after 72h. Strains placed in the upper extreme, on the box and whiskers plot, are considered as the more performant under different salt conditions. It is interesting to observe that the physiological diversity of LAB isolates correlates to the great inter- and intra-species diversity shown with RAPD-PCR.

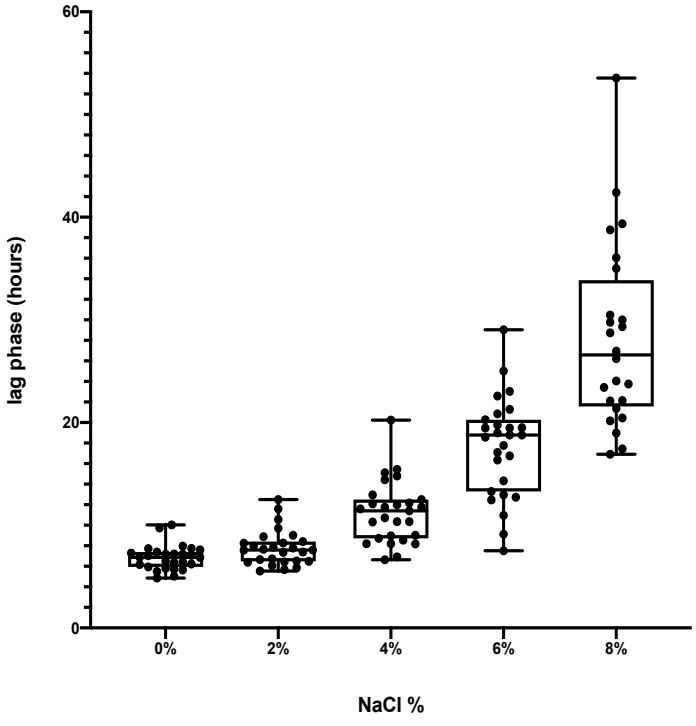


Figure 10. Growth performances of LAB isolates (lag phase)

The plot in Figure 10 shows different adaptation properties of the isolated strains (lag phase expressed in hours). In particular, when increased osmotic stress conditions are applied (6%

and 8% of NaCl), the strains require more time to adapt to the culture conditions (long lag phase). However, highly performant strains could be observed at 6% of NaCl with a short lag phase of 7.5 hours. A high heterogeneity among strains can be observed when higher osmotic stress is applied, with a lag-phase ranging from 16.92 to 53.55 hours. This confirms again a high physiological inter and intraspecies heterogeneity and potential technological traits of the indigenous meat-borne strains. When the salami fermentation process is considered, the first phase is run in a lower salt concentration that allows a selection of strains that are more performant under this condition. As the ripening process proceeds, the most salt tolerant strains become more performant. In this view, the NaCl tolerance is one of the major factors for starters and bioprotective cultures selection (Toldra, 2010). In a similar study, the autochthonous *L. sakei* strains have shown a higher growth lag phase that was shown between 14 and 22 h of incubation at 2.5% of NaCl (Ammor et al., 2005) in SB media. In accordance with the observations from the WGS of *L. sakei* 23K, all of the present sequenced strains possessed the genes that allow coping with the osmotic stress (Chaillou et al., 2005). In particular, in all genomes, genes encoding products belong to three ABC uptake systems that are involved in the accumulation of osmoprotectant molecules, like betaine, were found (Supplementary Material Table 1S).

The inhibition of pathogens outgrowth is important to be set at the very beginning of the fermentation process, considering that meat is a highly nutritious and perishable matrix. Also, some of the pathogens have short growth lag phase, as for instance *Salmonella* spp. (Birk et al., 2016), *Listeria monocytogenes* and *E. coli* (Pragalaki et al., 2013).

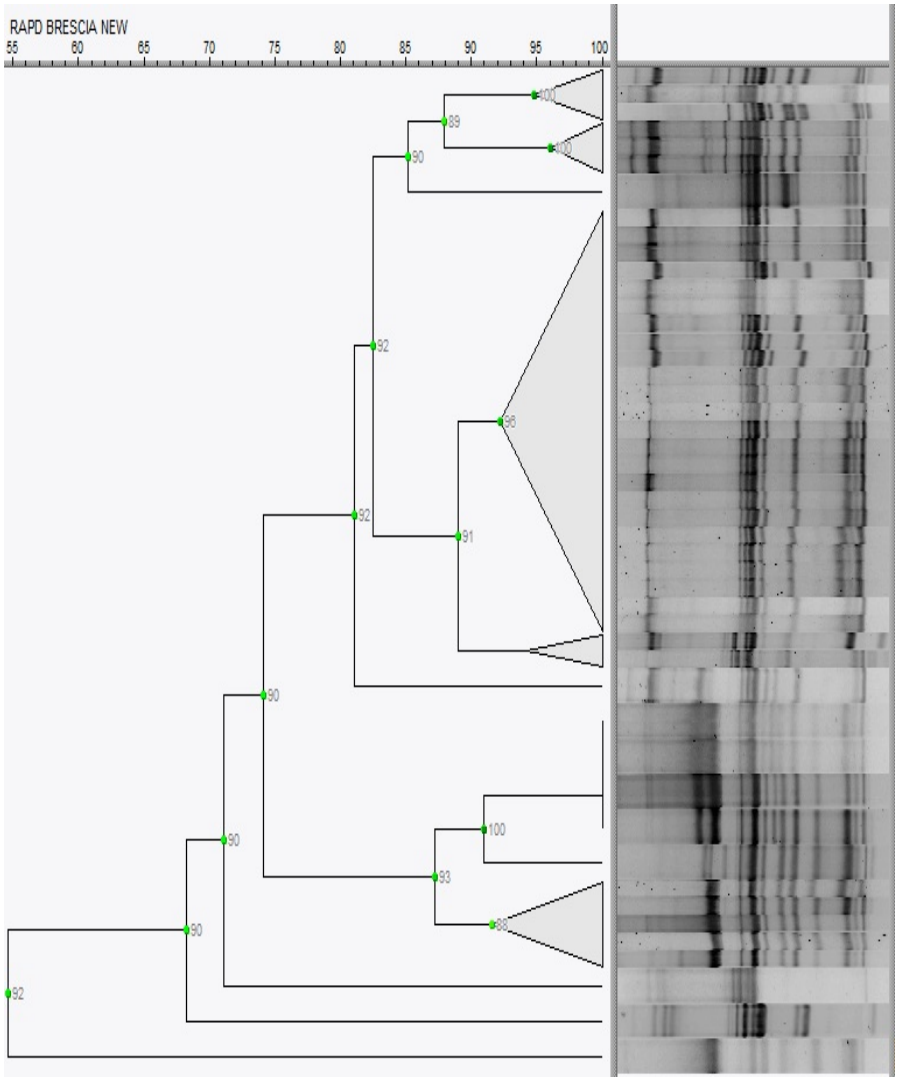
4. Conclusion

The present study allowed to select autochthonous meat-borne lactic acid bacteria, that are safe for their utilization in fermented meat production. Also, different number of strains were able to perform, both, as bioprotective as well as starter cultures, in *in-vitro* conditions. Considering the phenotypic-genotypic bias in bacteriogenic producers detection, further studies in targeted food models will elucidate better this aspect. However, the most performant strains from the present study will be further used in a development of bioprotective/starter cultures cocktail, and tested as a potential biopreservation approach in fermented salami production.

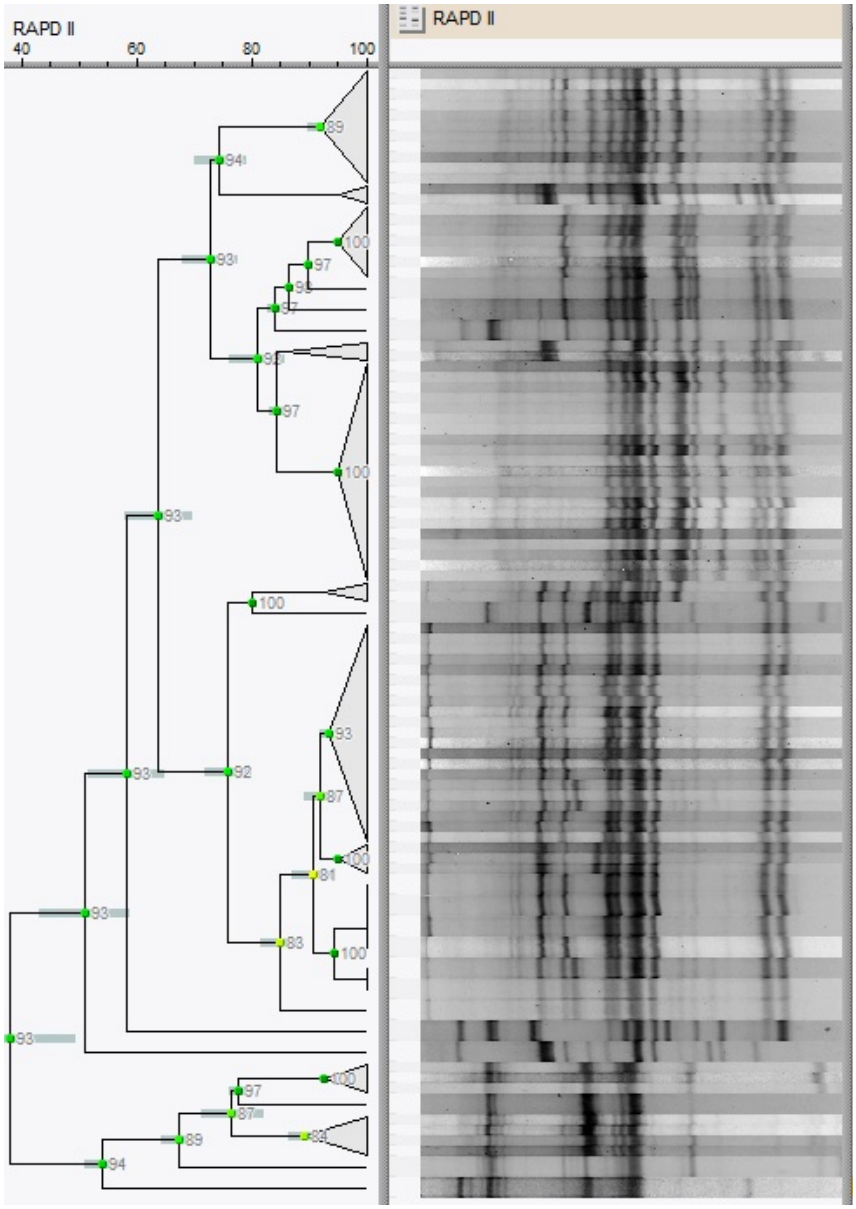
Supplementary material

Figure 1S. RAPD-profiles of 209 authoctounous lactic acid bacteria, isolated from three artisanal Italian salami.

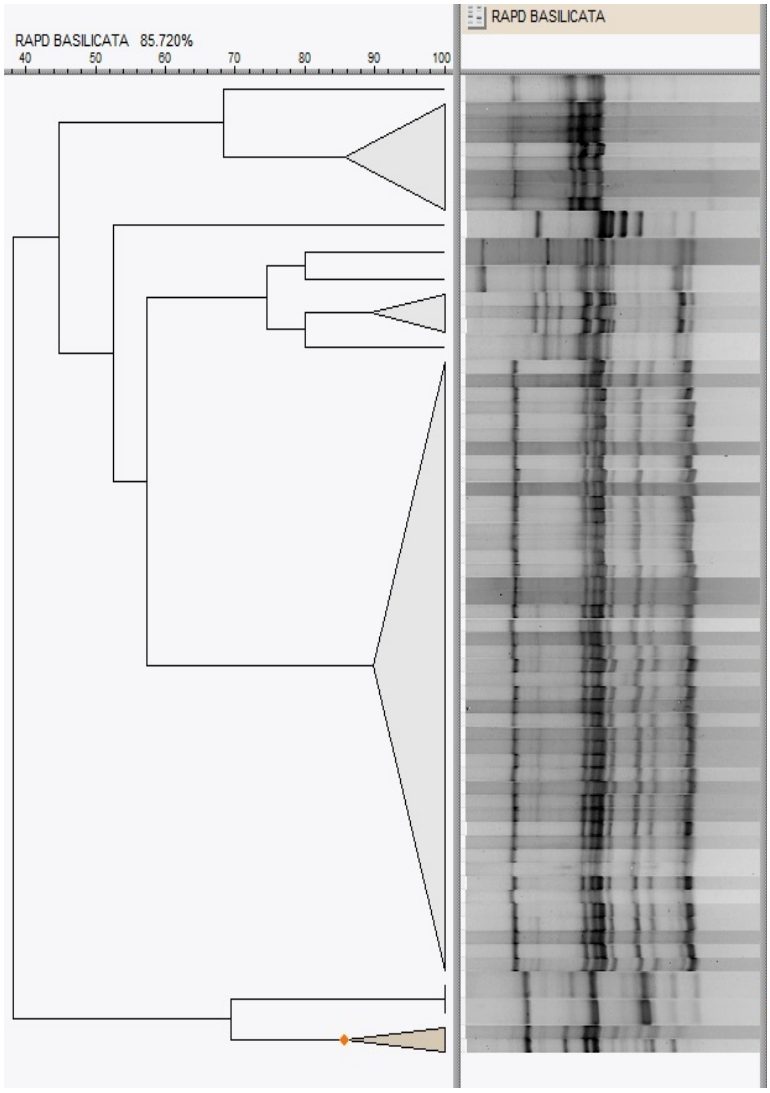
A). RAPD-PCR fingerprinting: Salame Bresciano



B). RAPD-PCR fingerprinting: Salame Romagnolo



C). RAPD-PCR fingerprinting: Salame Basilicata



Strains isolated from Salame Brescia	Antibiotic with the MIC (μg/mL)															
	Gm	Km	Sm	Nm	Tc	Em	Cl	Cm	Am	Pc	Va	Qda	Lz	Tm	Ci	Ri
C3B	1 (S)	4 (S)	16 (S)	1 (S)	4 (S)	0.12 (S)	0.12 (S)	4 (S)	2 (S)	1 (S)	128 (S)	2 (S)	2 (S)	4 (S)	8 (S)	≤0.12 (S)
C10B	1 (S)	8 (S)	16 (S)	1 (S)	2 (S)	0.25 (S)	≤0.03 (S)	2 (S)	2 (S)	1 (S)	16 (S)	1 (S)	2 (S)	8 (S)	2 (S)	≤0.12 (S)
C14B	1 (S)	8 (S)	16 (S)	2 (S)	4 (S)	0.12 (S)	0.06 (S)	1 (S)	2 (S)	1 (S)	128 (S)	1 (S)	1 (S)	4 (S)	2 (S)	0.25 (S)
C16B	≤0.5 (S)	≤2 (S)	8 (S)	2 (S)	4 (S)	0.12 (S)	0.06 (S)	2 (S)	0.5 (S)	0.25 (S)	16 (S)	1 (S)	2 (S)	0.5 (S)	16 (S)	2 (S)
C17B	1 (S)	4 (S)	16 (S)	4 (S)	4 (S)	0.5 (S)	0.25 (S)	4 (S)	2 (S)	1 (S)	>128 (R)	4 (S)	2 (S)	8 (S)	8 (S)	0.25 (S)
C21B	≤0.5 (S)	4 (S)	16 (S)	8 (S)	4 (S)	0.12 (S)	0.12 (S)	2 (S)	4 (S)	1 (S)	>128 (R)	1 (S)	2 (S)	32 (S)	8 (S)	0.5 (S)
C22B	2 (S)	4 (S)	16 (S)	2 (S)	4 (S)	0.25 (S)	0.06 (S)	4 (S)	4 (S)	1 (S)	>128 (R)	2 (S)	4 (S)	16 (S)	4 (S)	≤0.12 (S)
E3B	≤0.5 (S)	≤2 (S)	8 (S)	2 (S)	4 (S)	0.12 (S)	0.06 (S)	2 (S)	0.5 (S)	0.25 (S)	16 (S)	1 (S)	2 (S)	0.5 (S)	16 (S)	2 (S)
E7B	≤0.5 (S)	≤2 (S)	8 (S)	1 (S)	2 (S)	0.25 (S)	0.06 (S)	1 (S)	2 (S)	1 (S)	>128 (R)	2 (S)	2 (S)	2 (S)	8 (S)	0.25 (S)
E15B	1 (S)	4 (S)	16 (S)	1 (S)	4 (S)	0.12 (S)	0.06 (S)	2 (S)	2 (S)	1 (S)	>128 (R)	2 (S)	2 (S)	8 (S)	4 (S)	0.25 (S)
E23B	0.5 (S)	2 (S)	8 (S)	0.5 (S)	8 (S)	0.12 (S)	0.06 (S)	4 (S)	2 (S)	1 (S)	>128 (R)	2 (S)	4 (S)	>64 (R)	8 (S)	1 (S)

Strains isolated from Slame Romagnolo	Antibiotic with the MIC (μg/mL)															
	Gm	Km	Sm	Nm	Tc	Em	Cl	Cm	Am	Pc	Va	Qda	Lz	Tm	Ci	Ri
C10G	1 (S)	8 (S)	16 (S)	1 (S)	4 (S)	0.12 (S)	0.06 (S)	8 (S)	2 (S)	1 (S)	16 (S)	1 (S)	4 (S)	8 (S)	4 (S)	≤0.12 (S)
C12G	1 (S)	8 (S)	16 (S)	1 (S)	16 (S)	0.12 (S)	0.25 (S)	8 (S)	2 (S)	2 (S)	>128 (R)	4 (S)	8 (S)	>64 (R)	16 (S)	0.25 (S)
C16G	≤0.5 (S)	≤2 (S)	8 (S)	0.5 (S)	4 (S)	0.06 (S)	0.03 (S)	4 (S)	1 (S)	0.5 (S)	16 (S)	1 (S)	1 (S)	1 (S)	4 (S)	≤0.12 (S)
C21G	1 (S)	4 (S)	32 (S)	1 (S)	64 (S)	0.25 (S)	0.12 (S)	16 (S)	1 (S)	1 (S)	>128 (R)	1 (S)	16 (S)	>64 (R)	8 (S)	2 (S)
C22G	1 (S)	16 (S)	32 (S)	4 (S)	>64 (R)	0.12 (S)	0.25 (S)	4 (S)	>16 (R)	>16 (R)	128 (S)	4 (S)	4 (S)	>64 (R)	16 (S)	2 (S)
C26G	2 (S)	32 (S)	16 (S)	8 (S)	1 (S)	0.06 (S)	0.03 (S)	8 (S)	0.5 (S)	0.25 (S)	128 (S)	1 (S)	2 (S)	8 (S)	4 (S)	0.5 (S)
C27G	≤0.5 (S)	4 (S)	8 (S)	1 (S)	4 (S)	0.12 (S)	0.03 (S)	4 (S)	2 (S)	1 (S)	128 (S)	1 (S)	2 (S)	8 (S)	8 (S)	1 (S)
C45G	2 (S)	16 (S)	32 (S)	2 (S)	8 (S)	0.12 (S)	≤0.03 (S)	8 (S)	2 (S)	1 (S)	16 (S)	0.5 (S)	4 (S)	16 (S)	8 (S)	≤0.12 (S)
C48G	1 (S)	8 (S)	16 (S)	2 (S)	8 (S)	0.12 (S)	0.06 (S)	8 (S)	4 (S)	1 (S)	8 (S)	1 (S)	2 (S)	1 (S)	2 (S)	≤0.12 (S)
E13G	1 (S)	8 (S)	64 (S)	1 (S)	8 (S)	0.12 (S)	0.06 (S)	8 (S)	2 (S)	1 (S)	64 (S)	1 (S)	4 (S)	2 (S)	4 (S)	0.25 (S)
E15G	≤0.5 (S)	≤2 (S)	4 (S)	≤0.5 (S)	2 (S)	0.12 (S)	0.03 (S)	2 (S)	2 (S)	2 (S)	>128 (R)	1 (S)	1 (S)	16 (S)	8 (S)	≤0.12 (S)
E17G	≤0.5 (S)	4 (S)	8 (S)	≤0.5 (S)	8 (S)	0.12 (S)	0.06 (S)	8 (S)	2 (S)	1 (S)	128 (S)	1 (S)	4 (S)	16 (S)	4 (S)	≤0.12 (S)
E18G	≤0.5 (S)	≤2 (S)	4 (S)	1 (S)	4 (S)	0.06 (S)	0.06 (S)	2 (S)	0.5 (S)	0.25 (S)	>128 (R)	2 (S)	1 (S)	2 (S)	16 (S)	≤0.12 (S)
E19G	1 (S)	8 (S)	16 (S)	1 (S)	4 (S)	0.12 (S)	0.06 (S)	4 (S)	1 (S)	1 (S)	16 (S)	2 (S)	4 (S)	2 (S)	2 (S)	≤0.12 (S)
E1G	≤0.5 (S)	4 (S)	16 (S)	≤0.5 (S)	8 (S)	0.12 (S)	≤0.03 (S)	4 (S)	1 (S)	1 (S)	16 (S)	1 (S)	2 (S)	1 (S)	4 (S)	≤0.12 (S)
E22G	2 (S)	16 (S)	32 (S)	2 (S)	8 (S)	0.12 (S)	0.25 (S)	8 (S)	2 (S)	1 (S)	64 (S)	2 (S)	4 (S)	>64 (R)	16 (S)	4 (S)
E26G	≤0.5 (S)	≤2 (S)	8 (S)	≤0.5 (S)	4 (S)	0.06 (S)	≤0.03 (S)	4 (S)	1 (S)	0.5 (S)	16 (S)	0.5 (S)	2 (S)	2 (S)	4 (S)	≤0.12 (S)
E28G	0.5 (S)	≤2 (S)	4 (S)	0.5 (S)	8 (S)	0.12 (S)	0.03 (S)	4 (S)	1 (S)	0.5 (S)	32 (S)	1 (S)	2 (S)	1 (S)	2 (S)	0.25 (S)
E3G	2 (S)	16 (S)	32 (S)	4 (S)	16 (S)	0.12 (S)	0.5 (S)	8 (S)	>16 (R)	>16 (R)	128 (S)	4 (S)	4 (S)	>64 (R)	32 (S)	2 (S)
E8G	1 (S)	8 (S)	8 (S)	2 (S)	2 (S)	0.12 (S)	0.12 (S)	8 (S)	1 (S)	0.5 (S)	128 (S)	1 (S)	4 (S)	2 (S)	4 (S)	0.25 (S)

Strains isolated from Slame Basilicata	Antibiotic with the MIC (μg/mL)															
	Gm	Km	Sm	Nm	Tc	Em	Cl	Cm	Am	Pc	Va	Qda	Lz	Tm	Ci	Ri
BC17	1 (S)	≤2 (S)	16 (S)	≤0.5 (S)	1 (S)	0.12 (S)	≤0.03 (S)	2 (S)	4 (S)	1 (S)	16 (S)	1 (S)	1 (S)	16 (S)	8 (S)	≤0.12 (S)
BC5	≤0.5 (S)	≤2 (S)	8 (S)	≤0.5 (S)	4 (S)	0.12 (S)	≤0.03 (S)	4 (S)	0.12 (S)	0.25 (S)	>128 (R)	1 (S)	2 (S)	>64 (R)	4 (S)	2 (S)
BE28	2 (S)	8 (S)	16 (S)	4 (S)	1 (S)	0.12 (S)	≤0.03 (S)	2 (S)	1 (S)	0.5 (S)	>128 (R)	1 (S)	1 (S)	2 (S)	8 (S)	≤0.12 (S)
BC20	4 (S)	16 (S)	32 (S)	8 (S)	2 (S)	0.12 (S)	0.25 (S)	2 (S)	2 (S)	0.5 (S)	128 (S)	4 (S)	1 (S)	32 (S)	8 (S)	0.5 (S)
BC35	4 (S)	16 (S)	32 (S)	4 (S)	64 (S)	0.12 (S)	0.5 (S)	2 (S)	2 (S)	0.5 (S)	>128 (R)	4 (S)	2 (S)	32 (S)	8 (S)	1 (S)
BE23	2 (S)	8 (S)	16 (S)	4 (S)	32 (S)	0.12 (S)	0.06 (S)	2 (S)	2 (S)	1 (S)	>128 (R)	2 (S)	1 (S)	4 (S)	16 (S)	≤0.12 (S)
BC6	8 (S)	16 (S)	32 (S)	16 (S)	>64 (R)	0.12 (S)	0.5 (S)	2 (S)	2 (S)	0.5 (S)	>128 (R)	2 (S)	2 (S)	16 (S)	8 (S)	0.5 (S)
BC50	2 (S)	8 (S)	32 (S)	2 (S)	2 (S)	0.12 (S)	≤0.03 (S)	4 (S)	2 (S)	1 (S)	32 (S)	1 (S)	2 (S)	32 (S)	8 (S)	≤0.12 (S)
BE2	2 (S)	16 (S)	16 (S)	4 (S)	4 (S)	0.12 (S)	≤0.03 (S)	4 (S)	4 (S)	1 (S)	>128 (R)	1 (S)	2 (S)	>64 (R)	16 (S)	≤0.12 (S)
BE16	4 (S)	32 (S)	64 (S)	8 (S)	4 (S)	0.12 (S)	≤0.03 (S)	4 (S)	8 (S)	1 (S)	>128 (R)	2 (S)	4 (S)	>64 (R)	16 (S)	4 (S)
BC33	2 (S)	8 (S)	16 (S)	2 (S)	2 (S)	0.12 (S)	≤0.03 (S)	4 (S)	4 (S)	1 (S)	32 (S)	1 (S)	2 (S)	64 (S)	8 (S)	≤0.12 (S)
BC1	4 (S)	64 (S)	16 (S)	2 (S)	2 (S)	0.12 (S)	0.12 (S)	4 (S)	2 (S)	2 (S)	>128 (R)	1 (S)	2 (S)	8 (S)	16 (S)	0.5 (S)

Figure 2S. Phenotypic antibiotic resistance in LAB meat-borne strains

Table 1S. Salt resistance related genes in Whole Genome Sequenced strains

Genes related to a salt tolerance - osmoprotectant transport		**C21B**	**C22G**	**E13G**	**E28G**	**E23B**
1	glycine/betaine ABC transporter	FXV74_03700	FX991_04745	FX992_07275	FX990_03615	FX989_03440
	proline/glycine betaine ABC transporter permease	FXV74_03705	FX991_04750	FX992_07280	FX990_03620	FX989_03445
	glycine betaine/L-proline ABC transporter ATP-binding protein	FXV74_03710	FX991_04755	FX992_07285	FX990_03625	FX989_03450
2	betaine/proline/choline family ABC transporter ATP-binding protein	FXV74_02030	FX991_06710	FX992_03135	FX990_01810	FX989_02415
	ABC transporter permease	FXV74_02035	FX991_06715	FX992_03130	FX990_01815	FX989_02420
	osmoprotectant ABC transporter substrate-binding protein	FXV74_02040	FX991_06720	FX992_03125	FX990_01820	FX989_02425
	ABC transporter permease	FXV74_02045	FX991_06725	FX992_03120	FX990_01825	FX989_02430
3	glycine/betaine ABC transporter	FXV74_07140	FX991_07905	FX992_08350	FX990_06985	FX989_06880
4	amino acid ABC transporter permease	FXV74_01545	FX991_07905	FX992_01955	FX990_06985	FX989_01545

Genes related to a salt tolerance - N+/H+antiporters	**C21B**	**C22G**	**E13G**	**E28G**	**E23B**
sodium:proton antiporter		FX991_03405; FX991_08035	FX992_04270; FX992_05735; FX992_08220;	FX990_00425; FX990_06315; FX990_07510;	FX989_04520; FX989_07010; FX989_08410;
cation:proton antiporter	FXV74_03460; FXV74_04780; FXV74_07270; FXV74_08670; FXV74_09540;	FX991_04790	FX992_07320;	FX990_03580; FX990_03875;	FX989_03200; FX989_09150
Na+/H+ antiporter NhaC	FXV74_03965	FX991_07495	FX992_09485	FX990_04365	FX989_03705

CHAPTER 4

Role of biopreservation in a nitrite-reduced fermented salami: microbial impact on *Clostridium* spp. outgrowth control and on salami quality attributes

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Manuscript to be submitted

Abstract

The biopreservation approach, that considers a performant microbial cultures utilization, is a promising approach for a nitrite-reduced fermented salami production. Nitrates amount of 150 mg/kg is the maximal allowed amount in foods, currently regulated in the European countries due to their health safety concerns. In the present study we investigated the impact on the *Clostridium* spp. outgrowth control as well as on the fermented salami quality related aspect, of three nitrite concentrations: 0 mg/kg, 30 mg/kg and 75 mg/kg of $NaNO_2$, fermented spontaneously or with bioprotective/starter cultures addition. The combined approach that considers the bioprotective/starters used in the present study, together with 30 mg/kg is a promising approach in *Clostridium* spp. outgrowth control. Also, the cultures addition improved the product proteolysis as well as the proteins digestibility in an *in-vitro* gastro-intestinal digestion model.

1. Introduction

Intoxications and infections caused upon the consumption of contaminated meat products by clostridia are rare but severe and potentially fatal (Carter and Peck, 2015). *Clostridium* spp. spores occurs in soil, dust and in the animal's intestinal tract, and may eventually contaminate meat during slaughter or its processing (Keto-Timonen et al., 2012). The low-oxygen conditions stimulate spores germination and microbial proliferation, resulting in a neurotoxins release in the foods (Carter and Peck, 2015). Meat related *Clostridium* infections outbreaks are generally caused by homemade meat products whereas rarely are ascribed to commercial meat products (Keto-Timonen et al., 2012; Carter and Peck, 2015).

Raw meat is a great substrate for microbial growth due to its abundance of proteins, fats and fermentable glycogen, that together with the high moisture content may affect the microbiota of fermented meat products (Holck et al., 2017). The microbial composition of fermented meat products such as salami has a great impact on the products quality and safety. A wide variety of fermented salami is owed to the unique interactions between the primary material, microbiota composition and the processing conditions (Leroy et al., 2013; Tabanelli et al, 2012). Considering the perishable nature of fermented meat, different hurdle strategies are adopted. Lowering of the hurdle parameters as pH, water activity and the weight loss, is directly impacted by the meat-borne microbial proliferation and drying process. To control the meat microbiota composition, starter cultures are commonly used. *Lactobacillus* is mostly used genus for this purpose, due to its remarkable metabolic performances, where the lactic acid production from sugars is among them (Franciosa et al., 2018). The initial high water activity of the meat batter allows microbial proliferation, whereas the later a_w lowering by drying, is contributing for their inhibition as well as for the products hygienic stability (Leroy et al., 2006, Toldrà, 2010).

Curing with nitrites is an important hurdle barrier adopted in the salami production to prevent pathogen proliferation (Majou and Christieans, 2018, Alahakoon et al., 2015). This additive, besides its contribution to the flavour development, the red meat colour, and antioxidant activity that inhibits the lipids rancidity, is the most efficient preservative for the *Clostridium* spp. outgrowth control (Hospital et al., 2016). It was reported that the anti-clostridial activity increases with the increase of the added nitrite amount rather than with the residual one (Sindelar and Milkowski, 2012). Nitrites concentration to be added in meat products, is regulated due to the health safety concern of nitrosamine formation in the meat (IARC, 2010; EFSA, 2017a). The amount of 150 mg/kg of meat is the allowed amount in the majority of the EU countries (European Commission, 2018; EFSA, 2017a), whereas a more stringent restriction is in force in Denmark (100 mg/kg) as a maximal ingoing amount in fermented meats. However, EFSA, in the last re-evaluation report on nitrites safety, concluded that the maximal amount of 150 mg/kg, added in the meat products, is within the safety limits (EFSA, 2017a). However, the conclusion drawn by the International Agency for Research on Cancer (IARC) on the toxicity of nitrosamine was that 'under conditions that result in endogenous nitrosation, ingested nitrate or nitrite is probably carcinogenic to humans (Group 2A)' (IARC, 2010).

Therefore, there is a general concern about the addition of nitrite addition in meat and the residual nitrite concentration in the product. This concern is also supported by the continuous consumers demand for chemical free products.

A recent study investigated the impact of six starters on the residual nitrite content, concluding that when sausages were added with *Pediococcus pentosaceus* and *Staphylococcus carnosus* starter cultures, lowest residual nitrite amount was observed. In addition, significant lowering of the residual nitrite was observed in the gastric *in-vitro* phase, that was further lowered by *E. coli* and/or *L. casei* in the large intestine (Kim and Hur, 2018).

Over the last years, different studies were focused on a potential strategies of nitrite reduction. A biopreservation with *Lactobacillus* strains was recently described as a promising approach for *Clostridium* spp. outgrowth control *in vitro* and in *in carnis* model as well as against *Listeria monocytogenes* in a sausage model (Di Gioia et al., 2016, Nikodinoska et al., 2019). Furthermore, several studies have reported the 75 mg/kg as efficient amount for the control in a dry-fermented sausages model (Nikodinoska et al., 2019) as well as against *C. botulinum* in meats products (Keto-Timonen et al., 2012). Literature also reports that different amounts of nitrites are required for a specific quality related purpose (Wójciak et al., 2019). In particular, around 25 mg/kg is required for the typical cured meat colour (Sindelar and Milkowski, 2012) whereas 50 mg/kg for the cured flavour and oxidative stability (Toldrà et al., 2010).

The present study aims at understanding the effectiveness of a new developed bioprotective/starters cultures cocktail against *Clostridium* spp. outgrowth control in a fermented salami, without or added with different nitrites levels (i.e. 30 mg/kg and 75 mg/kg of $NaNO_2$). Along with the safety impact of the addition of microbial cultures and nitrites, the impact on the product quality, proteolysis, the degree of hydrolysis as well as on the proteins *in-vitro* digestion was also investigated. The obtained outcomes will contribute in understanding the anti-clostridia activity of the present bioprotective/starter cocktail and to what extent is possible to reduce the nitrites level in a fermented salami.

2. Material and methods

2.1 Bacteria preparation

2.1.1 Starter cultures: *Lactobacillus* spp. and *Staphylococcus* spp.

Starter cultures cocktail was prepared using two *Lactobacillus* strains, *L. plantarum* PCS20 and *L. sakei* E13G, and two commercially available *Staphylococcus* strains: *S. carnosus* and *S. xylosus* (Salum30, Probiotec, Italy). *L. plantarum* PCS20 possesses anticlostridial activity in a

meat-based model and antilisterial activity in salami prepared with a low nitrite amount (Di Gioia et al. 2016, Nikodinoska et al. 2019). *L. sakei* E13G is an indigenous meat-borne isolate, selected from an artisanal "Salame Romagnolo", and chosen due to its desirable techno-functional properties, as a short lag phase and high acidification rates in a meat-simulated environment.

Lactobacillus strains were separately grown at 30°C in 10 mL MRS broth (VWR, Radnor PA, USA). Bacterial cells, harvested after overnight growth, were washed twice with 0.9% (w/v) sterile physiological solution and finally resuspended in 10mL distilled H_2O (dH_2O) at a concentration of 10^9 cfu/mL. Regarding staphylococci inoculation, an aliquot of lyophilized mixed cultures was resuspended in a 0.9% sterile physiological solution with a final concentration of 10^9 cell/ml, stored overnight at 4°C, as recommended by the manufacturer. An aliquot of lactobacilli and staphylococci suspension containing 10^9 cfu/mL was resuspended in 150 mL sterile dH_2O, obtaining a final concentration of 10^6 CFU/g in the meat batter. Batches not inoculated with starter cultures were treated with only 150 mL sterile dH_2O.

2.1.2 *Clostridium* spore production and purification quantification

Clostridium sporogenes is a non-toxic Group I *C. botulinum* equivalent, commonly used as a surrogate organism due to its similar metabolic requirements (Carter and Peck, 2015). Sporulation of *Clostridium sporogenes* DSM532 strain was obtained according Yang et al. (2009) with some modifications. Briefly, 1 mL of actively growing *C. sporogenes* in TSB media (Oxoid, UK) was transferred in 75 mL of sporulation medium containing 3% trypticase peptone, 1% peptone and 1% $(NH4)_2SO4$. The inoculated medium was placed in a jar under anaerobic conditions generated with Anaerocult (Merck Millipore, USA) and incubated at a suboptimal temperature of 30°C for one week. The spore suspension was centrifuged at 1413g for 10 min and the obtained pellet washed twice with sterile distilled water and resuspended in a Phosphate Buffer Solution. Ultrasonication of 5 min was performed, to enhance spore release

from the cells, and centrifuged subsequently. After washing the pellet twice with dH_2O, the final re-suspension in dH_2O was considered as a stock solution and was stored at 4°C. The final spore purification step was performed each time before the experiment was performed. For this, 5 mL of the stock solution were washed 3 times with dH_2O at 1413g for 5 min. The pellet was resuspended in an ethanol:dH_2O 1:1 (v/v) solution and incubated for 1h and 30min at 4°C. Afterwards, the pellet was washed 3 times as previously described and resuspended in 2mL dH_2O. Spore suspension quality was checked by optical microscope. Also, the viable *Clostridium* counts were determined by plating 10-fold dilutions in RCA agar and incubated anaerobically at 37°C.

2. 2 Study design

The "Salame Cacciatore" prototype was prepared according to the traditional recipe and the ingredients were purchased from a local butcher. Meat for salami production consisted of 50% pork shoulder, 30% coppa (mixture of upper pork neck and pork shoulder) and 20% of pork fat. NaCl (2%) and dextrose (0.5%) were also added to the batter.

As shown in Figure 1, four meat batters were prepared: 1. Blank (B), without starters; 2. Starter (S), inoculated with starters; 3. *Clostridium* (C), inoculated with *C. sporogenes* (CS) and 4. Challenge (C), inoculated both with starters and *C. sporogenes*.

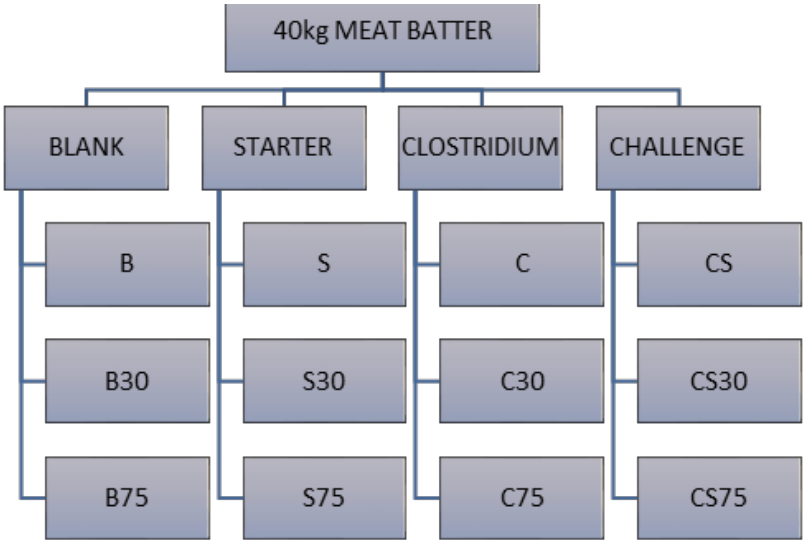


Figure 5. Schematic representation of the batches composition for the Challenge test and prototype fermented salami development

Each of the four batters was divided in three batches, with different amount of $NaNO_2$: a) no nitrites (B, S, C and CS); b) 30 mg/kg $NaNO_2$ (B30, S30, C30 and CS30); c) 75 mg/kg $NaNO_2$ (B75, S75, C75 and CS75) (Figure 1). After mixing the ingredients, the meat batter was homogenised and then placed in a horizontal manual salami filler machine. Natural swine casings, with a diameter of 40 mm, were used for each salami preparation. All steps of the salami trial were monitored by an experienced craft salami manufacturer. Three salami for each of the 12 treatments and for each sampling point were prepared.

The salami ripening process was performed in a temperature and humidity (%RH) controlled chamber, under the following processing conditions:

- Fermentation, 23°C for 48 h (Day 0-2) – RH <75%;

- Ripening, 15°C for 20/22 days (Day 3-60) – RH 85-90%

Temperature (°C) and humidity were recorded every 15 min during the entire experiment with a datalogger.

Samplings were performed at a Time 0 (before placing the homogenized meat batter in the filling machine), Time 2 (after 48h when the fermentation was finished); Time 3 (after 10 days) and Time 4 (after 60 days corresponding to the end of the ripening process). For each of the 12 different treatments (Table 1), 3 salami for each sampling point were analysed. The initial weight of each salami was recorded before their placement in the chamber.

Table 1. Microbial cultures and nitrites amount, added to Challenge test and prototype fermented salami batches

Treatment	Starter cocktail suspension (log cfu/g)	*Clostridium sporogenes* (log cfu/g)*	Nitrite ($NaNO_2$)
B	-	-	-
B30	-	-	30 mg/kg
B75	-	-	75 mg/kg
S	10^6	-	-
S30	10^6	-	30 mg/kg
S75	10^6	-	75 mg/kg
C	-	10^4	-
C30	-	10^4	30 mg/kg
C75	-	10^4	75 mg/kg
CS	10^6	10^4	-
CS30	10^6	10^4	30 mg/kg
CS75	10^6	10^4	75 mg/kg

* the cells number corresponds to the number of viable spores count, determined *in vitro* before the trial

2. 3 DNA extraction and qPCR microbial quantification

Quantification of total *Lactobacillus* spp. and *Clostridium* spp. (Group I) counts was performed with qPCR, for each salami biological replicate, per each treatment (n=3). Sampling was performed from different parts of the salami and the meat was homogenized. 250 mg were weighted for each sample in Eppendorf tubes, the weight was recorded and samples were stored at -80°C. DNeasy PowerSoil Kit (Qiagen) was used for DNA extraction, following manufacturer's instructions. Modifications were introduced in the first step, with the transferring of the PowerBeads to already weighted meat samples. Also, after the homogenization step in a bead beater, samples were centrifuged for 5 min. The centrifugation for 1 min and speed 21.952g was used for all further steps. Extracted DNA concentration and its purity were determined (NanoQuant, Tecan, Switzerland) and all samples were diluted to

5ng/µl. *Lactobcillus* spp. quantification was performed as described Di Gioia et al., 2016. Group I *Clostridium* spp. quantification was performed using CI-F1 and CI-R2 primers (Song et al., 2004). The enzymatic reaction was performed in 20µl final volume, using Fast SYBR® Green Master Mix chemistry (Applied Biosystems, USA), primers at a concentration of 0.2mM each and 2µl DNA obtained from the salami samples. Standard curve was created using 16S rRNA PCR product of *C. sporogenes* DSM532 as a result of serial dilutions of pathogens PCR product, using 10^2, 10^3, 10^4, 10^5, 10^6 for the qPCR reaction. The same reactions were performed for each run. Quantification was performed using StepOne Real-Time PCR Systems (Applied Biosystems) with a program as described Di Gioia et al., 2016, with a modification for *Clostridium* spp. annealing temperature, being 60°C for 30s. The obtained data were transformed to log cfu/g meat, considering the 16S rRNA copy number of each quantified genus (https://rrndb.umms.med.umich.edu).

2. 4 Preparation of DNA Libraries for Illumina MiSeq Sequencing and NGS data analysis

DNA samples were subjected to Illumina sequencing. The V3-V4 region of the 16S rRNA gene was amplified and sequenced. One sample was excluded as it did not pass the established quality threshold. The amplicons, approximately 460 bp in length, were generated using the forward and reverse primers, respectively: 5'-CCTACGGGNBGCASCAG-3' and 5'-GACTACNVGGGTATCAATCC-3' (Gaggia et al., 2015). The assays were performed using a previously published protocol with some modifications (Quagliariello et al., 2016). The sequencing process was outsourced at Macrogen Inc. (Next Generation Sequencing Division), Seul, Republic of Korea, using a 2x300 pair-end protocol.

Resulting 300 bp paired-end reads were assembled using QIIME tools (Caporaso et al., 2016) with the script "joined_paired_ends.py". Further sequence read processing was performed using QIIME ver. 1.9.1 (Caporaso et al., 2010) and ChimeraSlayer (Haas et al., 2017), including quality filtering based on a quality score of >25 and removal of mismatched barcodes

and sequences below length thresholds. Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH version 7 (Haas et al., 2011). OTU sequences were aligned using PyNAST (Edgar et al., 2010) and taxonomy assignment was determined using the SILVA SSU Ref database release 111 (Caporaso et al., 2010). Biodiversity indices analysis was performed using QIIME tools, in particular the script "core_diversity_analysis.py"; the phylogenetic classification of OTUs was carried out with the script "make_phylogeny.py" (fasttree). α-diversity was evaluated considering Chao, Observed OTU and PD whole tree metrics; β–diversity was evaluated using 'weighted_unifrac' method (Quast et al., 2013). Taxonomic analysis at family and genus level were manually annotated for bar charts and boxplot data presentation. OTUs accounting below 0.05 relative abundance have been grouped in "other".

2. 5 pH determination

pH was measured in all biological replicates per treatment (n=3) and with a pin electrode of a pHmeter inserted, randomly, three times into each sample.

2. 6 Water activity (a_w)

AquaLab (Aqualab CX3-TE (Labo-Scientifica, Parma, Italy) was used for the water activity (a_w) determination in the same replicates as previously described. Before measurements, the calibration was performed with standard solutions of known a_w.

2. 7 Weight loss

The weight of all salami samples was recorded at the beginning of the trial and at each sampling point. For this parameter determination the following formula was used:

$$\text{Weight loss (\%)} = \frac{\text{(Initial weight (kg) − weight after ripening(kg))}}{\text{Initial weight (kg)}} \times 100$$

2. 8 Protein content analysis

This analysis was outsourced at the Massey University, Palmerston North, New Zealand, what % of crude protein content was analysed according to AOAC 968.06 (Dumas method).

2. 9 Visual colour analysis of the salami samples

Triplicate of each salami treatment, was visually analysed for the colour differences.

2. 10 Degree of Hydrolysis (%DH) determination: Peptide extraction and OPA assay

Peptide extraction from the salami samples was performed in order to perform the OPA assay. For this purpose, 1g salami sample was homogenise with Ultraturex in 4mL 0.25% acetic acid and subsequently centrifuged at 20.000g at 4°C for 30 min. The obtained supernatant (~5 ml) was filtered through glass wool and collected in an Eppendorf tube (~1.5-2 ml). The obtained sample was diluted in 0.25% acetic acid and the % of Degree of Hydrolysis determination was performed as described by Nielsen et al., 2001.

2. 11 *In vitro* digestion of salami prototypes

2.11.1 Digestion fluids preparation

In vitro digestion study was aimed to understand the impact of starters and nitrite level of salami digestibility. Three sub-samples of each treatment were submitted to the *in*-vitro digestion. The *in-vitro* digestion static model, comprising simulated mouth, gastric and small intestine digestion was used. Compositions of the simulated juices of simulated saliva fluid (SSF), simulated gastric fluid (SGF) and simulated intestine fluid (SIF) are described in the Table 2.

Table 2. Composition of 1.25x electrolyte stock digestion fluids (SSF, SGF and SIF) is described in the table. Distilled water was used for adjustment to 400mL final volume. All the stock solutions were stored at 4°C. The addition of Ca^{2+} corresponds to the correct final concentration in the digestion mixture of simulated fluid and food.

	Digestion phase (concentration in the fluid in mmol L^{-1})		
Constituent	**SSF**	**SGF**	**SIF**
KCl	15.1	6.9	6.8
KH_2PO_4	3.7	0.9	0.8
$NaHCO_3$	13.6	25	85
NaCl	-	47.2	38.4
$MgCl2(H_2O)_6$	0.15	0.1	0.33
$(NH_4)_2CO_3$	0.06	0.5	-
$CaCl_2(H_2O)_2$	1.5(0.75*)	0.15(0.075*)	0.6(0.3*)
pH adjustment with:			
HCl (1 mol L^{-1})	pH 7	pH 2	-
NaOH (6 mol L^{-1})	-	-	pH 7

*Ca^{2+} concentration in the final digestion mixture in the gastric and intestinal phase

Enzyme solutions were prepared before their addition in the simulated digestion step. Pepsin 3200-4500 U/mg protein, (P6887, Sigma) was resuspending in SGF whereas Pancreatin (P8096, Sigma) in SGF.

2.11.2 Procedure and sampling points

All digestion phases were performed by incubation at 37°C in a shaking water bath. The digestive fluids were pre-warmed to 37°C. Freeze-dried salami samples were weighted, to approximate amount of 877mg protein, into a Schott bottle. 5mL of dH_2O was mixed with the sample, to rehydrate, plus 5mL of SSF phase. Samples were homogenized with Ultraturex.

a) Mouth phase: Schott bottles were incubated for 10 min. The first sampling was performed before starting the gastric phase, sample 0min.
b) Gastric phase: 7.5 mL of simulated gastric fluid plus 1.6 ml of 1.969 mg/ml pepsin solution were added to all samples when finished the mouth phase. Samples were incubated for 2h. Samplings were performed after 10min and 120min from the beginning of the gastric digestion.

c) Small intestine phase: the gastric chime was added with 11ml simulated intestinal fluid plus 5ml of 1.6mg/ml pancreatin (Acros, enzyme/substrate w/w in SIF). The samples were incubated for 2h and samplings performed at the end of the digestion, 240min.

2. 12 Samples treatment for SDS-PAGE

200µL were sampled for each sampling point (0 min, 10 min, 120min and 240min) for SDS-PAGE analysis. All samples were mixed by vortexing with 2x Sampling loading buffer. 2.5µL 6M NaOH were added to the gastric phase samples (10 min and 120min) and 2.5µL HCl to ones from the intestinal phase. All samples were incubated for 5min in a 95°C water bath followed by sonication for 10 min. Subsequently, dilutions were performed with to with 1X Sampling Buffer in order to be loaded in the gel 30µg proteins, based on the estimated concentration introduced into the digestion model. Diluted samples were centrifuged for 2min at 3000g before loading into ready Novex NuPAGE 10% Bis-Tris midi gels (#WG1202). NuPAGE MES SDS (#B0002) was used as running buffer with NuPAGE antioxidant (#NP0005) added to the upper chamber. After running the gels at 200 V for 35–40 min, staining in Coomassie Blue G-250 (Invitrogen #LC6060) overnight was performed. SDS PAGE gels were scanned with a CS-900 densitometer and the bands intensity analysed by Image Lab software (Bio-Rad).

2. 13 Statistic analysis

Significant effects of starters and nitrite addition on different parameters and their interactions were assessed by multi-factor analysis of variance (ANOVA). One-way ANOVA was also performed separately for each of the physio-chemical parameters. Differences among means were tested by Fisher's Least Significant Difference (LSD) (significance of $p<0.05$ was assumed where not indicated in the text). Statistica 8.0 (StatSoftInc., USA) was used and the

data are presented as mean value ± standard error. The relative digestibility and the proteolysis of the salami samples, was calculates as reported by Farouk et al. (2019). Briefly, relative digestibility was calculated by summing the line density above >10 kDa, and further normalizing by subtracting the summed density of line corresponding to the timepoint 0 (prior the digestion). Also, the same approach was used for the analysis of the treatments impact of the salami proteolysis. In this case, the time point 0 was considered as the end of fermentation, thus the normalized relative line densities were compared between treatments. Analysis of variance (ANOVA) was performed for the obtained data interpretation of n=3 biological replicates, using the above indicated software.

3. Results

3. 1 Challenge test results

3.1.1 *Clostridium* spp. growth in the salami at different nitrite concentrations (0, 30 and 75 mg/kg) with or without starters addition

Clostridium spp. growth in the presence of nitrite and bioprotective/starter cultures, observed at different time points, is shown in Figure 2. The initial *Clostridium* inoculum level was not significantly different among all the batches, being 3.97-4.49 4 log cfu/g. Considering the end of the fermentation (T2), an overall lower *Clostridium* counts were observed in batches added with starters (CS).

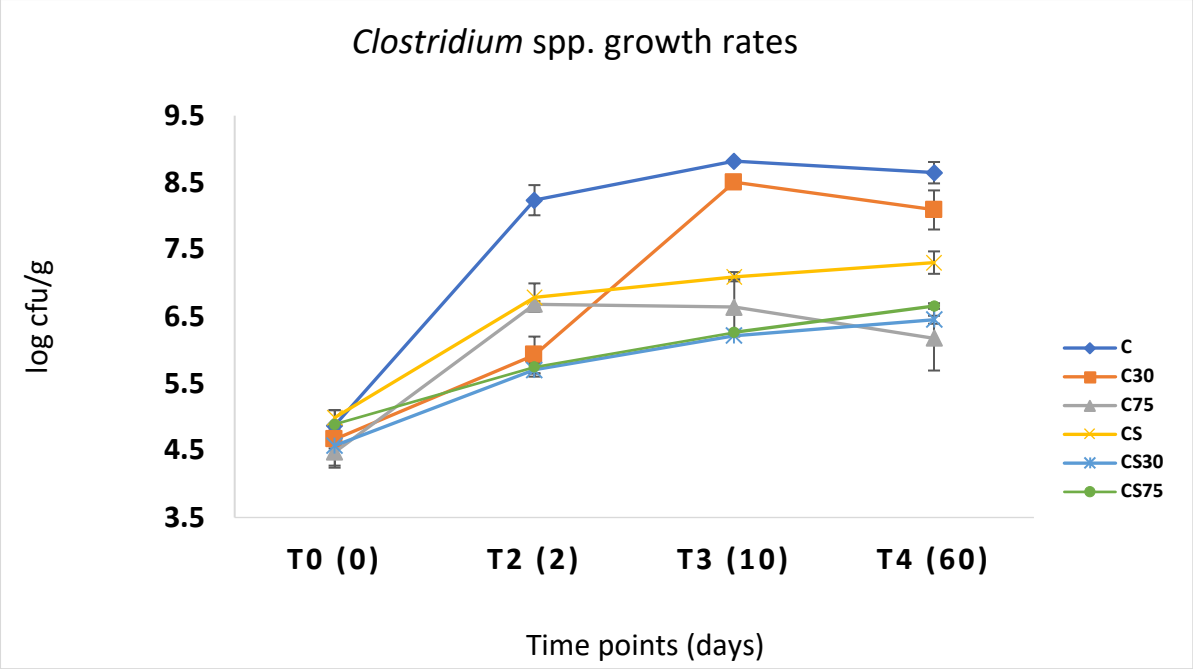


Figure 2. Effect of the bioprotective/starter cultures and different nitrites concentration addition on the *Clostridium* spp. growth. The mean and the standard error values are reported. Letters indicates the addition of microbial strains: only **C**=*C. sporogenes*, **CS**=*C. sporogenes* + bioprotective/starter cultures; numbers indicate the **$NaNO_2$ concentration: 0 mg/kg, 30 mg/kg, 75 mg/kg.**

Table 3. The *Clostridium* spp. growth in Clostridium and Challenge fermented salami batch at different time points.

Timepoint	Batch					
	Clostridium			Challenge		
	C	C30	C75	CS	CS30	CS75
T0	4.37 ± 0.03^{aA}	4.17 ± 0.14^{aA}	3.97 ± 0.23^{aA}	4.49 ± 0.11^{aA}	4.07 ± 0.30^{aA}	4.39 ± 0.21^{aA}
T2	7.74 ± 0.23^{dB}	5.43 ± 0.27^{bB}	6.18 ± 0.05^{cB}	6.28 ± 0.21^{cB}	5.21 ± 0.10^{abB}	5.25 ± 0.10^{abB}
T3	8.32 ± 0.01^{bC}	8.01 ± 0.09^{bD}	6.14 ± 0.42^{aB}	6.60 ± 0.07^{aB}	5.72 ± 0.01^{aBC}	5.77 ± 0.04^{aBC}
T4	8.15 ± 0.16^{cBC}	7.59 ± 0.29^{bcCD}	5.68 ± 0.49^{aB}	6.81 ± 0.17^{bB}	5.95 ± 0.06^{abC}	6.16 ± 0.04^{abC}

The mean and the standard error values are reported in the table. Nitrite, time point and treatment; nitrite x time point; nitrite x treatment; treatment x timepoint interactions are significant according to multifactorial ANOVA. $F_{(2, 72)}=40.97$, $p<0.0001$ (for nitrite factor); $F_{(3, 72)}=120.26$, $p<0.0001$ (for time factor); $F_{(1, 72)}=49.32$, $p<0.0001$ (for treatment factor); $F_{(6, 72)}=5.6$, $p<0.05$ (nitrite x time); $F_{(2, 72)}=9.17$, $p<0.05$ (nitrite x treatment); $F_{(3, 72)}=9.55$, $p<0.0001$ (time x treatment); $F_{(6, 72)}=3.47$, $p<0.05$ (nitrite x time x treatment). Letters indicates the addition of microbial strains: only **C**=*C. sporogenes*, **CS**=*C. sporogenes* + bioprotective/starter cultures; numbers indicate the **$NaNO_2$ concentration: 0 mg/kg, 30 mg/kg, 75 mg/kg**. Values followed by different small letter (ad) in the same row and different capital letter (AD) in the same column indicates significant differences according to LSD test.

In particular, in the absence of nitrites, significant lower counts were obtained in the CS batch compared to the batch without starter culture (C), at T2. The pathogen growth rates were not

significantly changed in the batch CS, until the end of the ripening (T3 and T4), whereas a significant increase was observed in the batch C. It is, however, important to highlight the bioprotective cultures effectiveness that was observed at the end of the ripening (T4) when a significantly lower *Clostridium* counts (1.34 log cfu/g) was obtained in CS batch compared to the C batch. The addition of 30 mg/kg $NaNO_2$ efficiently controlled the clostridia outgrowth at the end of the fermentation (T2). In particular, clostridia count, ranging from 5.21-5.43, were not significantly different between batches C30, CS30 and CS75. These counts were about 1 log cfu/g lower than those in C75 and CS, and 2.31 log cfu/g lower than the counts in C. However, 30 mg/kg nitrite was not able to control the *Clostridium* outgrowth in the batches without starter cultures addition (C30), observing 2.58 and 2.16 log cfu/g significant increase ($p<0.05$) in T3 and T4, respectively, compared to T2. Contrarily, the cultures addition (CS30) enabled more successful clostridia outgrowth control until T3, while a significant increase of 0.75 log cfu/g was observed in the same batch at the end of the ripening (T4), compared to T2 and T3. However, clostridia counts were 2 log cfu/g lower in CS30 batch compared to C30, whereas the same counts were not significantly different when higher nitrite amount was used (C75 and CS75), at T3 and T4. The addition of 75 mg/kg $NaNO_2$ significantly lowered the *Clostridium* spp., both in the batches with and without starters addition (CS75 and C75) at the end of the ripening process T4.

3.1.2 pH and a_W analysis in the Challenge test salami samples

The pH and the a_w trend in salami inoculated with *Clostridium sporogenes*, with or without bioprotective cultures addition and with different nitrite concentration is shown in the Figure 3 and Table 4, respectively. The initial pH in the meat batter, after the treatments, was in the range between 6-7 pH units, whereas the a_w was 0.97. The a_w data obtained from the multifactorial ANOVA analysis (Table 3) showed a significant impact when different nitrites concentrations were used, whereas the starters addition did not impact this parameter. Also,

significantly ($p<0.0001$) lower a_w was obtained at the T4<T3<T2, regardless starters or nitrite addition. However, exceptions were observed comparing different treatments, especially when nitrites were added. In particular, C30 and C75 at T2 showed a significantly lower a_w compared to C, whereas the CS75 was significantly lower than CS and CS30 at T3, but, contrarily, without starters, C75 showed significantly higher a_w compared to C and C30 at T3. At the end of the trial, all treatments in a clostridium batch showed significantly lower a_w compared to challenge batch. However, challenge batches added with nitrites (CS30 and CS75) showed significantly lower a_w compared to CS batch at T4.

Considering the pH rates, significantly lower pH, being 5.03-5.07, was observed in the challenge batches compared to 5.58-5.81 pH units in clostridium batches. at the end of the fermentation (T2). The addition of nitrites, did not significantly impacted the pH lowering in the challenge samples. Contrarily, the clostridia batches were impacted by both nitrites concentrations at T2, observed by the significantly higher pH in the batch C30 and C75 in comparison with the batch C. However, clostridium batches added with $NaNO_2$, showed significantly lower pH at T3 compared to the same treatments at T2. Considering the same time points comparison, the pH value of 5.02 was not significantly changed in the CS30, whereas significantly increased to 5.08 in the CS and significantly decreased to 5.05 in the CS75 batch. At the end of the ripening pH uniformly increased in all challenge batches, for a significant 0.3 pH units, compared to the T3. Also, the pH between 5.36-5.39 observed in the challenge batches, was significantly lower than the clostridium batches. In addition, the C75 was not significantly different from the CS30 batch at T4.

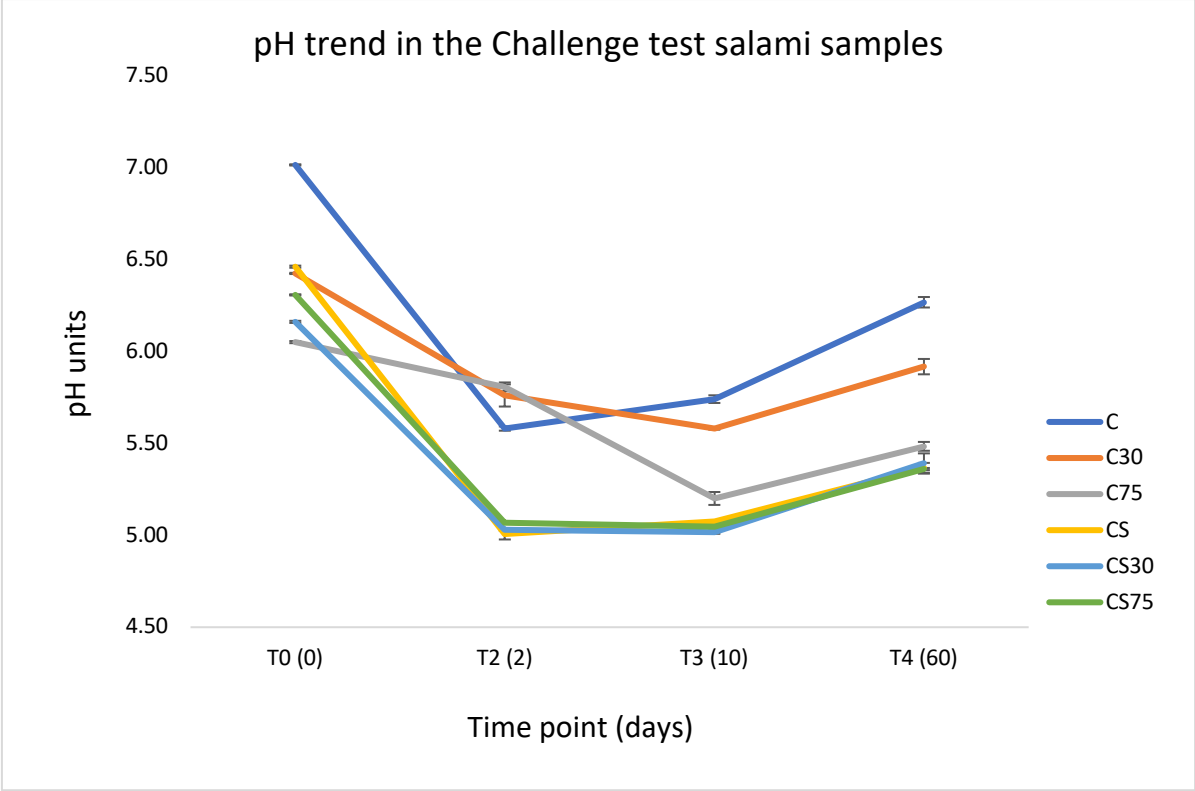


Figure 3. pH trend in salami inoculated with Clostridium sporogenes, with or without bioprotective cultures addition and with different nitrites concentrations. The mean and the standard error values are reported. Legend: letters indicates the addition of microbial strains: only **C**=*C. sporogenes*, **CS**=*C. sporogenes* + bioprotective/starter cultures; numbers indicate the **$NaNO_2$ concentration: 0 mg/kg, 30 mg/kg, 75 mg/kg**.

Table 4. The a_w trend in salami inoculated with *Clostridium sporogenes*, with or without bioprotective cultures addition and with different nitrites concentrations

Treatment	**Clostridium**			**Challenge**		
	$NaNO_2$ (mg/kg)			**$NaNO_2$ (mg/kg)**		
Timepoint (days)	**C**	**C30**	**C75**	**CS**	**CS30**	**CS75**
T2 (2)	0.971 ± 0.00^{Dc}	0.967 ± 0.00^{Cc}	0.964 ± 0.00^{ABCb}	0.961 ± 0.00^{Ac}	0.963 ± 0.00^{ABb}	0.965 ± 0.00^{BCc}
T3 (10)	0.961 ± 0.00^{ABb}	0.961 ± 0.00^{ABb}	0.963 ± 0.00^{Cb}	0.965 ± 0.00^{Cb}	0.963 ± 0.00^{BCb}	0.960 ± 0.00^{Ab}
T4 (60)	0.923 ± 0.00^{ABa}	0.920 ± 0.00^{Aa}	0.925 ± 0.00^{ABCa}	0.932 ± 0.00^{Da}	0.926 ± 0.00^{BCa}	0.929 ± 0.00^{CDa}

The mean and the standard error values are reported. Nitrite, time point and treatment; nitrite x time point; nitrite x treatment; treatment x timepoint interactions are significant according to multifactorial ANOVA. $F_{(2, 54)}=5.25$, $p<0.05$ (for nitrite factor); $F_{(2,54)}=1733.94$, $p<0.0001$ (for time factor); $F_{(1, 54)}=2.15$, $p>0.05$ (for treatment factor); $F_{(4, 54)}=1.49$, $p>0.05$ (nitrite x time); $F_{(2,54)}=0.21$, $p>0.05$ (nitrite x treatment); $F_{(2, 54)}=23.25$, $p<0.0001$ (time x treatment); $F_{(4,54)}=7.18$, $p<0.05$ (nitrite x time x treatment). Legend: letters indicates the addition of microbial strains: only **C**=*C. sporogenes*, **CS**=*C. sporogenes* + bioprotective/starter cultures; numbers indicate the **$NaNO_2$ concentration: 0 mg/kg, 30 mg/kg, 75 mg/kg**. Values followed by different small letter (ac) in the same column and different capital letter (AD) in the same row indicate significant differences according to LSD test.

3. 2 *Lactobacillus* spp. counts in the challenge test and in a prototype fermented salami

The *Lactobacillus* spp. growth under different conditions is shown in Table 5. After 48h of the fermentation process (T2), a significantly higher 2 log cfu/g counts were observed in all batches, compared to ones at the beginning of the trial (T0).

Observing all treatments per a single $NaNO_2$ concentration (0, 30 or 75 mg/kg), at a single sampling point (T0, T2, T3 or T4), *Lactobacillus* spp. counts were significantly higher in batches added with starters (S and CS) respectively the ones without starters addition (B, C). The same differences were not observed between batches added with starters, S vs CS. However, a single exception was observed comparing all treatments at T3: regardless the starters addition and when 75 mg/kg $NaNO_2$ were added, non-significant differences were obtained. At the T2 and T3, the lactobacilli growth rates were not significantly impacted by nitrites addition in the CS and S batches, with the exception of the S75 batch at the T2, where the counts were significantly lower than in the batches S. However, the *Lactobacillus* counts were not significantly impacted by nitrite addition at the end of the ripening (T4) when only starter was added (S).

In batches without starters addition (B), significantly higher lactobacilli counts were observed at time point T3 and T4 with respect to T2. *Lactobacillus* increase was significantly higher when 30 and 75 mg/kg nitrites were added with respect to no nitrite added.

A similar impact of the nitrites addition was observed also in the batches where *C. sporogenes* was inoculated. At the end of fermentation (T2), *Lactobacillus* spp. counts were significantly higher in treatments when 0 and 75 mg/kg $NaNO_2$ (C and C75) were added when compared with 30 mg/kg $NaNO_2$ addition (C30). At the end of the ripening, all level of nitrites were not found to have any impact on the lactobacilli growth.

Table 5. *Lactobaillus* spp. counts in the challenge and the prototype fermented salami trials

Timepoint (days)	**T0 (0)**			**T2 (2)**			**T3 (10)**			**T4 (60)**		
	$NaNO_2$ (mg/kg)			**$NaNO_2$ (mg/kg)**			**$NaNO_2$ (mg/kg)**			**$NaNO_2$ (mg/kg)**		
Treatment	**0**	**30**	**75**	**0**	**30**	**75**	**0**	**30**	**75**	**0**	**30**	**75**
C	3.88 ± 0.18^{aA}	$4.14\pm0.18^{a}_{A}$	5.15 ± 0.19^{bB}	7.36 ± 0.34^{deA}	6.70 ± 0.28^{cA}	7.82 ± 0.30^{efA}	7.24 ± 0.01^{cdA}	8.03 ± 0.26^{fA}	$8.21\pm0.26^{f}_{A}$	7.78 ± 0.14^{defB}	7.72 ± 0.18^{defA}	8.26 ± 0.27^{fA}
CS	6.34 ± 0.11^{aB}	$6.07\pm0.18^{a}_{B}$	$6.24\pm0.37^{a,C}$	8.53 ± 0.06^{bcdeB}	$8.62\pm0.07^{cde}_{B}$	8.20 ± 0.02^{bcB}	8.29 ± 0.06^{bcdeB}	8.35 ± 0.04^{bcdeB}	$8.11\pm0.02^{b}_{A}$	$8.24\pm0.11^{bcd}_{C}$	8.77 ± 0.24^{deB}	8.71 ± 0.20^{eB}
B	3.94 ± 0.22^{aA}	$4.04\pm0.22^{a}_{A}$	3.78 ± 0.22^{aA}	6.93 ± 0.10^{bcA}	6.70 ± 0.08^{bcA}	6.91 ± 0.26^{bcA}	7.31 ± 0.41^{cdA}	8.09 ± 0.40^{eA}	$8.22\pm0.55^{e}_{A}$	6.54 ± 0.15^{bA}	7.81 ± 0.38^{deA}	$8.49\pm0.52^{eA}_{B}$
S	6.48 ± 0.06^{aB}	$6.18\pm0.06^{a}_{B}$	6.38 ± 0.21^{aC}	8.73 ± 0.56^{cB}	8.31 ± 0.34^{bcB}	8.10 ± 0.18^{bB}	8.60 ± 0.08^{bcB}	8.47 ± 0.06^{bcB}	$8.12\pm0.02^{b}_{A}$	8.82 ± 0.07^{bcD}	8.56 ± 0.16^{bcB}	8.61 ± 0.13^{cB}

Nitrite, time point and treatment; nitrite x time point; nitrite x treatment; treatment x timepoint interactions are significant according to multifactorial ANOVA. $F_{(2, 108)}=6.23$, $p<0.05$ (for nitrate); $F_{(2, 108)}=17.04$, $p<0.0001$ (for time); $F_{(3, 108)}= 63.51$, $p<0.0001$ (for treatment); $F_{(4, 108)}=5.99$, $p<0.05$ (for nitrite x time); $F_{(6, 108)}=9.01$, $p<0.0001$ (for nitrite x treatment); $F_{(6, 108)}= 5.90$, $p<0.0001$ (for time x treatment); $F_{(12, 108)}=2.13$, $p<0.05$ (for nitrite x time x treatment). **C**=*C. sporogenes*, **CS**=*C. sporogenes*+bioprotective/starter cultures, **B**=no microorganisms added, **S**=bioprotective/starter cultures; numbers indicate the **$NaNO_2$ concentration: 0 mg/kg, 30 mg/kg or 75 mg/kg**. Values followed by different small letter (af) in the same row and different capital letter (AD) in the same column indicate significant differences according to LSD test.

3. 3 NGS data results

3.3.1 Metric analysis of NGS results

The α-diversity analyses of the bacterial communities in salami samples, in the presence of different nitrite levels and with or without bioprotective/starter cultures, were performed considering tree different metrics: the abundance-based indexes of species richness (Chao1), observed OTU and PD whole tree (Figure 4).

Based on the Chao1 and observed OTU estimators, the α-diversity showed the same trend at the end of the fermentation (T2) and the end of the ripening (T4) in *Clostridium* spp. inoculated salami. In particular, batches inoculated with starters showed significantly ($p<0.002$) lower indexes compared to the uninoculated batches, when lower nitrite levels were used (CS, CS30 vs C, C30). Contrarily, the addition of 75 mg/kg of $NaNO_2$ allowed to obtain the same Chao1 and observed OTU scores compared to batches inoculated with starters. The cultures addition brought to an almost identical Chao1 and observed OTU indexes reduction at all nitrite levels addition, both at T2 and T4. This reduction is due to starter addition and not to the nitrite level considering that there are not significant differences between nitrite concentrations (CS=CS30=CS75; T2 and T4). In uninoculated batches (C, C30, C75) a significant difference can be observed comparing low and high nitrite levels (C, C30 vs C75) confirming in this case the effect of 75 mg/kg nitrite addition in reducing the microbial diversity. This observation highlights the starters impact on the native microbial population reduction at the end of the fermentation and consequently at the end of the ripening. A similar scenario was also observed with the Phylogenetic Diversity (PD) whole tree estimator. In fact, the phylogenetic diversity in spontaneous fermented salami was significantly higher ($p<0.002$) respect to the fermentation driven by the added cultures, comparing CS vs C and CS30 vs C30 at T2. In addition, the PD estimator of C75 batch confirmed the same phylogenetic biodiversity of CS75. On the other

hand, the diversity of the PD whole tree index was not impacted by the nitrites level in the batches C and CS.

The Figure 4. shows also the microbial community β-diversity between salami samples treatments based on the weighted UniFrac estimator. Considering *Clostridium* treated batches, the different nitrite levels impacted the microbial community, contributing to the formation of defined clusters. However, the C75 cluster tends to group with the CS, CS30, CS75 clusters, confirming the α-diversity observations. In addition, nitrite levels did not impact on the β-diversity index within CS treatments, highlighting that cultures addition is the main contributor, shaping the matrix microbial ecology.

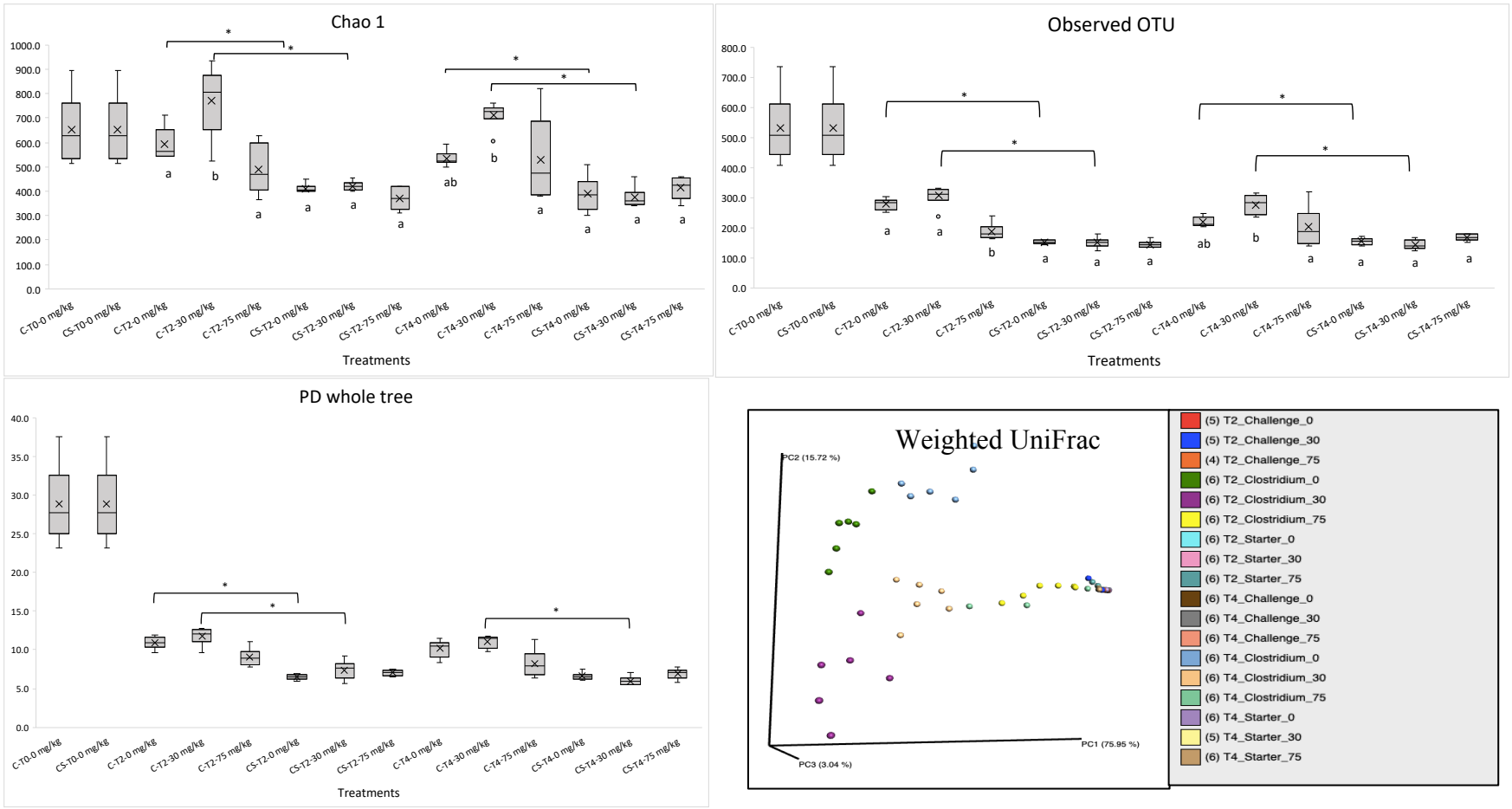


Figure 4. Box-plot shows the α-diversity and β-diversity indexes obtained from the NGS analysis on salami samples. The factors interactions are significant according to multifactorial ANOVA, using the Bonferroni correction for the 18 interactions considered. The letters indicate significant differences within the same batch (i.e. C vs C30 vs C75), whereas the asterisk represent significance between batches (i.e. C vs CS). The data are not significantly different where letters and asterisk are not used

3.3.2 Taxonomic analysis of the microbial diversity in fermented salami, inoculated with *Clostridium sporogenes*

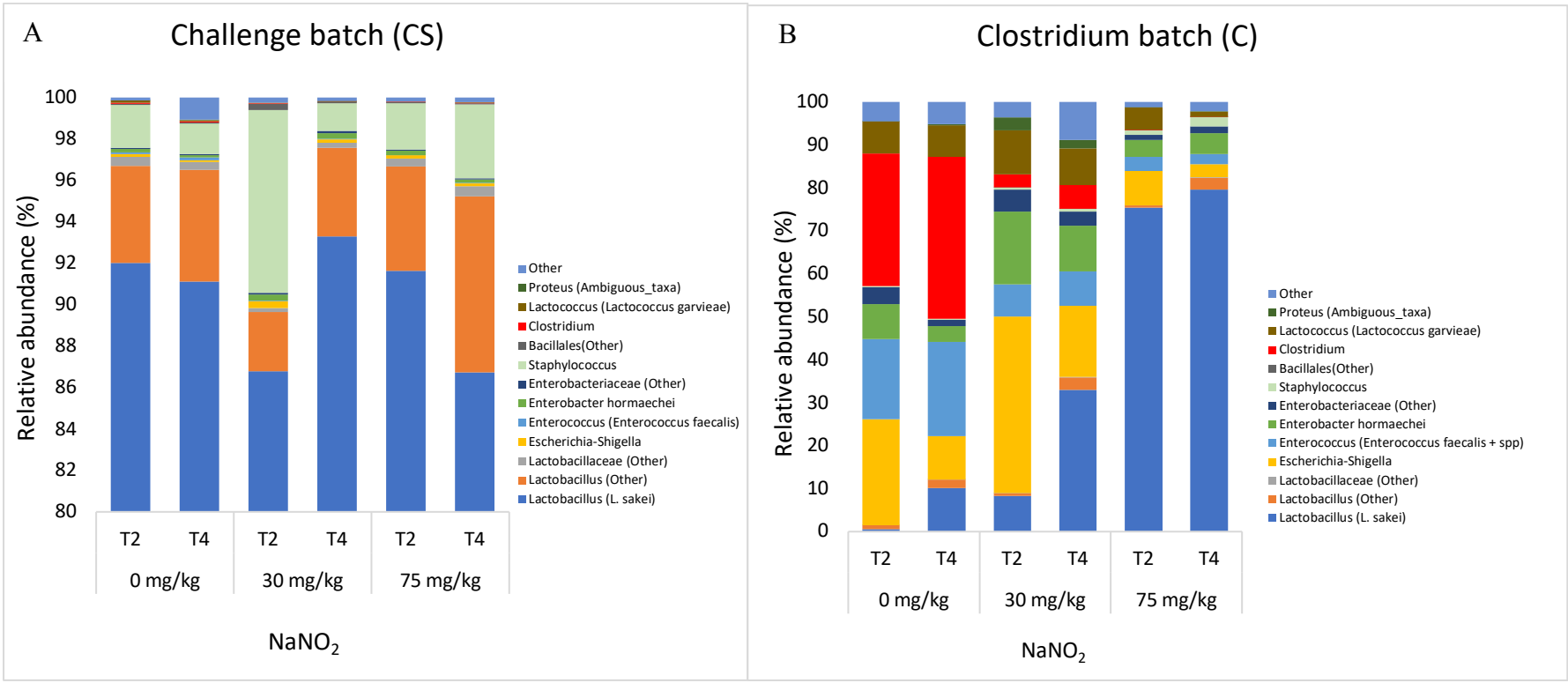


Figure 5. Genus relative abundances, expressed as a ratio between the sum of a single OTU reads and the total reads number, in salami samples inoculated with *C. sporogenes*, added with **(A)** or without bioprotective cultures **(B)** and nitrites at different level, at different time points.

High-throughput sequencing was used for the microbial diversity analysis in the salami prepared in different conditions. Figure 5 shows the microbial diversity observed in the salami samples, expressed as relative abundance (%) of microbial families and genera. For the present analysis, groups with a relative abundance higher than 5% were considered. Considering the major aim of the present study, i.e. the *Clostridium* spp. reduction using a bioprotective approach, the microbial diversity in the salami samples where this pathogen was inoculated were considered. The majority of the analysed OTUs, in the batches inoculated with the starter cultures, belonged to Lactobacillaceae and Staphylococcaceae at the family level and *Lactobacillus* and *Staphylococcus* at the genus level, regardless the amount of added nitrite. *Lactobacillus* and *Staphylococcus* account for 98% of the obtained reads in all the treatments of the challenge batches (CS) at T2 and T4; whereas in C batches the highest number of reads (75-80% of obtained reads) belonged to *Lactobacillus* only in C75 treatment, differently from C0 and C30, irrespectively of the timepoint. Possibly, the addition of 75 mg/Kg of nitrite, reducing OTUs and internal competition, favours the development of the autochtonous *Lactobacillus* population that reached high values as in batches inoculated with starter culture.

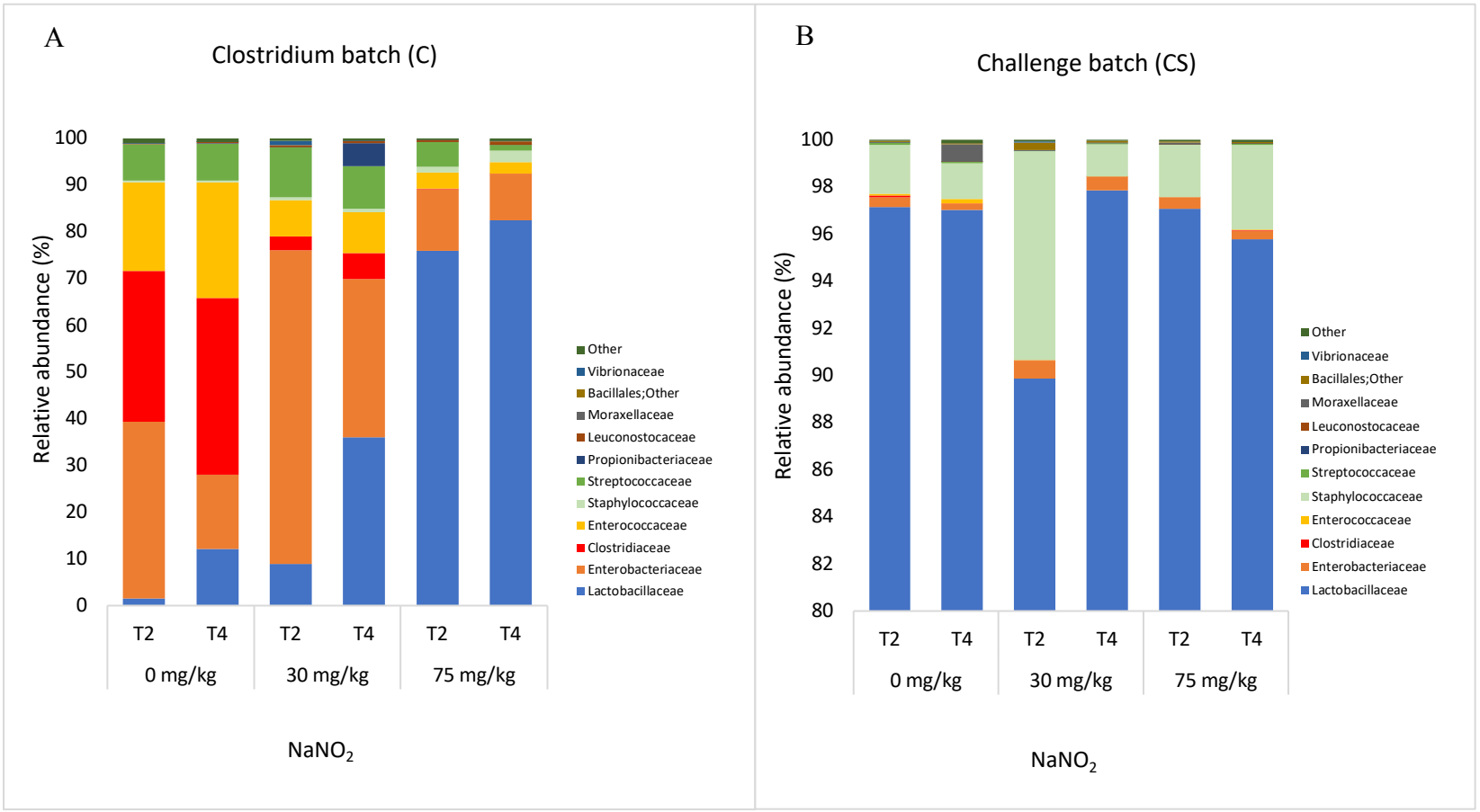


Figure 6. Families relative abundances, expressed as a ratio between the sum of a single OTU reads and the total reads number, in salami samples inoculated with *C. sporogenes*, added with **(A)** or without bioprotective cultures **(B)**, both added with nitrites at different level, at different time points.

The relative Clostridiaceae abundance is shown in the Figure 7.

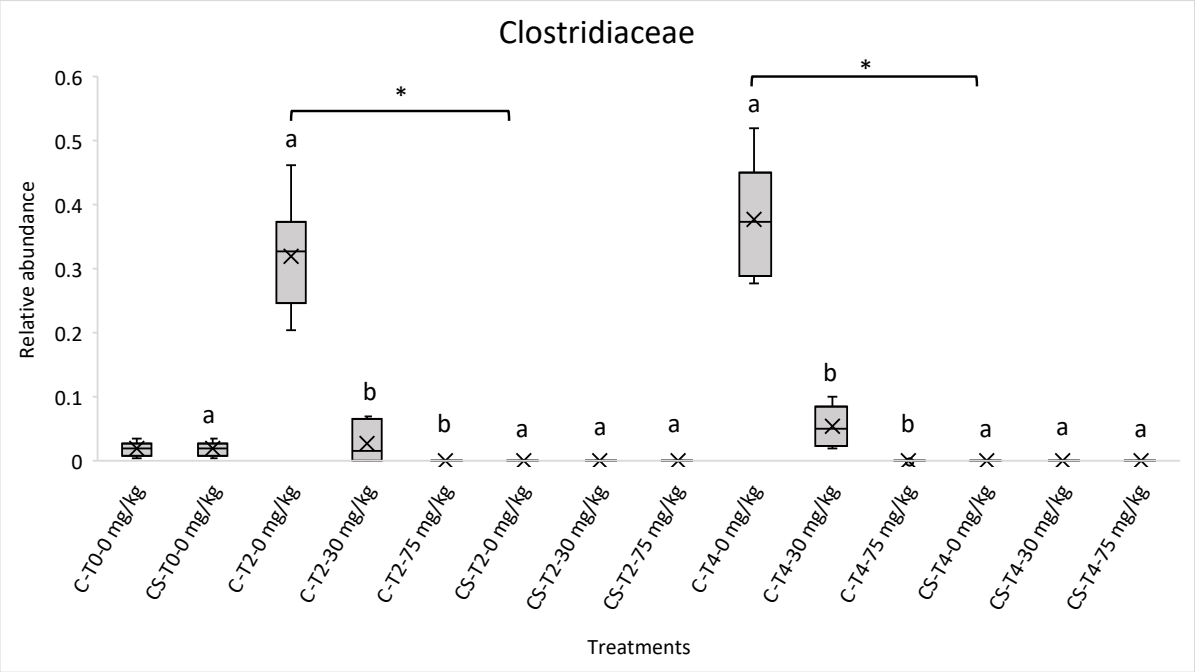


Figure 7. Box plots shows the relative OTU Clostridiaceae abundance observed in the Challenge and Clostridium batch. **C**=*C. sporogenes*, **CS**=*C. sporogenes* + bioprotective/starter cultures; numbers indicate the NaNO2 concentration: **0 mg/kg, 30 mg/kg, 75 mg/kg**; **T**=Time point. The letters indicate significant differences within the same batch (i.e. C vs C30 vs C75), whereas the asterisk represent significance between batches (i.e. C vs CS).

A significant ($p<0.002$) impact on this group was observed in batches where bioprotective/starter cultures were added compared to not inoculated batches (CS vs C). In particular, within the challenge batches, Clostridiaceae abundance was not significantly different at different nitrite levels, whereas in the C treatment it was significantly ($p<0.002$) higher compared to C30 and C75. Considering batches where nitrites were not added, the starter cultures addition significantly lowered Clostridiaceae relative abundance in challenge

batch compared to the clostridium batch (CS vs C), at both, T2 and T4. The same results have been obtained for *Clostridium* spp. relative abundance (Figure 8).

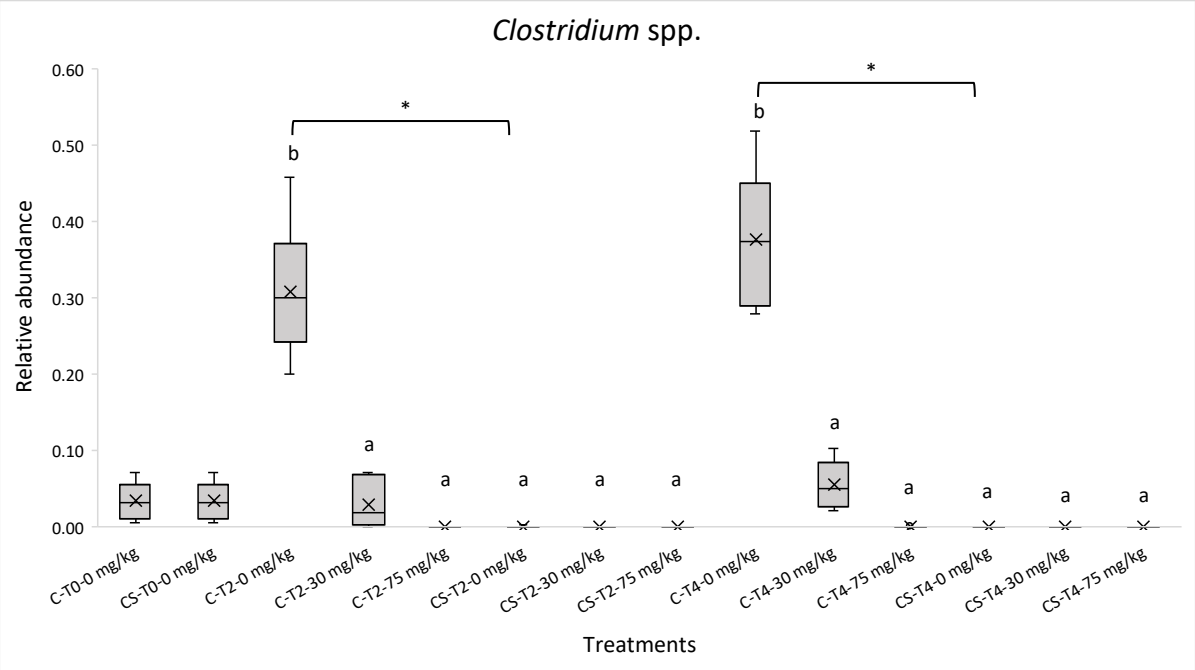


Figure 8. Box plots shows the relative OTU *Clostridium* spp. abundance observed in the Challenge and Clostridium batch. **C**=*C. sporogenes*, **CS**=*C. sporogenes* + bioprotective/starter cultures; numbers indicate the **$NaNO_2$ concentration: 0 mg/kg, 30 mg/kg, 75 mg/kg**; **T**=Time point. The letters indicate significant differences within the same batch (i.e. C vs C30 vs C75), whereas the asterisk represent significance between batches (i.e. C vs CS).

Figure 9 and Figure 10 show the Lactobacilaceae and *Lactobacillus sakei* relative abundance in batches added with *C. sporogenes* as well as with different treatments. Lactobacilaceae and *Lactobacillus sakei* relative abundances were significantly higher in batches inoculated with the starter cultures compared to not added ones, both at T2 and T4. Also, the addition of nitrites, positively impacted the same genus and family increase, observing higher abundance at 30 and 75 mg/kg nitrites in the *Clostridium* batches at both timepoints (T2 and T4), probably nitrites

reducing internal competition of autochthonous populations favour the development of *Lactobacillus*/Lactobacillaceae OTUs.

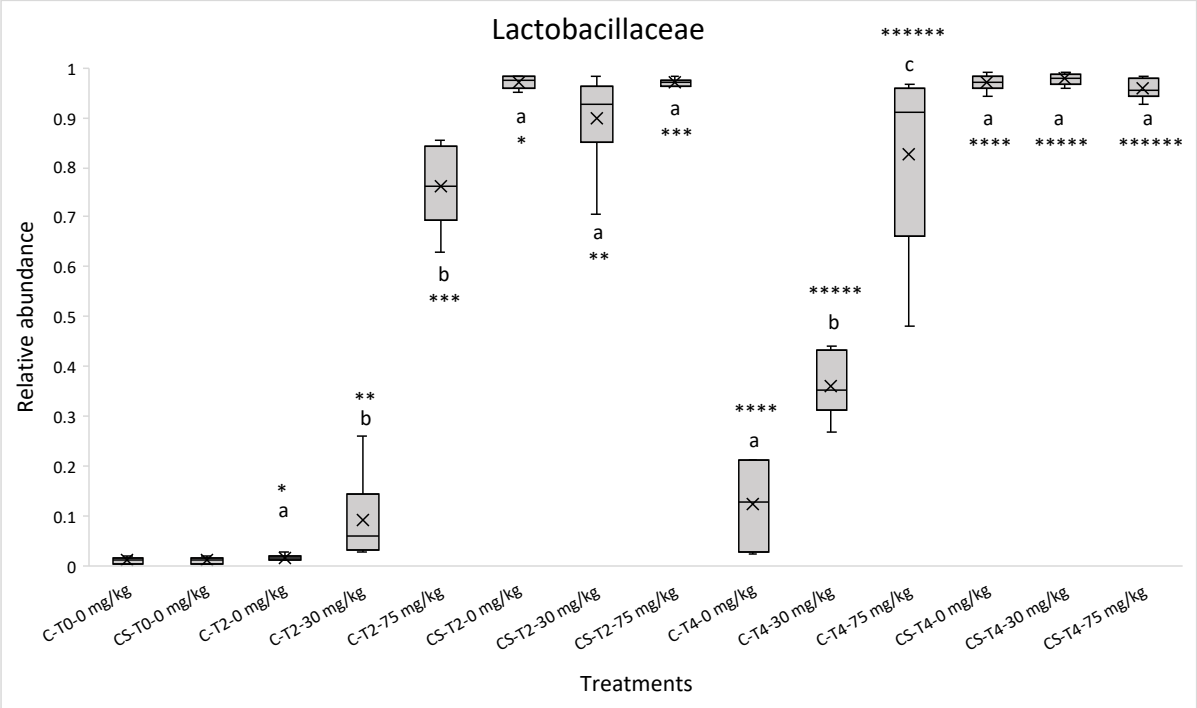


Figure 9. Box plots shows the relative OTU Lactobacillaceae abundance observed in the Challenge and Clostridium batch. **C**=*C. sporogenes*, **CS**=*C. sporogenes* + bioprotective/starter cultures; numbers indicate the $NaNO_2$ concentration: **0 mg/kg, 30 mg/kg, 75 mg/kg**; **T**=Time point. The letters indicate significant differences within the same batch (i.e. C vs C30 vs C75), whereas the asterisk represent significance between batches (i.e. C vs CS).

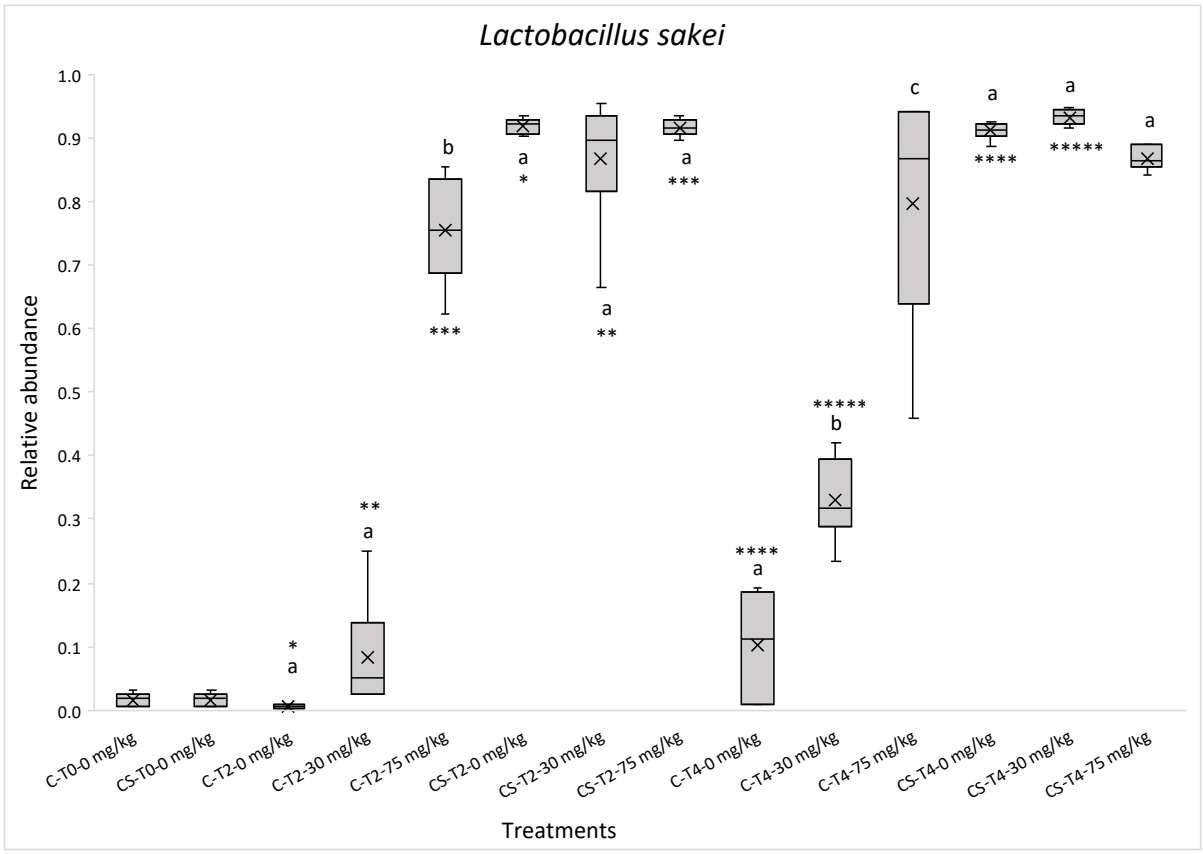


Figure 10. Box plots shows the relative OTU *Lactobacillus sakei* abundance observed in the Challenge and Clostridium batch. The data are not significantly different where letters and asterisk are not used. **C**=*C. sporogenes*, **CS**=*C. sporogenes* + bioprotective/starter cultures; numbers indicate the $NaNO_2$ concentration: **0 mg/kg, 30 mg/kg, 75 mg/kg**; **T**=Time point. The letters indicate significant differences within the same batch (i.e. C vs C30 vs C75), whereas the asterisk represent significance between batches (i.e. C vs CS).

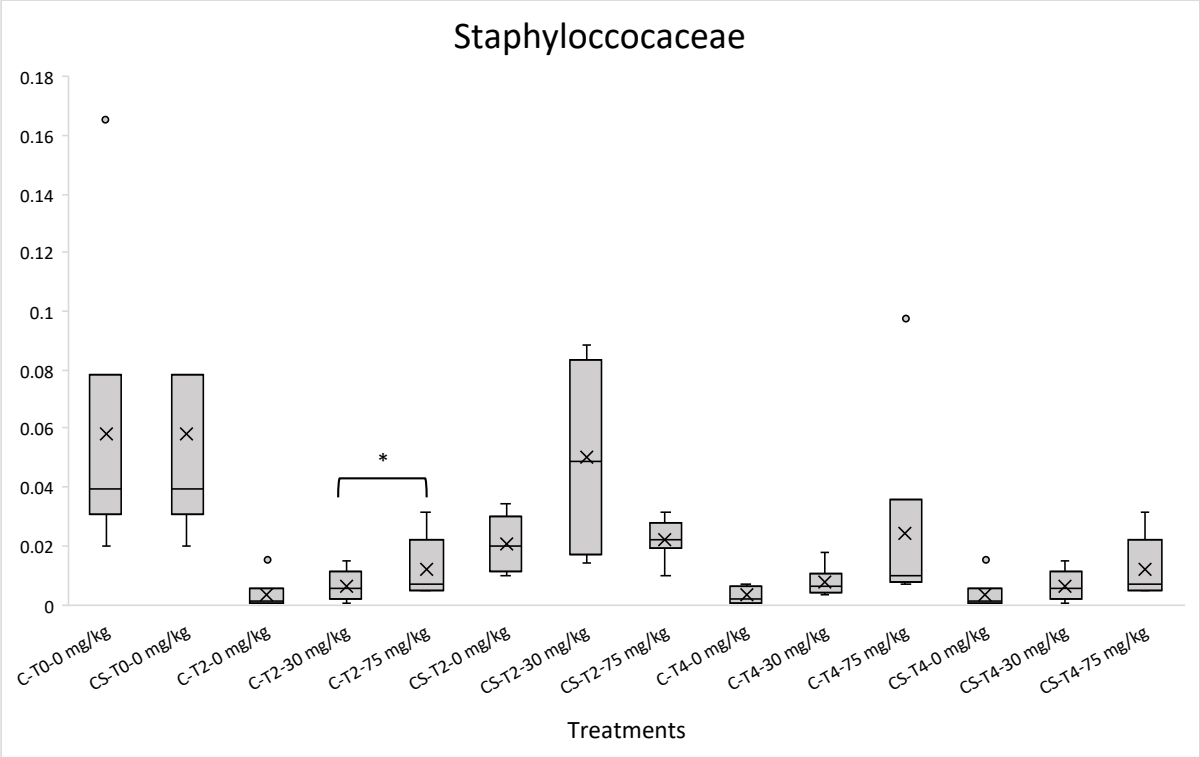


Figure 11. Box plots shows the relative OTU Staphyloccocaceae abundance observed in the Challenge and Clostridium batch. The letters indicate significant differences within the same batch (i.e. C vs C30 vs C75), whereas the asterisk represent significance between batches (i.e. C vs CS). The data are not significantly different where letters and asterisk are not used. **C**=*C. sporogenes*, **CS**=*C. sporogenes* + bioprotective/starter cultures; numbers indicate the $NaNO_2$ concentration: **0 mg/kg, 30 mg/kg, 75 mg/kg**; **T**=Time point.

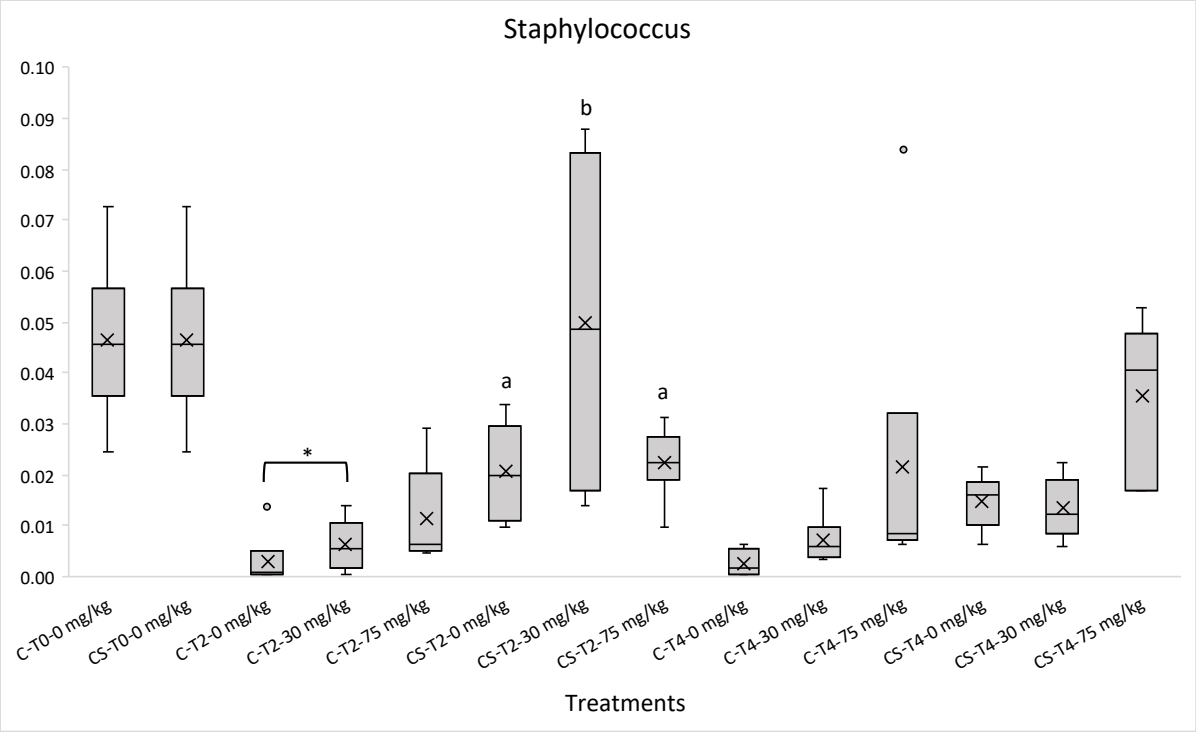
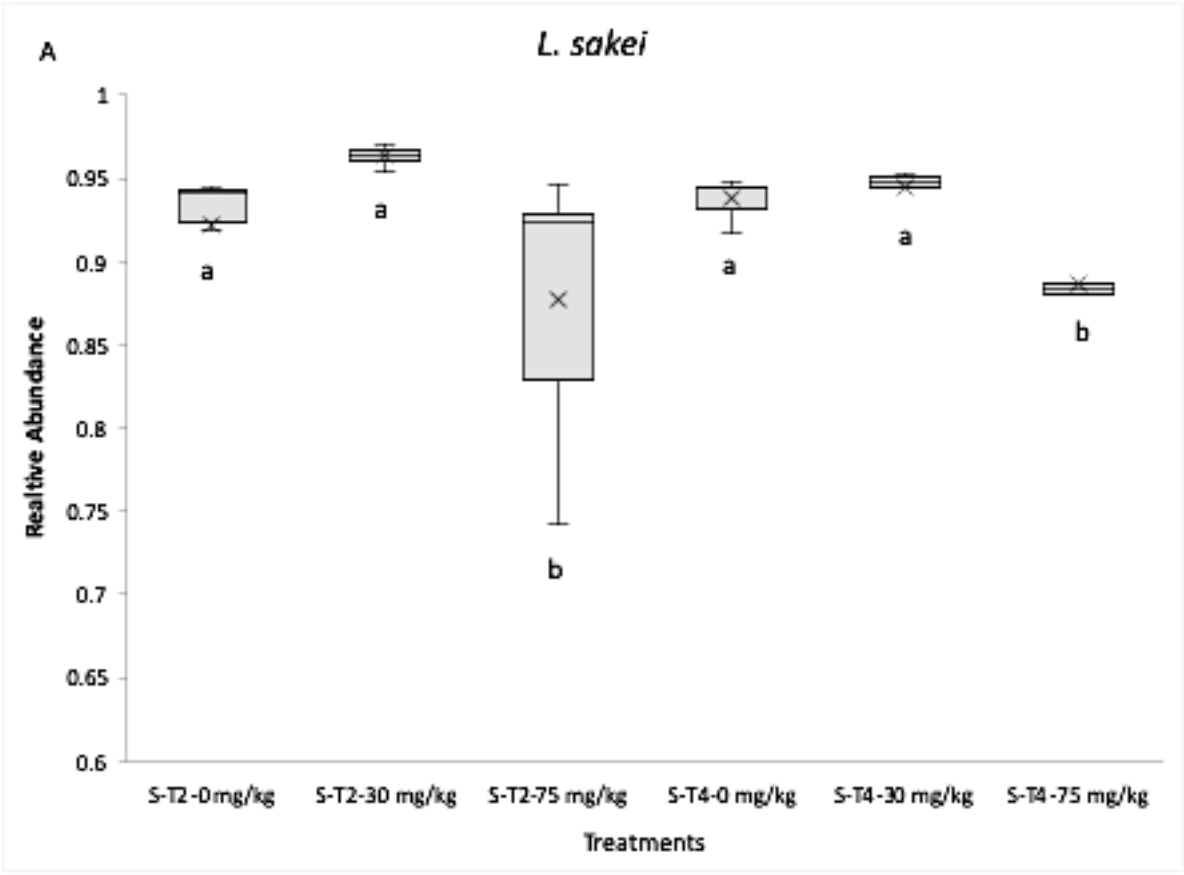


Figure 12. Box plots shows the relative OTU *Staphylococcus* abundance observed in the Challenge and Clostridium batch. The data are not significantly different where letters and asterisk are not used. **C**=*C. sporogenes*, **CS**=*C. sporogenes* + bioprotective/starter cultures; numbers indicate the $NaNO_2$ concentration: **0 mg/kg, 30 mg/kg, 75 mg/kg**; **T**=Time point. The letters indicate significant differences within the same batch (i.e. C vs C30 vs C75), whereas the asterisk represent significance between batches (i.e. C vs CS).

The analysis of Staphylococaceae related OTUs did not show significant differences between the starter non-inoculated and inoculated batches (C vs CS) and also within batches at different nitrites concentration. The same trend could be observed for *Staphylococcus*, a significant difference could be observed ($p<0.0001$) only comparing C30 and CS30 at T2 because *Staphylococcus* displayed the highest number of reads in that batch (CS30) also compared to all the other batches (>8%). The *Staphylococcus* population seems to scarcely compete in the meat matrix both in the presence of the autochthonous population and with the addition of starter cultures, maintaining values between 0.5 and 2.5%.

3.3.3 Taxonomic analysis of the microbial diversity in a prototype model of fermented salami

Figure 13. shows the *L. sakei* and Lactobacillaceae relative abundance in a fermented salami prototype model.



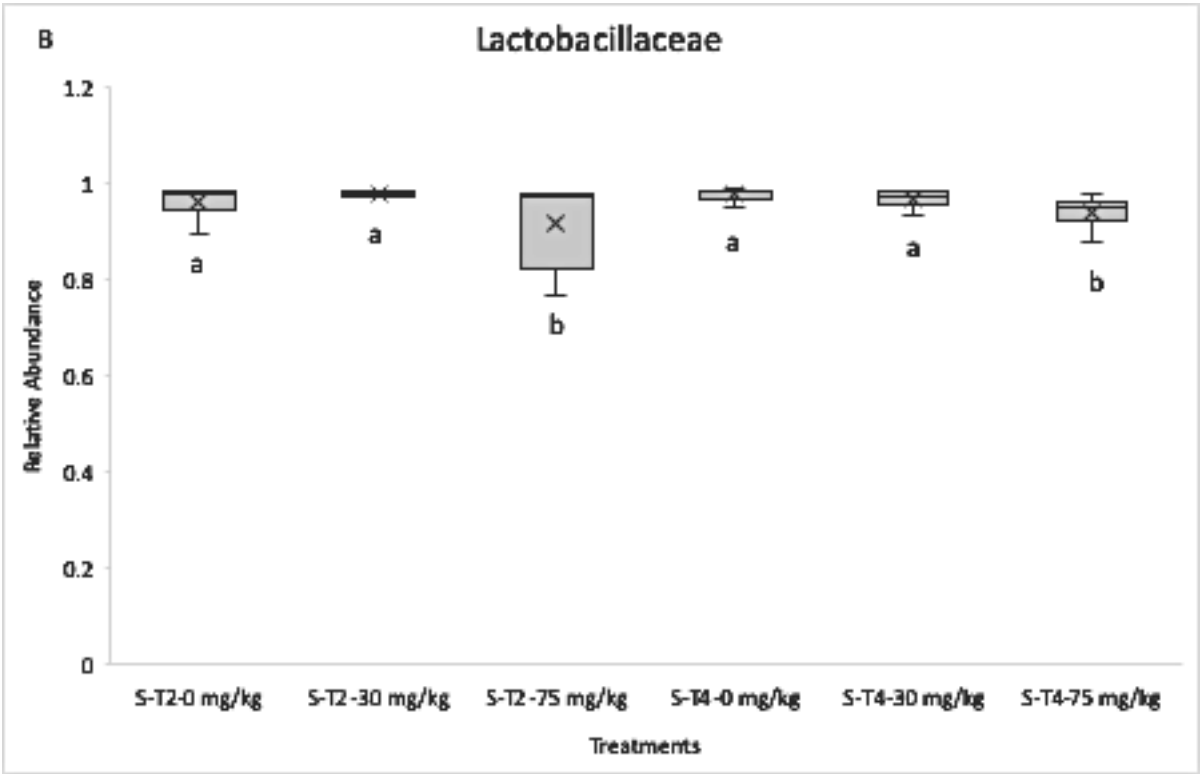
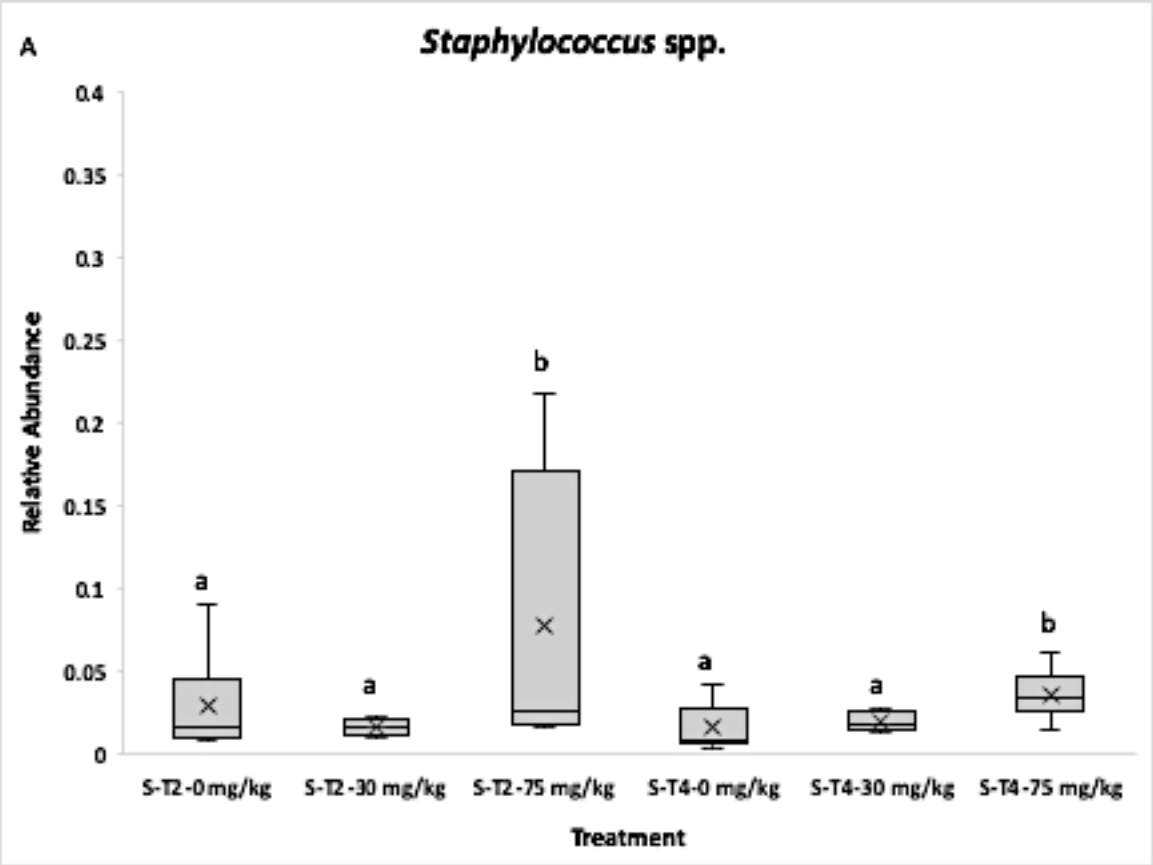


Figure 13 Box plots shows the relative OTU A) *L. sakei* and B) Lactobacillaceae abundance observed in the Starter batch. S=batch added with starter, T2,T4=Time, numbers indicate the NaNO2 concentration: 0 mg/kg, 30 mg/kg, 75 mg/kg.

Figure 14. shows the *Stephylococcus* spp. and Staphylococcaceae relative abundance in a fermented salami prototype model.

Considering the *L. sakei* relative abundance in batch 'Starter', nitrites level showed a significant (p=0.0003) impact on its behaviour. In particular, 75 mg/kg $NaNO_2$ lowered the *L. sakei* relative abundance respect to the 0 mg/kg and 30mg/kg. The observed dynamic was the same comparing after the salami fermentation (T2) and at the end of the study (T4). In addition, the Lactobacillaceae relative abundance results showed the same behaviour as *L. sakei*.



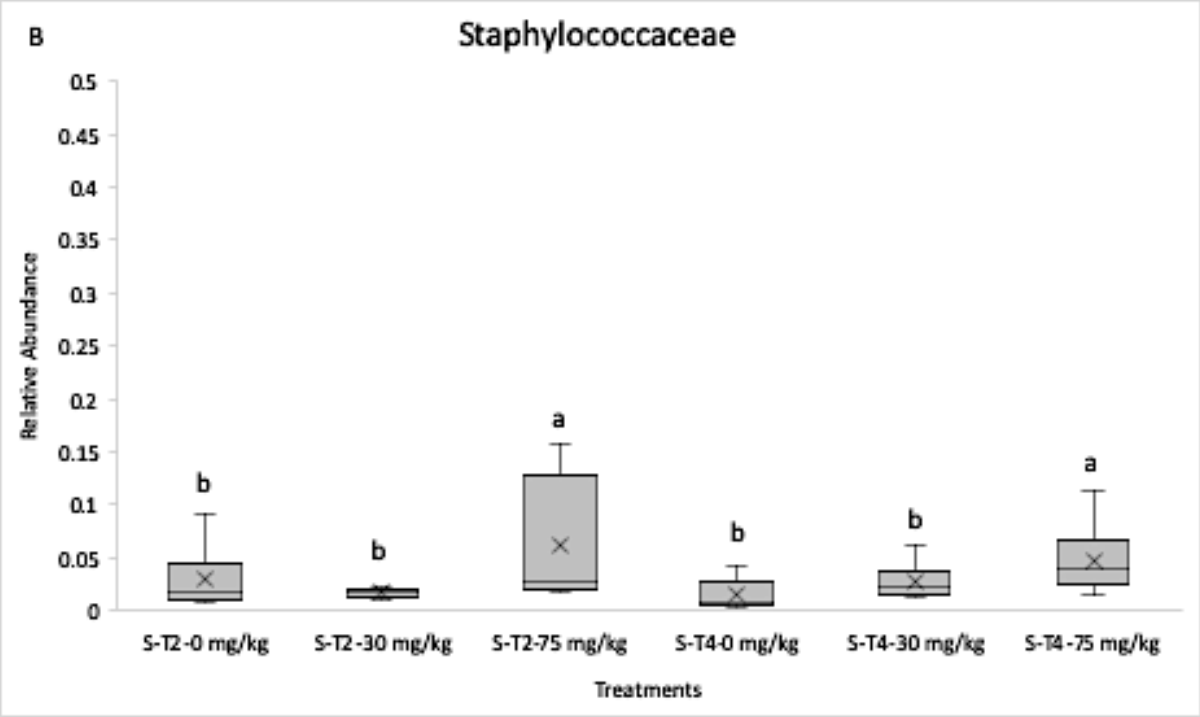


Figure 14 Box plots shows the relative OTU A) *Staphylococcus* spp. and B) Staphylococcaceae abundance observed in the Starter batch. S=batch added with starter, T2,T4=Time, numbers indicate the NaNO2 concentration: 0 mg/kg, 30 mg/kg, 75 mg/kg.

Considering the *Staphylococcus spp.* relative abundance in batch 'Starter', nitrites level showed a significant (p=0.04) impact on its behaviour. In particular, in the batch containing 75 mg/kg $NaNO_2$, *Staphylococcus* spp. relative abundance was significantly increased respect to the 0 mg/kg and 30mg/kg. The observed dynamic was the same comparing after the salami fermentation (T2) and at the end of the study (T4). In addition, the Staphylococcaceae relative abundance results showed the same behaviour as *Staphylococcus* spp.

3. 4 Quality parameters of the nitrite reduced prototype of fermented salami

3.4.1 Physio-chemical characteristics in the fermented salami

The pH, aW, weight loss and protein content data, obtained from the finished fermented salami prototype, with and without starters and different nitrite concentrations are shown in the Table 6.

Table 6. Physio-chemical characteristics and factors interactions in fermented salami

Batch	pH	aw	Weight loss (%)	Protein content (%)
B	6.09 ± 0.01^{c}	0.89 ± 0.00^{a}	41.60 ± 2.44^{ab}	45.07 ± 0.64^{a}
B30	6.09 ± 0.02^{c}	0.89 ± 0.00^{a}	44.87 ± 0.44^{b}	45.33 ± 0.39^{ab}
B75	5.54 ± 0.06^{b}	0.90 ± 0.01^{ab}	38.79 ± 1.71^{a}	47.30 ± 0.75^{bc}
S	5.65 ± 0.06^{b}	0.90 ± 0.00^{ab}	42.31 ± 1.19^{ab}	46.87 ± 0.39^{abc}
S30	5.48 ± 0.04^{ab}	0.91 ± 0.01^{b}	39.14 ± 1.43^{a}	48.03 ± 0.30^{c}
S75	5.38 ± 0.04^{a}	0.92 ± 0.00^{b}	37.29 ± 1.92^{a}	47.70 ± 1.11^{c}
Source				
Starters (A)	<0.0001	0.0005	0.131	
Nitrites (B)	<0.0001	0.123	0.052	
Interactions				
A*B	0.00076	0.639	0.181	

The mean and the standard error values are reported. Nitrite, starters and starters x nitrite interactions are significant according to multifactorial ANOVA. Letters indicates the addition of microbial strains: **B**=no microorganisms added, **S**=samples added with bioprotective/starter cultures; numbers indicate the **$NaNO_2$ concentration: 0 mg/kg, 30 mg/kg or 75 mg/kg**. Means with the same letter in the column are not statistically different ($p>0.05$).

Considering the interactions between starters and nitrite addition, the pH values were impacted by both factors. ($p=0.00076$) (Table 6). A significantly higher pH values, being 6.09, in the batches fermented without starters (B) and with 30mg/kg of nitrites (B30) in comparison with the ones added with 75 mg/kg. As expected, starter cultures showed a significantly higher acidification property in a salami samples (S), ranging from 5.38-5.65 to pH units, compared to samples without starters addition (B). Also, the different amount of added nitrites (30 mg/kg and 75 mg/kg), did not significantly impacted the pH decrease in salami fermented with starters. However, the addition of $NaNO_2$ at 75 mg/kg also significantly lowered pH (B75 and S75) compared with samples not added or with 30 mg/kg of nitrites. On the other hand, there were no differences on pH reduction in salami fermented with starters between the difference levels of nitrites added (i.e. 30 mg/kg vs 75 mg/kg).

When considered the water activity, the addition of starter was the main factor that influenced this parameter (Table 6). A significantly lower a_w reduction was observed in spontaneously fermented salami (batches B), when compared with the ones added with starters. Proportional with the a_w behaviour was the salami weight loss parameter, where a significantly higher loss was observed in batches with lower a_w parameter, i.e. 44.87% in B30 versus 39.14% in S30. The visual colour evaluation of the salami samples, at the end of the ripening, showed equally intense red colour in samples treated with 30 mg/kg or 75 mg/kg $NaNO_2$ compared to samples not added with nitrites (Figure 15).

Figure 15. Photos are showing the colour of salami samples with starter cultures and different nitrite concentration (left) and salami prototype (right)

3.4.2 Salami proteolysis and degree of hydrolysis

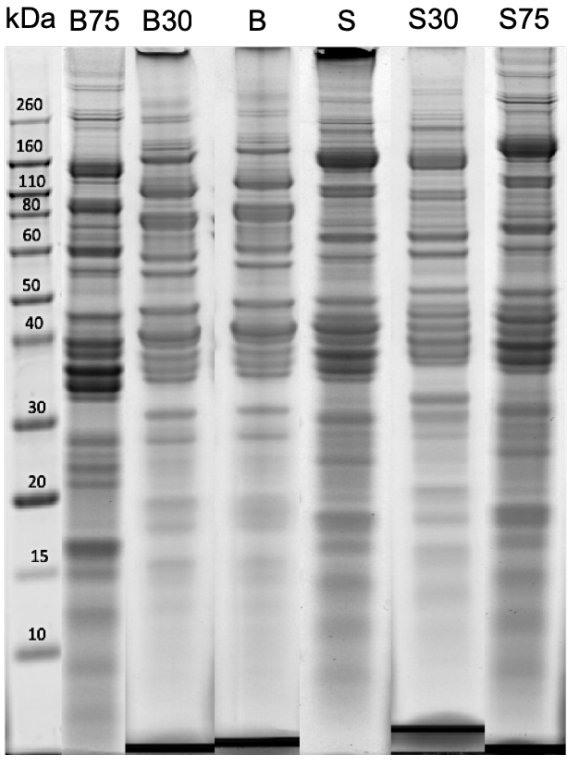


Figure 16. Protein patterns in salami, fermented with and without cultures addition and with a and without nitrites additives. Letters indicates the addition of microbial strains: **B**=no microorganisms added, **S**=samples added with bioprotective/starter cultures; numbers indicate the **$NaNO_2$ concentration: 0 mg/kg, 30 mg/kg or 75 mg/kg**.

In the Figure 16 are shown the protein profiles at the end of the salami ripening (profiles taken from time 0 from in vitro digestion model). It can be observed that proteins and polypeptides profiles are similar in the samples added with starters (S, S30, S75) whereas a slight difference in bands intensity were observed in samples without starters. In the spontaneously fermented salami (B, B30 and B75) can be observed an overall less polypeptides bands and more intense bands in the samples added with nitrites (B30 and B75), in particular at the range between 160 kDa and 110 kDa.

3.4.3 Degree of hydrolysis in salami samples with or without addition of starters and/or nitrites

The Figure 17. shows the results obtained from the degree of hydrolysis of the fermented salami prototype.

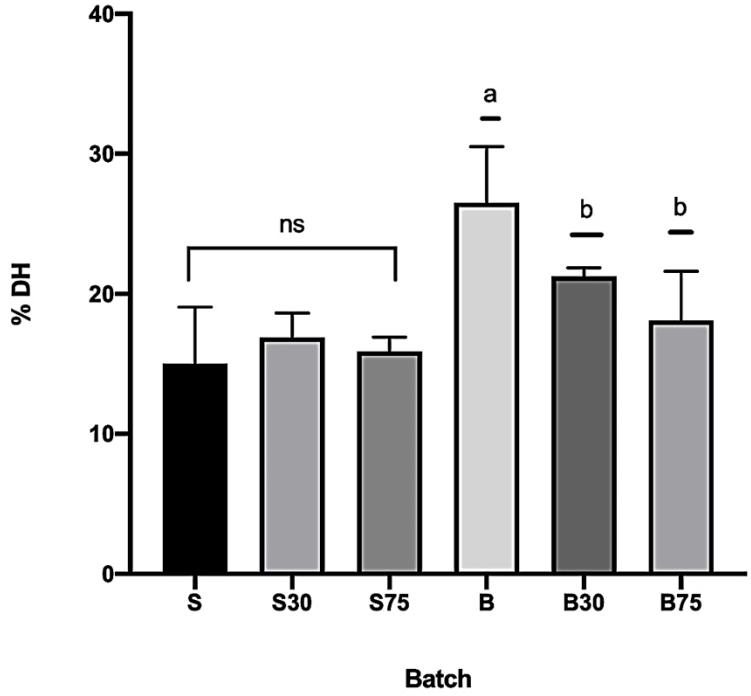


Figure 17. Degree of hydrolysis (%) in a fermented salami. Letters indicates the addition of microbial strains: **B**=no microorganisms added, **S**=samples added with bioprotective/starter cultures; numbers indicate the **$NaNO_2$ concentration: 0 mg/kg, 30 mg/kg or 75 mg/kg**. The letters indicate significant differences within the same batch (i.e. B vs B30 vs B75); ns=not significant.

When considered salami fermented with starters (S, S30 and S75), no significant differences were observed in the proteins hydrolysis whereas significant differences were observed in spontaneously fermented salami (B, B30 and B75). Although not significant differences were observed when different nitrite concentrations are concerned (B30 and B75), a significantly higher % DH was observed in spontaneously fermented salami without nitrites (B) with when compared with nitrites batches.

3.4.4 *In-vitro* digestion

Differences in salami samples proteolysis, after ripening and upon *in-vitro* digestion was evaluated using SDS-PAGE and the results are shown in the Figure 18. *In-vitro* digestion sampling point (0 min) was considered as an indicator of salami proteolysis after ripening process.

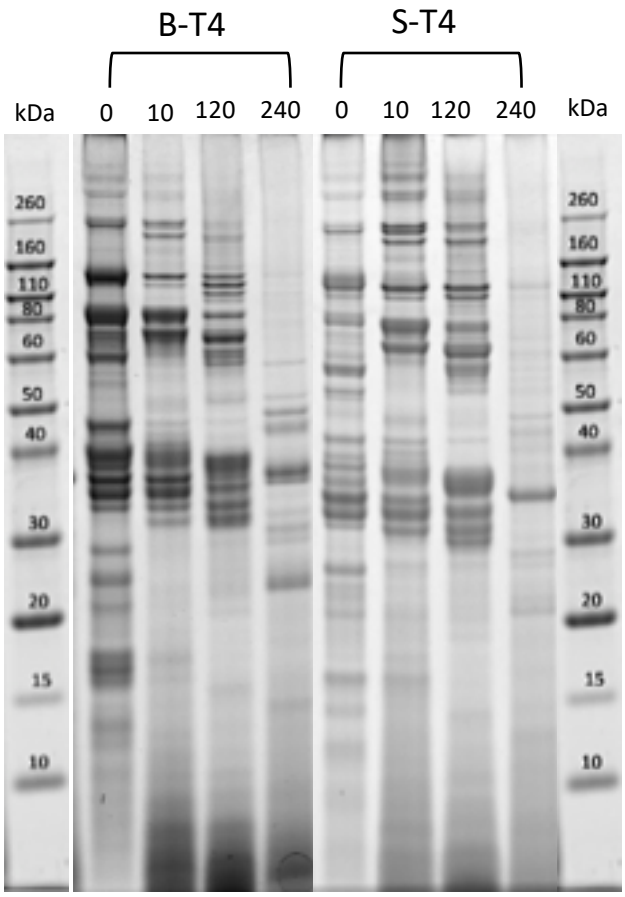


Figure 18. Comparison of protein digestibility of salami fermented with (B) and without starters (S) at the end of the ripening (T4)

When compared batches with and without starters addition (S and B, respectively), at time 0 min, more intense bands were observed in the sample fermented without starters (B), suggesting a better proteolysis when starters were used.

Some differences between the B and S samples were also observed during the two gastric phase sampling points (10 min and 120 min) in the range between 110-60 kDa. At the protein bands between 30-40 kDa, the intensity of bands belonging to tropomyosin complex (e.g. tropomyosin-α-chain and β-chain, 33 kDa and 37 kDa respectively) and troponin T (35 kDa) was more pronounced in the sample fermented without starter cultures. Proteins in the range of 30-10 kDa were digested in both samples at the end of the gastric phase.

In the intestinal phase, a pronounced resistant band, ~40kDa was observed in both samples. The same band was previously identified as a complex of a fragments belonging to myosin isoforms 1, 2 and 7 (Farouk et al., 2019). In both samples a high molecular weight protein was digested whereas

pronounced bands, at different level from 60kDa to ~25kDa, were observed in sample fermented without starters, compared when the same were added.

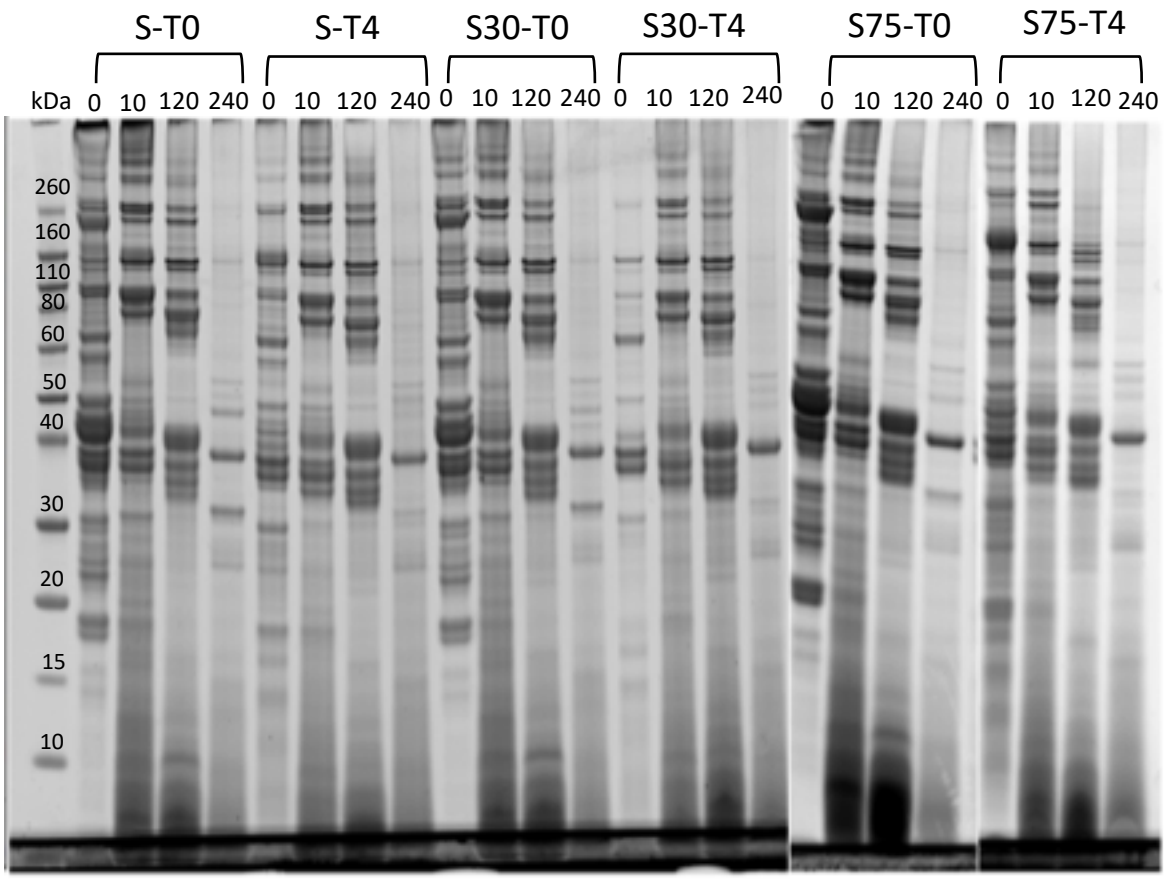


Figure 19. In-vitro digestion of meat batter and fermented salami without and with different nitrite addition. Letters indicates the addition of microbial strains: **S**=samples added with bioprotective/starter cultures; numbers indicate the **$NaNO_2$ concentration: 0 mg/kg, 30 mg/kg or 75 mg/kg**.

Salami samples before fermentation (T0), with different nitrite concentration, were also subjected to *in vitro* digestion for the assessment of nitrates impact on the digestion. Figure 19 shows the SDS-PAGE protein profiles of salami samples with or without starter cultures, containing 0, 30 and 75 mg/kg $NaNO_2$, before the fermentation (T0) and at the end of the ripening (T4). It can be observed that nitrites have not impacted the digestibility of the non-fermented meat batter. The results show that there were no differences between the protein digestion profiles of salami samples without starter cultures at different levels of nitrite addition. Interestingly, with the increase of the nitrite

concentration, a slight decrease intensity of two bands was observed, at the level of ~160 kDa at the end of the gastric phase (120min) in salami samples: in the order of S-T4<S30-T4<S75-T4. The same was not observed in the samples before the fermentation (T0 samples). These results suggest that nitrite addition influences the protein digestibility of fermented meat.

4. Discussion

In this study, meat fermentation for salami production was performed with the use of targeted protective/starter cultures, at low nitrite dosages, with and without *Clostridium* inoculation. The salami microbiota in the different experimental conditions was studied as well as physio-chemical parameters of the salami and *in-vitro* digestion products.

The fermentation is one of the crucial moment for the pathogens outgrowth control and the meat microbiota has a key role in pathogen inhibition. However, to ensure safety, nitrites are usually added. Some studies have focused on the use of protective cultures as a feasible solution for the reduction of nitrite in fermented meat products. (Nieto-Lozano et al., 2010, Nikodinoska et al., 2019). Acidic pH and low water activity are additional parameters for both, safety and quality aspects (Majou and Christieans, 2018, Toldrà, 2010). The results in the present work showed that the combination of two hurdles approaches, bioprotective/starters cultures addition with a reduced nitrite concentration with respect to the currently maximum allowed amount of 150 mg/kg, have a strong impact on the *Clostridium* spp. outgrowth in fermented salami. In particular, it was observed that the combination of microbial cultures plus 30 mg/kg or 75 mg/kg $NaNO_2$ (CS30 or CS75) as well as the addition of only 75 mg/kg $NaNO_2$ (C75) were equally effective on *Clostridium* outgrowth. Keto-Timonen et al. (2012) reported that *C. botulinum* type B toxigenesis could be observed in nitrite-free products during storage at 8°C, whereas the spores germination was inhibited in the presence of 75 or 120 mg/kg nitrite, concluding the same group of pathogens can be controlled with a lower $NaNO_2$ concentration with respect to the maximum allowed amount. In addition to the effect on *Clostridium*, we also investigated to what extent different nitrite concentration impacts the meat native microbiota and the

added bioprotective/starter cultures. The obtained results from the challenge and clostridia batches, showed that nitrites addition inhibited the total autochthonous *Lactobacillus* spp. population in the spontaneously fermented salami (C30 and C75), at the end of the fermentation period (T2), whereas the same growth was not inhibited when the starter cultures were added (CS vs CS30 and CS75). This was supported also by pH results, which were the highest in C30 and C75 batches at T2. Differently, only in C30 but not in C75 a low a_w was measured. It is important to highlight that the pH was lowered to around 5.05 in batches inoculated with bioprotective cultures, the reason why these cultures are also referred to 'bioprotective/starters' in the present work. At pH 5 nitrites are in undissociated state that can exert their antimicrobial activity (Majou and Christieans, 2018). Among all treatments, the highest *Lactobacillus* spp. counts were obtained when starters were used with 30 mg/kg $NaNO_2$ (batch CS30), whereas the lowest when only 30 mg/kg $NaNO_2$ were added in the meats (C30), at T2. This observation might explain the same clostridia inhibition when 30 mg/kg of nitrites were used (C30 and CS30 batches), at T2, whereas at the end of the ripening, 1.64 log cfu/g higher clostridia counts were obtained in C30 compared to CS30. The lower a_w value measured in C30 compared to CS30 was not sufficient for clostridia inhibition that might also suggest the greater antimicrobial impact of the lower pH compared to a_w parameter, as observed in CS30 at T4. Interestingly, with the lactobacilli increase in C75, the clostridia counts efficiently decreased as in C75 batch at T4. The hereby reported starters and the nitrites interaction ($p<0.001$) is in line with the hurdle technology concept, where the reduction of an individual component concentration, in a combined treatment, may cause synergistic effects in reduction of pathogenic bacteria in food products (Zhou et al., 2010). Similarly, Ghabraie et al. (2016), reported that the anti-clostridial effect of reduced nitrite, 100 mg/kg, as more efficient compared to 200 mg/kg, only when combined with the essential oils or with organic acid salts, in a sausage model.

The study of microbial composition in a *Clostridium* added batches with NGS approach, confirmed the observations regarding the *Clostridium* spp. and *Lactobacillus* spp. dynamic in different treatments. In fact, it could be speculated that the addition of 75mg/kg $NaNO_2$ in uninoculated batches

(C) in some way "simulate" the bioprotective/starter culture effect in reducing the diversity of the microbiota. The lactobacilli dominance in the meat matrix in C75 treatment, confirmed the anti-clostridia activity observation, i.e. 30mg/kg $NaNO_2$ are not as efficient as when combined with cultures; whereas the 75 mg/kg $NaNO_2$ are able to control the *Clostridium* spp. outgrowth.

From the technological point of view, acidification has a strong impact on the muscle proteins denaturation and their coagulation that along with the proteolysis contributes to the flavour and texture development. In addition, during the drying process, the removed water from the products, contributes to decrease of the mass and volume, resulting in a harder texture and in aroma compounds development typical for fermented salami (Toldrà, 2010).

In the present study both variables, starters and nitrites, showed an impact on the physio-chemical salami parameters. The addition of cultures contributed to an important pH decrease to reach the value 5.05 during the fermentation, regardless the nitrite level, followed by final pH increase to 5.38-5.65, as a consequence of the accumulation of proteolysis compounds (Hospital et al., 2015). Regarding the nitrites impact on the physio chemical parameters, the concentration of 75 mg/kg $NaNO_2$ showed a higher pH lowering activity both in B75 and S75 treatments.

A recent study reported that the increased nitrite content could stimulate and/or inhibit a certain microbial population (Cardinali et al., 2018). However, in the same study the same pH lowering ability of the cocktail starters composed of *Pediococcus pentosaceus* and *Staphylococcus xylosus* was not observed. Interestingly, in the present study, pH was significantly lower in B75 and in S75 compared to the respective lower nitrite level batches (B0, B30 and S0, S30, respectivelly). In B75 and S75 the *Lactobacillus* spp. counts were also higher, showing that lactobacilli are the main contributors in pH lowering in the meat fermentation. In particular, *L. sakei* was attributed as a dominant species irrespectively of nitrate/nitrite concentration (Cardinali et al., 2018).

The significantly higher pH and lower a_w, observed in spontaneously fermented salami (batches B), when compared with salami additioned with starters, are in line with previous observation showing that lactic acid addition in salami fermentation determined lower pH and higher a_w values (Toldrà,

2010). The salami weight loss was consistent with the a_w values, increasing the former when the latter diminished.

Literature reports that some traditional dried salami, usually manufactured without cultures addition, as well as some salami types, have a pH around 6.0 and a_w lower than 0.9 (Toldra et al., 2010; Cenci-Goga et al., 2012). This evidence is in line with our observations when considered batches spontaneously fermented (B) and when low nitrite concentration was added (B30). This suggests that microbiota development is different when 30 mg/kg or 75 mg/kg nitrites are added. However, naturally selected microbiota in a reduced nitrite environment (B and B30), will have more diversity compared to the one shaped on the bases of the nitrite resistance.

The microbiota diversity, with their specific metabolic requirements, has an impact on products final characteristics. Interestingly, batches that are spontaneously fermented showed a lower protein content and a significantly higher degree of hydrolysis, compared to ones where starters and nitrites were added. The meat proteolysis in fermented salami is guided by the meat proteases (chatepsins), prevalently during the first stage, and further by microbial proteases. It should be however stressed that the higher pH values can be result of proteolysis, resting in an increased safety hazard, due to the pathogens and histidine decarboxylase-positive strains like *Pseudomonas* spp., staphylococci, micrococci and enterococci (Hospital et al., 2015, Cenci-Goga et al., 2012).

In a previous study it was hypothesized that a chemical or conformational change in proteins could be influenced by the nitrite addition, resulting in a decreased proteolysis. In addition, amino acids modifications in a digestive proteolytic enzymes cleavage site, might be influenced by the nitrite derivates (de La Pomélie et al., 2018). However, the present study showed that, overall, the protein digestion was not impacted by the nitrite addition what was confirmed with the not significant results from relative density analysis.

It is known the fact that the nitrites dissociate in acidic condition and further react with secondary ammines, resulting in nitrosamine formation (Kim and Hur, 2018). Considering this, nitrites might have quite short time as a free compound and a low affinity for gastric enzymes. Also, Alahakona et

al. (2015), reported that only 10-20 % of nitrites might be detected after processing and the highest loss is upon the bond with proteins that is 20-30%. However, residual 0.2 mg/kg of nitrites were found only in the salami sample fermented with 75 mg/kg (data not shown), whereas in the digested juices this was not detected (data not shown).

Considering the overall *in-vitro* digestion of the samples, it can be concluded that the high-molecular-mass proteins (260-110kDa), including myosin heavy chain (MHC) and α-actinin (~100kDa) were completely digested at the end of the two-stages enzymatic digestion, in all samples, whereas, myosin light chains (~15-25kDa) were completely digested at the end of the gastric phase, indicating physiological relevance of the *in-vitro* conditions. The hereby used bioprotective/starter cultures contributed to the improved digestibility, that was especially evident at the end of the digestion, with a number of bands, ranging from 60kDa to ~25kDa, respectively, without and with bioprotective/starter culture addition. Ge et al. (2019) have recently described that the addition of *Lactobacillus plantarum* NJAU-01 enabled a reduction of protein oxidation during the fermentation and ripening of fermented sausages, suggesting its potential use as an antioxidant starter. Many factors such as protein oxidation may lead to a reduced digestibility and a reduced nutritional products value, it is possible to speculate that added cultures may possess antioxidant activity that prevents protein oxidation, as observed by Estevez, 2011.

5. Conclusion

The present study showed that is possible to lower the nitrites level of the described fermented pork meat salami model to 30 mg/kg when combined with a bioprotective/starter cultures cocktail, as a valid anti-clostridia outgrowth control. The addition of 75 mg/kg is able to control *Clostridium* spp., regardless the cultures addition.

Considering the proteolysis feature, hereby used cultures were more performant and were not impacted form the nitrite addition, that was not the case in spontaneously fermented salami where the proteolysis was impacted by the nitrite amount. Also, *in vitro* digestion study showed that protein digestibility of fermented salami was not impacted by ingoing nitrite level.

Findings in our study suggests that careful selection of bioprotective/starter cultures combined with a low nitrite amount can contribute to a successful lowering of nitrites content within the quality and safety limits.

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