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FROM FOOD WASTE TO FOOD PRESERVATION: PRODUCTION AND APPLICATION OF SAKACIN A

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Introduction to the thesis project

This thesis has been conceived in the frame of a broader project named “NANOSAK- Nanocellulose–sakacin A conjugates for food packaging purposes”, financend by Fondazione Cariplo and developed in collaboration with several divisions of the Department of Food, Environmental and Nutritional Sciences (DeFENS), with the Department of Environmental Science and Policy (ESP) of the Università degli Studi di Milano and together with the Department of Food quality and preservation of the Instituto de Agroquímica y Tecnología de Alimentos (IATA), CSIC (Valencia, Spain).

NANOSAK aims at exploring the use of cheese whey and/or their derivatives as cheap substrates for growth of bacterial species that produce two families of molecules: a specific bacteriocin sakacin A, and bacterial cellulose (BC), that will be turned into nanocellulose (BNC) using sustainable procedures.

In Italy most of the liquid whey is exported, often after an ultrafiltration (UF) step. The by-product of whey UF, namely permeate, still represents a valuable raw material due to the high content of lactose, minerals and vitamins. This permeate from the dairy industry is a low-cost alternative for industrial production, which can provide high yields of the antimicrobial agent. Both the molecules, sakacin A and bacterial cellulose, expected to be produced by microorganisms through whey utilization can be considered products of high added value. Bacterial cellulose also represents an emerging material with excellent intrinsic properties due to its high crystallinity, tensile strength and water holding capacity (Shoda and Sugano, 2005). On a subsequent step, NANOSAK will also aim at using sustainable procedures for turning BC into nanocellulose, in fact, bacterial cellulose has potential to be turned into bacterial nanocellulose by chemical modification, thus forming an innovative material that finds applications across several industrial sectors, including the use in the food packaging sector. Recently, more attention was given to the development of packaging materials based on bacterial nanocellulose as functional nanofiller in papers and in coatings for plastic matrices. An interesting application is related to the use of cellulose nanocomposites to extend shelf-life and enhance the quality of perishable foods, not only acting as barriers against moisture, water vapor and gasses but also serving as a carrier of active substances, such antimicrobials, in bioactive packaging (Lee et al., 2013). In particular, stand-alone BNC films and coatings incorporating sakacin A will be developed, using food-compatible biopolymers and aqueous chemistry. BNCs/sakacin A conjugates will also be applied to paper by surface sizing. The functional properties of the nanocellulose/sakacin A films and coatings will be assessed, as well as the kinetics of sakacin A release in several food simulants, in order to establish suitable mathematical release models. The results will contribute to increase shelf-life and quality of perishable food.

The main significance of NANOSAK is the demonstration that application of industrial biotechnologies will achieve innovative and highly sustainable bioprocesses. The bioeconomic model approach developed by the project would give original opportunities to improve the sustainability of Lombardy economy, in the general food sector, and for what attains food packaging operators.

In particular, the focus of this thesis is the optimization of the production of the bacteriocin sakacin A using cheese whey permeate, its food-grade isolation and purification and the development of active packaging solutions based on the antimicrobial activity of this molecules.

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Chapter 1:
STATE OF THE ART

1. State of the art

1.1. Overview on *Listeria monocytogenes* and listeriosis

1.1.1. *Listeria monocytogenes*

Listeria is a gram-positive, facultative anaerobe, motile, non-spore forming, small (0.5 µm diameter and 1 to 2 µm length), rod shaped bacterium. The genus *Listeria* was composed of ten species until 2014, when five new species were identified by den Bakker and co-authors. They also divided the species in four clades. Within the genus, only *L. monocytogenes* and *L. ivanovii* are pathogenic to humans and animals respectively, and belong to the same clade (Ryser and Buchanan, 2013; den Bakker et al., 2014; Rothrock et al., 2017). This organism is psychrotrophic and has the ability to grow over a wide temperature range of 0 to 45 °C, with an optimum around 37 °C, and in a pH levels between 4.4 and 9.4 (FAO/WHO, 2004).

1.1.2. Listeriosis

L. monocytogenes has been known as the causative agent of listeriosis, a highly fatal opportunistic foodborne infection. While the modes of transmission for *L. monocytogenes* can include vertical, zoonotic and nosocomial transmission, it is now widely recognized that most cases of human listeriosis involve foodborne transmission.

Although 12 serotypes can cause disease, at least 95% of *L. monocytogenes* strains isolated from human listeriosis belong to three serotypes: ½ a, ½ b and 4 b; however, the reason for the apparent prevalence of these serotypes has not clearly identified yet (Kathariou, 2002).

Evidence for foodborne transmission of *L. monocytogenes* first emerged in an outbreak associated with contaminated coleslaw in 1981 in Canada (Ryser and Marth, 2007; Garner and Kathariou, 2016). Invasive listeriosis starts with the infection of the intestinal tissue and arrives to nervous system or blood causing primarily septicaemia, meningitis and meningoencephalitis with fatality rates of 20 to 30% among hospitalised patients. Listeriosis is relatively rare but among foodborne diseases is the one with the highest social and economic impact, representing the third-leading cause of death among foodborne bacterial pathogens, with death rates higher than those caused by *Salmonella* or *Clostridium botulinum*; nota that almost all (97%) reported listeriosis cases were hospitalised (Vazquez-Boland et al., 2001; FAO/WHO, 2004; Álvarez-Ordóñez et al., 2015).

Table 1.1: Listeriosis characteristics and manifestation (Adley & Ryan, 2016).

Organism	Common name of Illness	Onset Time After Ingestion	Signs and Symptoms	Duration	Food Source
Listeria monocytogenes	Listeriosis	9-48 h for gastrointestinal symptoms 2-6 weeks for invasive disease	Fever, muscle aches, and nausea or diarrhea; pregnant women may have mild flu-like illness, and infection can lead to premature delivery or stillbirth; elderly or immunocompromised patients may develop bacteremia or meningitis	Variable	Unpasteurized milk, soft cheeses made with unpasteurized milk, ready-to-eat deli meats

Listeriosis occurs more often in well-defined high-risk groups including elderly people as reported in Figure 1.1, new-borns, immunocompromised adults and pregnant women where it can lead to miscarriage (Ryser and Buchanan, 2013). Incubation of invasive listeriosis can last from two to three weeks before illness manifestation, whereas non-invasive listeriosis occurs when high doses of *L. monocytogenes* are ingested by healthy subjects: in this case, after a short incubation time, symptoms of gastroenteritis, fever and headache arise (FAO/WHO, 2004).

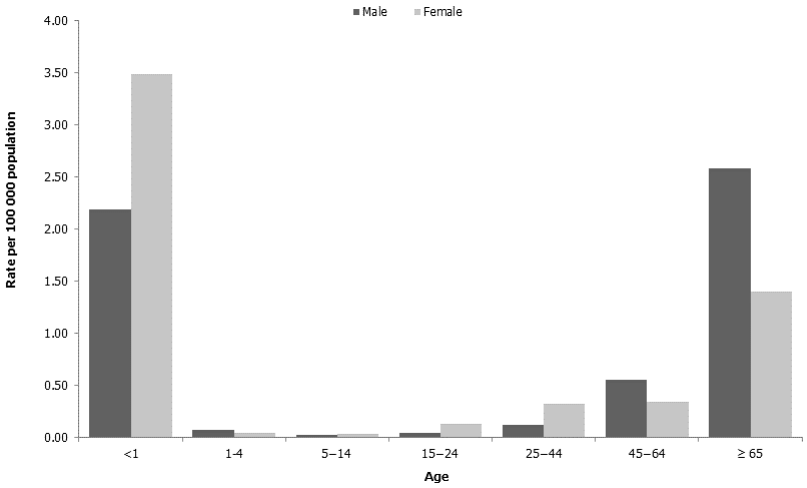

Figure 1.1: Confirmed listeriosis cases: rate per 100000 population, by age and gender, EU/EEA 2014. (European Centre for Disease Prevention and Control ECDC, 2018)

The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks reports 2536 confirmed invasive human cases of listeriosis for the year 2016. The EU notification rate was 0.47 cases per 100,000 populations, with an increase of 9.3% compared to 2015. Confirmed listeriosis in the EU/EEA has showed a statistically significant increasing trend of cases with 247 deaths reported due to listeriosis in 2016 and a case fatality rate confirmed around 20%. In general, listeriosis is the only food-borne zoonosis, which continues to show a significant increasing trend in the EU/EEA in the last 5 years (2012–2016) as reported in Tables 1.2 and 1.3 (EFSA Journal, 2017).

Table 1.2: Summary statistics of human invasive *L. monocytogenes* infections and *L. monocytogenes* occurrence in the major RTE food categories in the EU, 2012–2016. (ECDC, 2018).

Humans	***2016***	***2015***	***2014***	***2013***	***2012***
Total number of confirmed cases	2536	2206	2242	1883	1720
Total number of confirmed cases/100000 population (notification rates)	0.47	0.43	0.46	0.39	0.36

Table 1.3: Reported cases of human invasive listeriosis and notification rates per 100000 in Italy by year, 2012-2016. (ECDC, 2018).

Year	**2016**		**2015**		**2014**		**2013**		**2012**	
	Cases	Rate	Cases	Rate	Cases	Rate	Cases	Rate	Cases	Rate
ITALY	179	0.30	153	0.25	132	0.22	143	0.24	112	0.19

The infection dose of *L. monocytogenes* depends upon several factors such as the host, the type of the ingested food, the strain virulence and the number of organisms consumed, however data about foodborne listeriosis indicate that contaminated food generally contained more than 100 CFU/g.
The ubiquitous distribution of this bacterium in the environment, the ability to grow in the cold and its pathogenic potential make this pathogen particular concern for the safety of refrigerated and Ready To Eat (RTE) food (Kathariou, 2002). However, *L. monocytogenes* has been isolated in several raw foods such as milk, cheeses, ice-creams, vegetables, meats (raw or fermented), poultry and seafood, but also in cooked or pasteurized foods as a result of a post-processing contamination (Ryser and Marth, 1991; FAO/WHO, 2004).
Generally, the source of contamination has typically been the food processing environment where this microorganism may enter by various routes and can colonize the processing plant and equipment and can persist sometimes for years. *Listeria* is involved in the formation of biofilms, as it is able to adhere to surfaces including stainless steel, glass, plastic and rubber. For these reasons, contamination occurs widely in food processing environments where *Listeria* can be transferred from surfaces to food and *vice versa* (FAO/WHO, 2004; Ryser and Marth, 2007; Giaouris, et al., 2014).
Its ability to grow over a wide range of unfavourable environmental conditions makes the control of this foodborne pathogen difficult, since it overcomes many food preservation and safety barriers, posing an actual risk to human health.
In contaminated food *L. monocytogenes* is initially present in low amount, nevertheless it is able to grow at refrigeration temperature (2-4 °C, but -1.5 °C is the lower growth limit), low pH (till 4.2-4.3), high salinity (0.5% NaCl) and low activity water (0.91-0.93, but some strains resist at a_w as low as 0.83) (FAO/WHO, 2004; Ryser and Buchanan, 2013; Giaouris et al., 2014; Beaufort et al., 2014).

Even if a lot of food may be contaminated by *L. monocytogenes*, listeriosis is predominately associated with RTE products since they are defined as "*food intended by the producer or the manufacturer for direct human consumption without the need for cooking or other processing effective to eliminate or reduce to an acceptable level micro-organisms of concern*" (Reg. (EC) No 2073/2005); thus, they are characterized by an extended shelf-life at refrigeration temperature and they are in general consumed in their raw state without further cooking or processing (FAO/WHO, 2004; Iannetti et al., 2016). Regulation (EC) No 2073/2005 covers primarily RTE food products and specifies the following food safety criteria for *L. monocytogenes* (Tab. 1.4).

Table 1.4: Food safety criteria for *Listeria monocytogenes* (Reg (EC) No 2073/2005).

Food category	Microorganisms/ their toxins/ metabolites	Sampling plan		Limits		Analytical reference method	Stage where the criterion applies
		n	c	m	M		
1.1. Ready-to-eat foods intended for infants and ready-to-eat foods for special medical purposes (4)	*L. monocytogenes*	10	0	Absence in 25 g		EN/ISO 11290-1	Products placed on the market during their shelf-life
1.2. Ready-to-eat foods able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes	*L. monocytogenes*	5	0	100 cfu/g		EN/ISO 11290-2	Products placed on the market during their shelf-life
		5	0	Absence in 25 g		EN/ISO 11290-1	Before the food has left the immediate control of the food business operator, who has produced it
1.3. Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes (4) (8)	*L. monocytogenes*	5	0	100 cfu/g		EN/ISO 11290-2	Products placed on the market during their shelf-life

(4) Regular testing against the criterion is not useful in normal circumstances for the following ready-to-eat foods: — those which have received heat treatment or other processing effective to eliminate *L. monocytogenes*, when recontamination is not possible after this treatment (e.g. products heat treated in their final package), — fresh, uncut and unprocessed vegetables and fruits, excluding sprouted seeds, — bread, biscuits and similar products, — bottled or packed waters, soft drinks, beer, cider, wine, spirits and similar products, — sugar, honey and confectionery, including cocoa and chocolate products, — live bivalve molluscs.
(8) Products with pH ≤ 4,4 or aw ≤ 0,92, products with pH ≤ 5,0 and aw ≤ 0,94, products with a shelf-life of less than five days are automatically considered to belong to this category. Other categories of products can also belong to this category, subject to scientific justification.

In 2016, among the different RTE food categories and across all sampling stages, *L. monocytogenes* was most frequently detected in 'fishery products', 'fish', 'pork meat products other than fermented sausages' and in 'soft and semi-soft cheeses made from raw milk'. Since they support its growth indeed, they are used as a target food in the zoonoses monitoring activity carried out by the European Centre for Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA) (EFSA Journal, 2017).

Despite the control of *L. monocytogenes* is difficult, due to its widespread presence in nature, intrinsic physiologic resistance, adaptive capacity and ability to grow at low temperature, several strategies are available to prevent microbial growth responsible of this foodborne diseases.
To aid pathogen prevention, the ideal preservation techniques should increase the lag time of bacterial growth while causing little alteration to the food product itself.
Different technologies are applicable, traditional methods like drying, freezing, heating and salting, preserve foods through the elimination of bacteria involving a physical change in the composition of foods making it unsuitable for the pathogenic microorganism. Modern techniques use technology to eliminate bacteria while keeping the food product unaltered, meeting the consumers' request for limited processed

food and/or containing only natural additives: irradiation, modified atmosphere packaging, high pressure processing, ultrasound technology and biopreservation can thus be considered (Fu et al, 2016).
In this frame biopreservation strategies, i.e. using microorganisms or their natural products, such as bacteriocins, to inhibit or inactivate undesired microorganisms in foods, may be effectively exploited to control *L. monocytogenes* in RTE products (Appendini and Hotchkiss, 2002); most commonly utilized bacteria in biopreservation are lactic acid bacteria (LAB), indeed LAB excretes bacteriocins which possess interesting antimicrobial activity.

1.2. Bacteriocins and their application for food security

Foodborne diseases, such as listeriosis, are a global public health issue and food safety becomes an increasingly important international concern. Due to these reasons, the interest on the application of natural antimicrobial agents that target food pathogens is increasing and represents an alternative to the use of traditional methods for food preservation (Cleveland et al., 2001; Fu et al, 2016).
Almost all the bacteria (up to 99%) produce antimicrobial compounds, and in particular bacteriocins. They were first identified in 1925 and defined as "ribosomally synthesised peptides that inhibit the growth of closely related bacteria strains" (Balciunas et al., 2013; Cavera et al., 2015; Etayash et al., 2016). However, producer organisms are immune to their own bacteriocins, a property mediated by specific immunity proteins (Cotter et al., 2005)
The first description of bacteriocins activity was reported in Gram-negative bacteria with the identification of an inhibitory activity of what was later identified as colicin, a bacteriocin produced by *Escherichia coli*. Colicin constitute a diverse group of antibacterial proteins able to kill closely related bacteria by permeabilizing the membrane of the target cell and inhibiting cell wall synthesis (Cleveland et al., 2001; Snyder and Worobo, 2013).
Despite all bacteria produce these antimicrobial compounds, bacteriocins produced by Gram-positive have found large interest for food application. In fact, many bacteriocins are produced by food-grade Lactic Acid Bacteria (LAB) and have been extensively investigated due to their potential as natural food preservatives from safe origin (Cleveland et al., 2001). In particular increasing interest in their applications is due to the fact that bacteriocins have been shown to be: safe for consumption since they are completely digested in the gastrointestinal tract, highly potent (10^3 to 10^6 times more than several antimicrobials) and resistant to the common thermal treatments for pasteurization or in some cases even sterilization (De Vuyst and Leroy, 2007; Bedard and Biron, 2018).
Currently, pediocin and nisin are the most studied bacteriocins and are commercially used as natural preservatives, even if only nisin, produced by *Lactococcus lactis* has been licensed as food preservative; this molecule was recognized as GRAS and approved by the Food and Drug Administration in 1988 and also admitted into the European food additive list as E234. Nisin was first commercialized as Nisaplin, a bacteriocin concentrate manufactured by DuPont Danisco and by now has been largely used in food industry as antibotulinic agent in cheese, liquid eggs and sauces; it exhibits antimicrobial activity also against *L. monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus* and other pathogens (Balciunas et al., 2013). However, the use of pediocin has also been commercially exploited in the form of a food ingredient generated from the pediocin-producing strain *Pediococcus acidilactici* (Mapelli et al., 2018).

1.2.1. Synthesis and mode of action

Bacteriocins are an heterogeneous group of antimicrobial peptides ribosomally synthesized that kill closely related bacteria. Exceptions to this general definition exist in terms of inhibitory spectrum and composition. During years' researchers have discovered and characterized bacteriocins with a relatively broad inhibitory activity spectrum: typically, they can target members of the same species, whereas few of them display broader activity spectra (targeting other species and genera) (Cotter et al., 2013; Etayash et al., 2015).

The mostly known bacteriocins are peptides with around 20-60 amino acid residues in length, the initial synthesis being carried out by the ribosome although the structure may be modified following translation (Snyder and Worobo, 2014). So, these peptides can be defined as small, heat-stable, general cationic, amphiphilic molecules, active against other bacteria and to which the producer organism possesses a specific immunity mechanism.

In general, bacteriocins act via interactions with a specific receptor on the target cell but many distinct mechanisms of action have been found. The interaction with the cell leads to permeabilization of the cell membrane of the target cell, triggering outflow of intracellular components and disintegration of the proton-motive force (Etayash et al., 2015). Bacteriocins are positively charged molecules with hydrophobic patches. Electrostatic interactions with negatively charged phosphate groups on target cell membrane are thought to contribute the initial binding with the target molecule (Cleveland et al., 2001). Some bacteriocins, especially the ones inhibiting Gram-positive bacteria, binds to lipid II on the cell membrane blocking peptidoglycan synthesis or forming pores to restrict or kill their target bacterium, while many bacteriocins that inhibit Gram-negative bacteria can control their target by interfering with DNA, RNA and protein metabolism (Fig. 1.2) (Cotter et al., 2013).

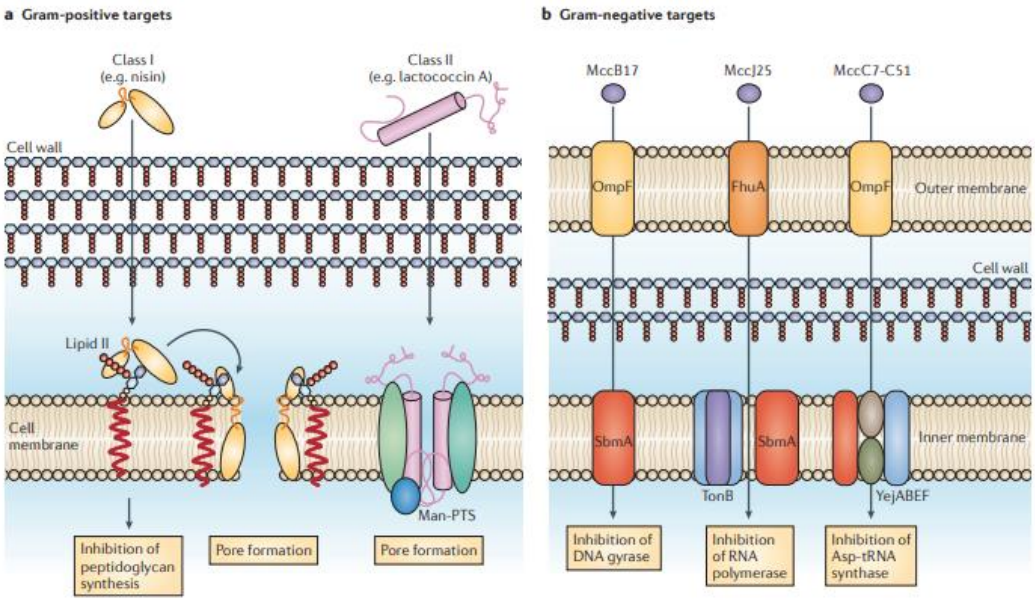

Figure 1.2: Mechanism of action (Cotter et al., 2013).

1.2.2. Bacteriocins classifications

There have been multiple classifications for bacteriocins based on their structure, mode of action, biochemical properties and post-translational modifications. A brief summary of the most common classifications proposed in the last 25 years is reported in Table 1.5.

Class I and II bacteriocins are the most studied and generally described as follows (Eijsink et al., 1998; Cleveland et al., 2001; Drider et al., 2006; Snyder and Worobo, 2013; Etayash et al., 2015):

Class I: small, heat stable, membrane-active antimicrobial peptides with molecular sizes < 5 kDa and containing generally between 19 to more than 50 amino acids. They are called post translationally modified bacteriocins because of the presence of unusual amino acids such as lanthionine or β-methyl lanthionine, dehyudrobutyrine and dehydroalanine. Lantibiotics (class Ia) are typically produced by Gram-positive bacteria, mainly LAB, to target other bacteria species such as *Listeria monocytogenes*, *Clostridium botulinum* and *Enterococcus* spp. They act by membrane pore formation, causing membrane potential dissipation and resulting in metabolite efflux. Molecules are exported outside the cell by a dedicate ABC transporter. The most studied bacteriocin of this class is Nisin which is produced by some strain of *Lactococcus lactis* and is composed of 34 aminoacidic residues.

Class II: small, heat stable, non post-translationally modified peptides, between 30-60 aminoacidic residues. In general, this group is divided into subclasses: IIa, defined as pediocin-like antilisterial bacteriocins with a conserved N-terminal sequence Tyr-Gly-Asn-Gly-Val and two cysteines forming a S-S bridges in the N-terminal half of the peptide; IIb, two components bacteriocins comprised of separate peptide domains, two different peptides are required to form an active poration complex; IIc, thiol-containing bacteriocins resulting in cyclic structures. The main representative bacteriocins of this class are Pediocin synthesized from different species of LAB as *Pediococcus acidilactici*, *Pediococcus pentosaceus Lactobacillus plantarum*; Sakacin produced by *Lactobacillus sakei*; Leucocin produced by *Leuconostoc mesenteroides* and Lactocin produced by *Lactobacillus sakei*, *Lactobacillus casei*, *Lactobacillus rhamnosus*.

Table 1.5: Historic evolution of bacteriocins classification over the last 25 years (Mapelli et al., 2018).

	Klaenhammer (1993)	Cleveland et al. (2001)	Drider et al. (2006)	Cotter et al. (2013)
Class I	**Low molecular weight (<5 kDa), presence of lanthionine and derivatives**	**Low molecular weight (<5 kDa), presence of lanthionine and derivatives**	**Lanthionine or peptide containing** **β-lanthionine** **Sub-classes:** **Ia:** linear molecules **Ib:** globular molecules	**Post-translational modification** **Sub-classes:** lantibiotics linaridins proteusins linearazole- or azoline-containing peptides cyanobactins thiopeptides lasso peptides sactibiotics bottromycins glycocins modified microcins
Class II	**Small thermostable peptides (<10 kDa)** **Sub-classes:** **IIa:** *Listeria* active peptides **IIb:** activity related to two peptides **IIc:** containing an activated thiol	**Small thermostable peptides (<10 kDa)** **Sub-classes:** **IIa:** pediocin-like *Listeria* active peptides **IIb:** composed of two peptides	**Small thermostable peptide (<10 kDa)** **Sub-classes:** **IIa:** high specifity against *L. monocytogenes* **IIb:** composed of two peptides **IIc:** covalent bond between C and N terminals	**Unmodified or cyclic peptides** **Sub-classes:** **IIa:** possess a conserved YGNGV motif **IIb:** two peptides **IIc:** cyclic peptides **IId:** unmodified, linear, non-pediocin-like, single-peptide **IIe:** contain a serine-rich carboxy-terminal region with a non-ribosomal siderophore-type modification
Class III	**High molecular weight (>30 kDa) thermolabile peptides**	**High molecular weight (>30 kDa) thermolabile peptides**	**Large thermolabile peptides (>30 kDa)**	*Not contemplated*
Class IV	**Large peptides complexed with carbohydrates or lipids**	*Not contemplated*	*Not contemplated*	*Not contemplated*

1.3. Class IIa

Within class II bacteriocins, the largest and most extensively studied sub-group is represented by class IIa, that are strong inhibitors of the foodborne pathogen *L. monocytogenes*. Because of this antilisterial effectiveness and because produced by LAB these molecules have emerged as one of the most interesting group of antimicrobial peptides and have significant potential as biopreservatives in a large numer of foods (Ennahar et al., 2000; Drider et al., 2006). In fact, all class IIa bacteriocins are produced by food-associated strains of LAB and some bacteria produce more than one bacteriocin. These bacteriocins have been indeed isolated from a wide variety of LAB, as *Pediococcus spp*., *Leuconostoc spp*., *Lactobacillus spp*., *Streptococcus spp*. and *Enterococcus spp*. in various environments including dairy products, fermented meat and vegetables (Cui et al., 2012).

1.3.1. General nature of the class

Class IIa bacteriocins are 37-48 aminoacidic residues basic peptides (isoelectric point varying from 8.3 to 10.0) showing high sequence similarity (40-60%). The molecular structure is amphipathic, with a highly conserved, hydrophilic, and positively charged N-terminal region containing the consensus sequence - YGNGV - and a C-terminal region having a high content of nonpolar and small amino acid residues and a low sequence similarity (Ennahar et al., 2000), Figure 1.3 reported the aminoacidic sequences of the main studied bacteriocins. It was suggested that this conserved region is a part of the recognition sequence for the membrane-bound protein receptor.

They are also characterized by the presence of two conserved cysteine residues forming a disulphide bridge, that seems to be related to the antimicrobial activity exerted (Ennahar et al., 2000; Nes and Holo 2000; Drider et al., 2006). The N-terminal part is believed to consist of a β-sheets like domain which is structurally stabilized by the N-terminal disulphide bridge while the C-terminal domain has been predicted to adopt an amphiphilic α-helix conformation.

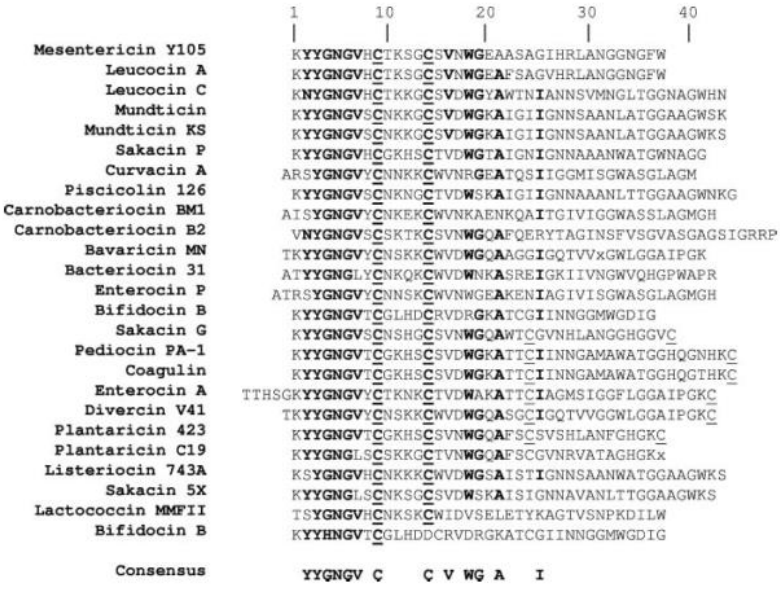

Figure 1.3: Sequence alignment of mature class IIa bacteriocins (Drider et al., 2006).

Some class IIa bacteriocins, like pediocin PA-1 and enterocin A, present an additional disulfide bridge and show a broader spectrum of action than other members of this class, however they also show a dramatic decrease of their antimicrobial activity after addition of reducing agents like β-mercaptoethanol or dithiothreitol, revealing that this additional disulfide bridge may be crucial for bacteriocin activity (Ennahar et al., 2000; Drider et al., 2006); the exact role of disulfide bridges in influencing stability, spectrum of action and bacteriocins activity, should be further investigated as it is not yet fully understood.

1.3.2. Biosynthesis and secretion

Bacteriocin production is often correlated with the presence of a plasmids, genes have been shown to be located on chromosome fragments. At least four genes organized in operons are required for the production of class IIa bacteriocins: a bacteriocin structural gene encoding a prebacteriocin, an immunity gene encoding an immunity protein, a gene encoding an ABC (ATP-binding cassette) transporter necessary for the secretion and a gene encoding accessory protein for bacteriocin extracellular traslocation (Drider et al., 2006; Cui et al., 2012).

Class IIa bacteriocins are ribosomally synthesized pre-peptides that are biologically inactive. During their export, N-terminal extensions, called leader sequences, are cleaved off to generate the biologically active peptides and transferred across the cytoplasmatic membrane by adenosine triphosphate-binding cassette transporters (ABC transporters) and their accessory proteins (Ennahar et al., 2000; Drider et al., 2006; Bali et al., 2015). The presence of two conserved glycine residues in these leaders may serve as a recognition signal for protein processing and secretion. In fact, Drider et al. (2006), supposed that the pre-sequence plays a dual role in biosynthesis: a protective role at the cytosolic side by keeping the bacteriocin inactive and protecting the producer organism; and also as the recognition signal during export.

Production is regulated by a Quorum Sensing systems, a cell density-dependent mechanism, composed of three gene products, typically include an inducer factor, a histidine protein kinase and a response regulator as reported in the figure below (Fig. 1.4).

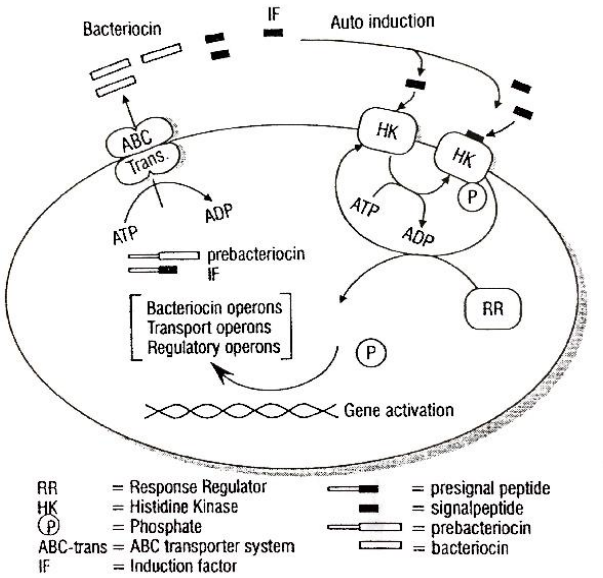

Figure 1.4: Schematic overview of the regulation/production of class II bacteriocins in a cell (Nes et al., 1996).

Nevertheless, environmental factors can affect the biosynthesis of several class IIa bacteriocins like growth temperature, ionic strength and pH; for examples bacteriocins production seems to be highest at temperature near 20 °C and strongly decreased to zero at temperature higher than 35 °C.
As regards the last immunity gene, in all the investigated cases it was reported the presence of the immunity proteins that protect the bacteriocin producer against its cognate bacteriocin. Usually, immunity proteins consist of 50 to 150 aminoacids residues.

Bacteriocins are produced by microbial fermentation but, in most cases, the low and problematic purification yields seriously limit their use on a large scale. To overcome these limitations, chemical synthesis has been proposed and recent advances in peptide synthesis methodologies have allowed for the preparation of several bacteriocins. However, as class II bacteriocins are peptides containing 25-70 aminoacids, this approach results currently difficult: generally, the synthetic approach is more appropriate and efficient for low-molecular weight peptides (<6 kDa); several studies reported the possibility to use a synthetic way for bacteriocins production, nevertheless complete details about the synthesis protocol are often missing. Recently, Bedard and Biron (2018) summarized the advances made for class II bacteriocins chemically synthetized and reported the most successful methodologies employed to date; an example is reported in brief in Table 1.6.

Table 1.6: Synthetic class IIa bacteriocins and methods used for their production (Bedard and Biron, 2018).

Bacteriocin	AA	Sequence	Method	Yield[a]
Sakacin P	43	KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK	Boc-SPPS	10%[b]
Curvacin A	41	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	Boc-SPPS	3%[b]
Leucocin A	37	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	Boc-SPPS	3%[b]
			Fmoc-SPPS + couplings at 50°C	6%[c]
			NCL	12%[d]
Leucocin A analogs	37	KYYGNGVHXTKSGXSVNWGEAFSAGVHRLANGGNGFW	Fmoc-SPPS + Pseudoproline	<1–3%[e,f]
Pediocin PA-1	44	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC	Boc-SPPS	1%[b]
[M31Nle]-Pediocin PA-1 and its analogs	44	KYYGNGVTXGKHSXSVDWGKATTCIINNGAZAWATGGHQGNHKC	Fmoc-SPPS + couplings at 50°C	3.8%[g]
			Fmoc-SPPS + Pseudoproline	<1%[f]
Mesentericin Y105	37	KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW	Fmoc-SPPS	n.r.[h]
Enterocin CRL35	43	KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS	Fmoc-SPPS	n.r.[i]
Lactococcin MMFFII	37	TSYGNGVHCNKSKCWIDVSELETYKAGTVSNPKDILW	Fmoc-SPPS	n.r.[j]

n.r.: not reported; X = allylglycine, Ser, Phe, norvaline, or Cys; Z = Norleucine (Nle). [a]Overall isolated yield for purified bacteriocins; [b]sequence analysis revealed 85–95% purity for pediocin PA-1 and leucocin A and 70–80% purity for sakacin P and curvacin A (Fimland et al., 1996); [c]leucocin A enantiomer (all-D leucocin A) and amino acids after position 20 were coupled with HATU at 50°C in DMF (Yan et al., 2000); [d]calculated overall yield based on yields obtained for each step (fragment 1 (33%) × fragment 2 (55%) × NCL (98%) × cyclization (70%)) (Bodapati et al., 2013); [e]overall yields based on resin loading (Derksen et al., 2006); [f]overall yields based on resin loading (Derksen et al., 2008); [g]yield for [31Nle]-pediocin PA-1 (Kaur et al., 2004); [h](Fleury et al., 1996; Castano et al., 2005); [i](Masias et al., 2017); [j](Ferchichi et al., 2001).

1.3.3. Mode of action

Bacteriocins are able to permeabilize the membranes of target microorganisms through the formation of pores by their hydrophobic C-terminus, a schematic representation of this mechanism is reported in Fig. 1.5. A consequence is the leakage of intracellular material and the dissipation of the proton motive force. Class IIa bacteriocins provoke a total dissipation of the pH gradient and a partial dissipation of the transmembrane potential. A depletion of ATP is observed probably due to an accelerated consumption in order to restore the proton motive force and to the phosphate efflux.

Permeabilization follows bacteriocin-membrane interaction and involves the formation of membrane channels by a multi-step process of binding, insertion, aggregation and finally the formation of poration complex (Klaenhammer et al., 1993).

The initial step is generally believed to be an electrostatic binding: the positive charges of class IIa bacteriocins interacted with anionic phospholipids of target cells, allowing the insertion of an amphipatic portion in the membrane. After the binding, an interaction between the hydrophobic domain within the C-terminal and the lipid acyl chains occurs; this binding seems to be crucial for the pore formation process (Ennahar et al., 2000; Drider et al., 2006).

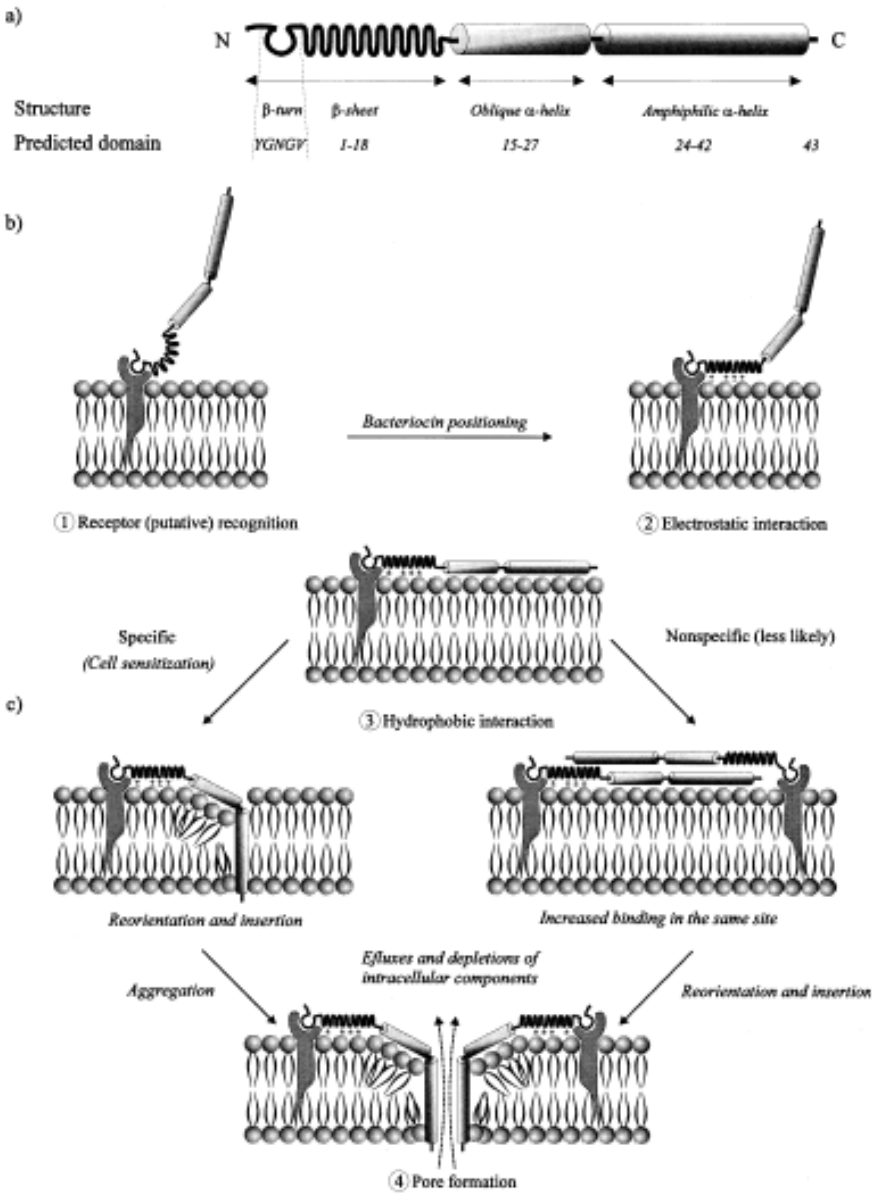

Figure 1.5: Schematic representation of class IIa bacteriocins structure and the predicted location into target cell membrane: a) bacteriocins structural domains, b) possible interaction with membrane surface and c) bacteriocin insertion and pore formation (Ennhar et al., 2000).

Ríos Colombo et al. (2018) proposed the following mechanism of action (Fig. 1.6): bacteriocins remain unstructured in the extracellular medium, positive charged residues mediate the initial non-specific

binding with the negatively charged phospholipids of the target. Subsequently two methods are proposed: a) the bacteriocin would bind the receptor, leading to the irreversible formation of an intrinsic channel; or b) the bacteriocin would employ the receptor as a docking molecule to bring the peptide closer to the plasma membrane, allowing the subsequent bacteriocin insertion and oligomerization to form a pore.

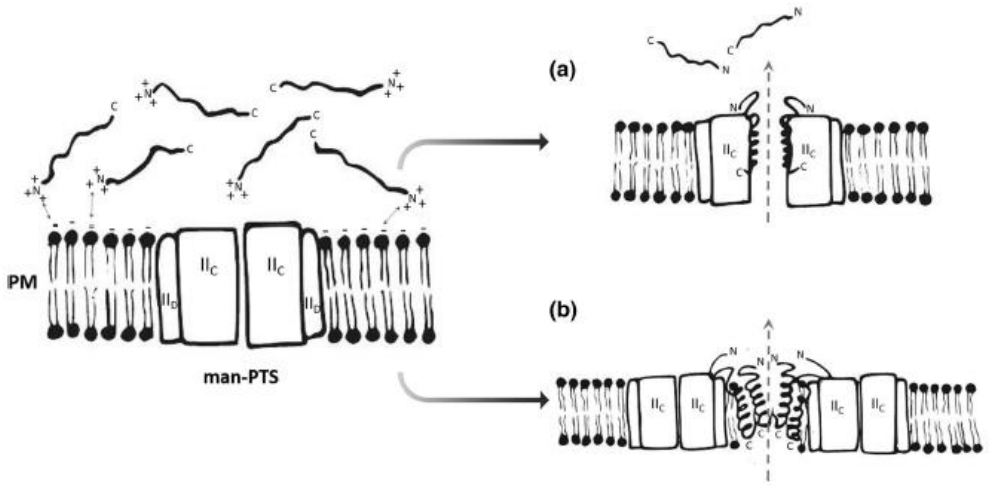

Figure 1.6: Proposed model for class IIa bacteriocins mechanism of action (Colombo et al., 2018).

1.3.4. Spectrum of activity

The antimicrobial spectrum of activity is defined as the range of bacterial types against which the bacteriocin is effective. The list of target pathogenic microorganisms includes *Clostridium perfrigens, C. botulinum, S. aureus* and in particular species of the genus *Listeria* (Balciunas et al., 2013). In general, a lot of species have been reported to be sensitive as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, *Staphylococcus*, *Streptococcus*, *Clostridium* and *Bacillus*. Nevertheless, the spectrum of activity depends on each specific bacteriocin. Some bacteriocins have been shows to prevent the outgrowth of spores and vegetative cells of *Clostridium spp*. All the class IIa are described to be active against *Listeria monocytogenes* (Ennhar et al., 2000).

1.3.5. Food applications

Because of their antilisterial effectiveness, class IIa bacteriocins possess a significant potential as biopreservatives. The use of bacteriocins in food preservation may offer several benefits such as safety and their use can reduce the need of chemical preservatives and thermal treatments on food products, thus meeting the consumers request for minimally-processed food containing natural additives (Campos et al., 2013). For this reason, they are used in several applications, among which are biopreservation, shelf-life extension, clinical antimicrobial action and control of fermentation flora (Balciunas et al., 2013).
The source of bacteriocins for food security application can be either the purified compound, the crude bacterial grown culture, as well as the bacteriocin-producing organism itself.
There are two basic methods of applying bacteriocins to food:

- *in situ*, by adding bacteriocin producing bacteria directly on food product;
- *ex situ* with the addition of semi-purified or purified peptides, however this last technique requires specific approval as preservatives from the legislative viewpoint.

Food can also be supplemented with bacteriocins produced in the form of raw concentrate obtained by cultivation of the producer strain in a food-grade substrate; in this case the preparation may be regarded as food additive or ingredient by-passing any legal limitations (Cotter et al., 2005; Balciunas et al., 2013).
To the best of our knowledge, pediocin is the only purified class IIa bacteriocins commercialized up to now in a concentrated form (i.e. available from Sigma-Aldrich); nevertheless, many bacteriocins containing products are commercially available as food ingredients that can serve as an effective barrier to help control the development of *Listeria monocytogenes* and other susceptible microorganisms. Kerry Bioscience produced "ALTA 2341", described by the manufacturer as a crude fermentation LAB product containing Pediocin (Drider et al., 2006; Lopez-Cuellar et al., 2016). Different bacteriocins sources, including class IIa bacteriocins are available from Chr. Hansen: "Bactoferm™ F-LC" a mixed culture contains *L. curvatus*, the producer of curvacin A (identical to sakacin A under a molecular point of view), whereas "Bactoferm™ B-2" and "Bactoferm™ B-FM" contain *L. sakei*. DuPont Danisco also produces antimicrobial fermentates under the trade mark "MicroGard™" with antilisterial and anti-Gram-negatives activity. To date, no sakacin has been commercialized in a pure, concentrate or enriched form.
Once released, the activity of bacteriocins may not be uniform and constant, depending on the chemical composition and physical conditions of food. It mainly depends on pH, on presence of NaCl, on the formation of binding to food component as fat, on activity of proteases or other enzymes (Balciunas et al., 2013)
In this field, an interesting solution for applying class IIa bacteriocins to food is to deliver them as a part of an active packaging; this mechanism may represent an efficient solution to control the release of the antimicrobial agent during food storage, maintaining its antimicrobial activity over time (Papagianni et a., 2003).

1.4. Active antimicrobial packaging in brief

Active packaging was defined as "packaging in which subsidiary constituents have been deliberately included in or on either the packaging material or the package headspace to enhance the performance of the package system" (Robertson, 2006). It is the results of innovation in packaging required by consumers for packaging which could provide quality maintenance, shelf-life extension and safety assurance on food. Active packaging solutions can be classified in two main categories: scavengers, aim to adsorb specific elements responsible for the degradation of food such as oxygen or ethylene; and release systems, aimed at releasing substances into the environment surrounding the food, such as carbon dioxide or antibacterial molecules (Lavoine et al., 2015).
The majority of research publications on emerging active packaging technologies have been focused on antimicrobial packaging. Suppakul et al. (2003) defined antimicrobial packaging as "a subset of active packaging in which the package, the product and the environment interact to extend the lag phase and/or reduce the growth rate of microorganisms in order to prolong shelf-life". Antimicrobial packaging is obtained by adding antimicrobial substances to "raw" packaging materials to extend the lag phase and reduce the growth rate of microorganism. Antimicrobial packaging can be classified according to the packaging material and the origin of antimicrobial substances used. It is possible to find polymeric, biopolymeric and cellulose-based package materials or films incorporated with different substances generally selected on the bases of their spectrum of activity, chemical composition, and mode of action. For examples:

- organic acids: acetic, lactic, sorbic acid;

- chemicals: potassium sorbate, sodium lactate, chelating agents (EDTA);
- metals: silver, titanium;
- essential oils and/or their components: thymol, cinnamaldehyde, allicin, carvacrol;
- enzymes: lysozyme, peroxidase;
- bacteriocins: nisin, pediocin;
- surfactants: lauric arginate (LAE).

Antimicrobials packaging systems can be developed into different ways depending on the correlation between the active substances and the matrix: 1. antimicrobial coatings onto packaging structure surfaces, 2. direct incorporation of antimicrobial agent into packaging matrix, 3. chemical immobilization of antimicrobial to polymers by ion or covalent linkage, 4. addition of sachet/pads containing the volatile substance into the package (Sung et al, 2013; Miranda et al., 2016).

One of the advantage of active packaging may be represented by the higher antimicrobial efficiency observed rather than the direct addition of the agent into food, and by the continuous antimicrobial effect during food shelf-life.

In this frame, bacteriocins produced by LAB have gradually gained popularity due to their ability to resist to high temperature and acid conditions and due to their GRAS classification.

Several experiment studies have been published on this topic as reported in Table 1.7.

Table 1.7: Active packaging solution containing class IIa bacteriocins

Bacteriocin	Type of packaging	Bacteriocin application	Tested food	References
Sakacin A	Polyethylene-coated paper	Gelatin coating	Thin-cut veal meat slices	Barbiroli et al. 2017
Pediocin ALTA™ 2341	Cellulose acetate	Incorporation	Sliced bologna	Espitia et al. 2013
Sakacin A	Pullulan	Incorporation	Sliced turkey deli meat	Trinetta et al. 2010
Pediocin ALTA™ 2341	Cellulose acetate	Incorporation	Sliced ham	Santiago-Silva et al. 2009
Bac162W produced by *Lactobacillus curvatus* and BacAM09 produced by *L. plantarum*	Polyethylene	Coating	*In-vitro*	La Storia et al. 2008
Enterocins A and B, Sakacin K	Polypropylene Polyamide interleaves	Spreading	Sliced cooked ham	Jofré et al. 2007
Bacteriocin produced by *L. curvatus* 32Y	Polyethylene	Spray coating	Pork frankfurters	Ercolini et al. 2006
Bacteriocin produced by *L. curvatus* 32Y	Polyethylene	Spray coating	Pork steaks, hamburgers	Mauriello et al. 2004
Pediocin	Plastic packaging bags	Coating	*In-vitro*	Ming et al. 1997

1.5. The case of study: bacteriocin Sakacin A

Sakacin A is a class IIa bacteriocin produced by the LAB *Lactobacillus sakei* DSMZ 6333 (Lb706) (DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) that has been shown to inhibit lactic acid bacteria and *Listeria monocytogenes*.

1.5.1. *Lactobacillus sakei*

L. sakei has been isolated from several raw food products, is one of the most important lactic acid bacteria of meat and play an important role in the fermentation of meat products. First described almost a century ago as a contaminant of the rice wine "saké", this microorganism is able to colonize different habitats: it has often been isolated from food of vegetable sources like flours, sourdoughs, or fermented cabbages but is systematically associated with meat products and exhibits properties that allow for better preservation and storage of fresh meats and fish; in fact, its capacity to adapt to meat environmental and its use for biopreservation or as starter for fermented meat products are remarkable. *L. sakei* is a Gram-positive bacterium, facultative anaerobic, heterofermentant, which groups by pair or in short chains.

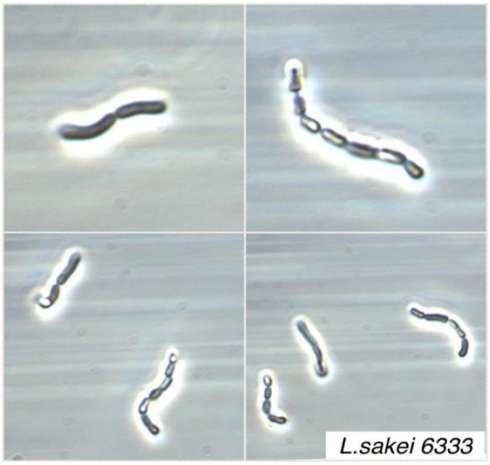

Figure 1.7: Genus*: Lactobacillus*; Species: *sakei*. Picture obtained under an Optical Microscope (Zeiss, 100X).

Generally, it ferments glucose and fructose: the hexose fermentation is homolactic and proceeds through the glycolysis and with the formation of lactic acid, however, meat is a relatively poor substrate for sugars and richness in aminoacid and peptides. Arginine, inosine and adenosine can be used as energy sources by all the strains. Nevertheless, *L. sakei* is not able to use arginine as the sole carbon source for growth; arginine degradation is governed by the arginine deiminase pathway which leads to NH_3 and ATP production. This pathway is composed of three enzymes: arginine deiminase, catabolic ornithine transferase, carbamate kinase and also an arginine/ornithine antiporter is present. This pathway seems to not confer an advantage for the growth, however it enhances the survival of microorganism because of the ATP produced and not as a consequence of ammonia production (Zagorec and Champomier-Vergès, 2017). *L. sakei's* metabolism is reported in detail in the figure below (Fig. 1.8).

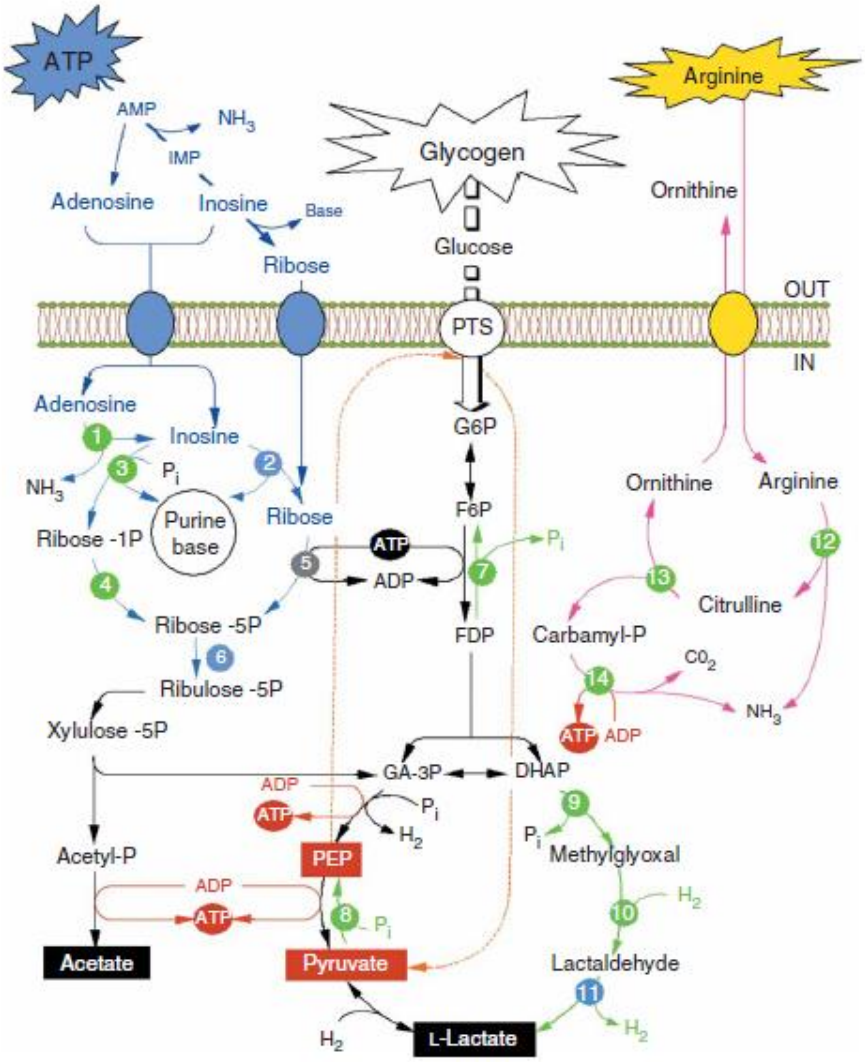

Figure 1.8: Energetic pathway *L. sakei* 23K (Chaillou et al., 2005).

L. sakei has been studied for the production of different bacteriocins. Three bacteriocins, sakacin A, sakacin P and lactocin S, have been isolated and well characterized in *L. sakei* (Tab. 1.8) (Champomier-Vergès et al., 2002).

Nevertheless, in the literature sakacins nomenclature is almost confused: different authors often named differently what represents at the end the same molecule; for this reason, literature comparison is difficult. To the best of our knowledge the difference between the different sakacins is probably ascribable only to a variability in the aminoacidic sequence or to differences in the spectrum of activity.

Table 1.8: Biochemical characteristics and inhibitory spectrum of bacteriocins produced by *L. sakei* (Champomier-Vergès et al., 2002)

Bacteriocin	Producers	Biochemical characteristics	Inhibitory spectrum
Sakacin A *(Curvacin A)*	*L. sakei* L706 *L. curvatus* LTH1174	Class IIa 4309 Da 41 aminoacids hydrophobic	*Listeria monocytogenes,* *L. sakei, L. curvatus,* *Staphylococcus aureus,* *Lactobacillus brevi,* *Lactobacillus cremoris,* *Enterococcus spp.,* *Leuconostoc paramesenteroides*
Sakacin P	*L. sakei* LTH673 *L. sakei* L674	Class IIa 4436 Da 43 aminoacids hydrophobic	*L. sakei, L. curvatus,* *Lactobacillus delbruecki,* *Lactobacillus pentosus,* *Lactobacillus plantarum,* *Lactobacillus reuteri,* *Listeria ivanovii,* *L. monocytogenes,* *E. faecalis*
Sakacin M *(Lactocin S)*	*L. sakei* L45 *L. sakei* 148	3769 Da 33 aminoacids 50% nonpolar AA	*Lactobacillus spp.* *Leuconostoc spp.* *Pediococcus spp.*

In the present research, sakacin A was chosen as the focus of the entire project.

1.5.2. Molecular characteristics

Sakacin A coding sequence is located onto the *L. sakei* largest plasmid (60 kb) (Holck et al., 1992). The structural gene coding for sakacin A spans an open reading frame of 177 bp and is referred to as *sapA*. Putative promoter sequences, designated P1, were found upstream of *sapA*. The gene is located in the strongly expressed operon upstream to the *saiA* gene, coding for a 90 amino acids peptide whose function is related to sakacin A immunity (Axelsson and Holck 1995).

The protein encoded by *sapA* gene, sakacin A indeed, is a 59 amino acids prepeptide whose first 18 residues are proteolytically cleaved to give rise to the mature form. The resulting 41 amino acids peptide shows a molecular mass of 4308.9 Da (Holck et al., 1992). The mature form isoelectric point is 9.31 with the charged amino acids located in the N-terminal hydrophilic region. Thus, sakacin A shows a net charge of +3 in a wide range of pH (Ennahar et al., 2000). The C-terminal part of the peptide consists of pairs of hydrophobic amino acid residues regularly spaced by glycine and serine (Fig. 1.9). Cysteine 28 and Cysteine 33 are involved in a disulfide bridge, however, their oxidation state only moderately influence protein activity indicating that these residues are not directly involved in protein function (Holck et al., 1992).

```
 1  Ala-Arg-Ser-Tyr-Gly-Asn-Gly-Val-Tyr-Cys-  10
11  Asn-Asn-Lys-Lys-Cys-Trp-Val-Asn-Arg-Gly-  20
21  Glu-Ala-Thr-Gln-Ser-Ile-Ile-Gly-Gly-Met-  30
31  Ile-Ser-Gly-Trp-Ala-Ser-Gly-Leu-Ala-Gly-  40
41  Met-***
```

Figure 1.9: Aminoacid sequence of sakacin A (Holck et al., 1992).

Sakacin A presents a 100% sequence identity with the bacteriocin produced from *L. curvatus*, curvacin A; although these two proteins could be considered equivalent under a molecular point of view, small differences are reported in the gene cluster organization by Axelsson and colleagues (1995). *L. sakei* and *L. curvatus* are clearly separated at the genomic level (40 to 50% identity) but are phenotypically highly related and difficult to separate (Champomier-Vergès et al., 2002). Moreover, curvacin A seems to be expressed only in the late exponential phase (Tichaczek et al., 1993), whereas sakacin A is expressed during the whole exponential growth phase. With these premises, structural and functional information obtained on the curvacin A peptide can be transferred to describe sakacin A. Figure 1.10 reports the protein structure available for curvacin A (Haugen et al., 2005) where the C-terminal hydrophobic α-helical part is highlighted (A) as well as the disulfide bridge in the N-terminal region (B).

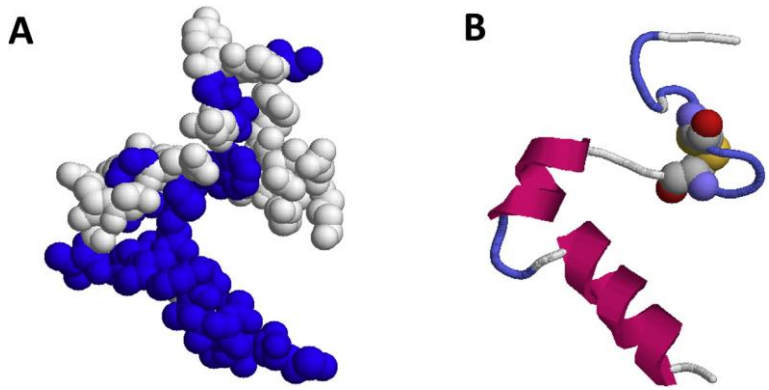

Figure 1.10: Spacefill (A) and cartoon (B) representations of curvacin A (i.e. sakacin A) as inserted in the membrane. Hydrophobic and hydrophilic residues are in blue and gray, respectively. Alpha-helixes are in magenta. Cysteines are shown as spacefill and CPK colored. PDB 2A2B file resolved by Haugen et al. (2005).

1.5.3. Mechanism of action

Sakacin A is a secreted protein, whose extracellular fate is determined by the cleaved recognition signal, which is fundamental for the transmembrane translocation of the mature molecule (Havarstein et al., 1995).

Sakacin A, like other class IIa bacteriocins, acts primarily by permeabilizing *Listeria* membrane, dissipating transmembrane potential (ΔΨ) and pH gradient (ΔpH) through the formation of pores. This leads to the almost complete depletion of intracellular ATP pool. The hypothesized mechanism of action follows the 'barrel-stave model': the initial step is supposed to be an electrostatic interaction between the hydrophilic, positively charged portion of sakacin A and the anionic phospholipid heads in the membrane, putatively through the involvement of a receptor molecule that could easily bind the protein YGNGV motif. Subsequently the hydrophobic C-terminal half interacts with the lipid acyl chains inserting in the membrane, ultimately leading to the pore formation (Ennahar et al., 2000).

Permeabilization of cell membrane is not the only mechanism of action of sakacin A since also cell wall is reported to be a target for the bacteriocin. Indeed, sakacin A could also act by breaking the bond formed by the N-acetyl muramic acid (NAM)-linked L-alanine residue nearest to the polysaccharide chain. This

activity accompanies the capability of sakacin A in hydrolyzing the β-1,4 glycosidic bond in NAM-NAG units, ultimately complementing the effects on the sensitive bacteria (Trinetta et al., 2012).

1.5.4. Production process

Sakacin A can be produced by *L. sakei* in stationary liquid cultures: the maximum production levels are reached during the exponential growth phase and decreased as the cells entered the stationary phase; sakacin A production is thus growth-related and following the primary metabolite kinetic, can be classified as type I Gaden fermentation process (Leroy and de Vuyst, 1999). *L. sakei* is generally grown in MRS broth (DeMan-Rogosa-Sharpe) at incubation temperature between 25 and 30 °C (Holck et al., 1992; Trinetta et al., 2008); however, this medium is expensive and not suitable for industrial application; Trinetta and co-authors (2008) developed a cheaper alternative culture medium formulation containing raw ingredients of industrial use that allowed also to increase sakacin A yield, expressed in terms of Activity Units (AU/mL) (Trinetta et al., 2008).

1.5.5. Sakacin applications

Besides the use as starters in meat products, the evidenced antibacterial, bacteriostatic and bactericidal properties of *L. sakei* have been explored and developed. Some studies have already demonstrated the antilisterial effect of sakacin applied *in situ* in food products. Bredholt et al. (1999 and 2001) exploited these properties against *L. monocytogenes* and *Escherichia coli* O157:H7 employing this LAB strain as protective cultures in cooked ham and sausage. Jones et al. (2009 and 2010) tested the anti–*Listeria* properties of several *Lactobacillus sakei* strains inoculated in vacuum-packaged lamb and beef during 12 weeks, lamb inoculated with the Sakacin A producer *L. sakei* Lb706 had lower *Listeria monocytogenes* populations than lamb inoculated with a bacteriocin-negative variant. Martinez et al. (2015) demonstrated the antilisterial effect of *Lactobacillus sakei* subsp. *sakei* 2a inoculated in a potentially symbiotic cheese spread for 28 days.

As regards *ex situ* application Trinetta et al. (2010) was the first study that demonstrated the possibility to incorporate sakacin A in active packaging solution, in this study authors set up a sakacin A-containing pullulan films and proved the reduction of *L. monocytogenes* population intentionally inoculated in sliced turkey breast after 3 weeks under refrigerated storage. Also Barbiroli et al. (2017) incorporated a sakacin A preparation into a gelatine coating and applied it on the plastic side of a polyethylene-coated paper wrapping, the efficacy of the developed active solutions was demonstrated on thin-cut veat meat slices intentionally inoculated with *Listeria innocua*.

The use of sakacin A, not only as food starter in meat sector, but also as food preservatives is still in its infancy: even if *in situ* application is up to now the only authorized approach, *ex situ* application represents a promising alternative solution.

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Chapter 2:
OBJECTIVES

2. Objective of the thesis

The main objective of the present Doctoral Thesis is the exploration of the use of whey permeate as a cheap substrate for the growth of the bacterial species *Lactobacillus sakei*, able to produce the bacteriocin sakacin A; the optimization of its production process as well as its food-grade isolation was set-up, for the bacteriocin application in active packaging solutions.

2.1. Milestones

Here follows a list of the specific milestones achieved:

- *L. sakei* growth characterization and sakacin A production in MRS medium.
- Development of a low cost and food-grade culture medium formulation using whey permeate, a valuable raw material, and characterization of the bacteriocin production.
- Optimization of sakacin A purification yields and sakacin A recovery.
- Production and purification of sakacin A extract in valuable amounts in a pilot-scale submerged bioreactor applying a food-grade enrichment process.
- Development of different strategies to apply sakacin A in active packaging solutions using cellulose nanofibers.
- Evaluation of the influence of the conjugation between sakacin A and cellulose nanofibers on the retention of the antimicrobial activity.
- Application of the developed active material solutions in *in vitro* trials against the food pathogen surrogate *Listeria innocua* as well as in *in vivo* experiments on smoked salmon fillets.

This thesis has been structured in two main sections: the first relates to all that concerns sakacin A production and the recovery of a protein extract that exhibits an antimicrobial activity; the second section groups all the tested applications of this extract in several antimicrobial packaging solutions employing strategies possessing future feasibility for food systems.

Chapter 3:

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3.1. Sakacin A production and optimization

Abstract

In the present research *Lactobacillus sakei* Lb 706 growth and its bacteriocin production, sakacin A, were characterized in MRS medium and in SAK media, comparatively. This last is a new low cost culture medium formulated employing whey permeate, a food waste from the dairy industry, developed in order to reduce sakacin A process cost and reach a food-grade production. Sakacin A production was evaluated in both media in terms of Activity Units/mL.

A protein extract enriched in sakacin A was recovered using two different methods: a classical ammonium sulphate purification and reverse phase (RP) chromatography with a PoraPack TM RXN RP cartridge. This last purification method showed a higher yield that allowed to recover 53% of activity units initially present in culture medium while only 25% were recovered applying the classical technique. Moreover, RP chromatography resulted cheaper than traditional one with a production cost estimated around 0.06 €/1000 AU *vs.* 0.115 €/1000 AU. The production process was studied and optimized in order to improve final yield and obtain high amount of sakacin A for food industrial application, as for example for incorporation in packaging materials.

3.2. Introduction

The new concept of healthy food promotes scientists to search for new antibacterial compounds for food preservation. Food preservation is carried out to maintain the quality of raw materials, their physicochemical properties as well as the functional quality of the final product, providing safe and stable food items (Bharti et al., 2015). Bacteriocins and bacteriocins-producing strains can represent a very attractive alternative to the currently used preservatives due to their bactericidal or bacteriostatic effects, without any toxic consequence to human health or change in sensory features of food (Garsa et al., 2014; Sidooski et al., 2018). Bacteriocins are of very high perspective because they may be used not only for food biopreservation, but also as antibiotics, in animal health care and in marine environment (Cotter et al., 2013; Kaskoniene et al., 2017). Among the more than 230 bacteriocins from Lactic Acid Bacteria (LAB) have been isolated and characterized, nisin is the most popular and extensively investigated probably because it is the only bacteriocin approved as additive in several food categories. In general, bacteriocins from LAB have been isolated from different food and non-food matrices and exhibit a broad spectrum of antimicrobial activity at low concentration against many food spoilage and pathogenic bacteria. In addition, they are heat tolerant, active at acidic pH and do not modify the flavour when used in food systems (Garsa et al., 2014; Kaskoniene et al., 2017).

Nevertheless, one of the main concerns related to the application of bacteriocins as food biopreservatives is represented by their low fermentation yields and high production and purification costs. In fact, bacteriocins producers use complex and expensive media, that cannot often be classified as food-grade; moreover, they are formulated with a high protein content that can interfere in the downstream purification process. Several studies have compared different media suitable for bacteriocins production and have identified MRS (de Man, Rogosa and Sharpe) as the best formulation for LAB growth, nevertheless this medium is unsuitable for industrial production due to its high formulation cost. An attractive option under development is the use of food-grade media formulated by using ingredients representing residues or by-products of the dairy and/or agri-food industry to produce food-grade bacteriocins (Garsa et al., 2014).

Currently there is an interest in the use of whey and whey permeate as the basis of food-grade inexpensive fermentations by LAB that require minimum nutritional supplementation. Guerra et al. (2001) investigated the ability of *Lactococcus lactis* subsp. *lactis* and *Pediococcus acidilactici* to produce bacteriocins on both diluted and concentrated whey in batch fermentations; Halami et al. (2005) demonstrated the potentiality of a native isolate *Pediococcus acidilactici* C20 to produce quantifiable amounts of pediocin C20 on whey permeate; Liu et al. (2005) studied the production of nisin by immobilized cell of *Lactococcus lactis* subsp. *lactis* in whey permeate supplemented with yeast extract or casein hydrolysate. It was also demonstrated that yeast extract is one of the most efficient components allowing to increase bacteriocin production, because of the presence of aminoacids and peptides that can promote cell growth and induce bacteriocins production (Sidooski et al., 2018).

Whey is obtained after milk coagulation during cheese manufacturing (Fig. 3.1), being the watery fraction correspondent to the 90% volume of the original milk. It contains soluble milk components like lactose, soluble proteins, peptides and aminoacids, minerals and vitamins in traces. Whey proteins can be recovered by ultrafiltration, thus generating a new by-product, named whey permeate, that represents a production waste with high disposal costs posing serious environmental impact. Nevertheless, this permeate still remains a valuable raw material due to the high content of lactose around 80% of the solid content, free aminoacids, minerals and vitamins (Tab. 3.1).

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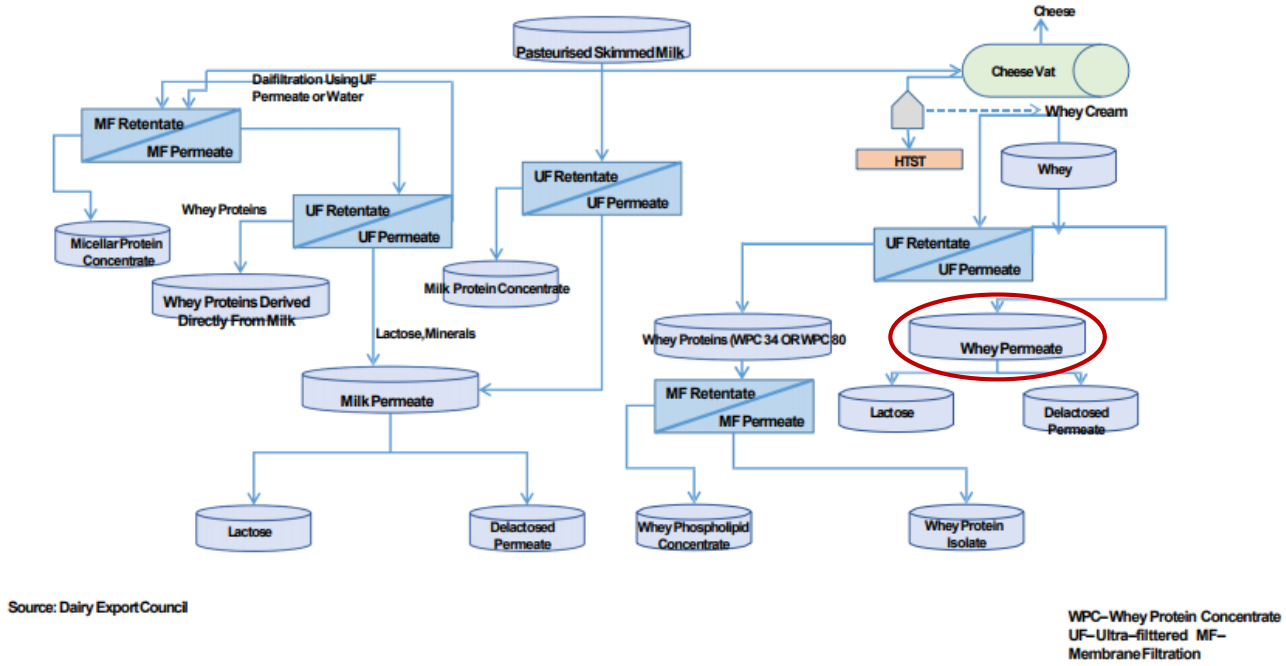

Figure 3.1: Dairy permeate powder manufacturing process. Source: Dairy Expert Council.

Table 3.1: Composition of Whey Permeate Powder (%).

Component	Whey permeate %
Protein	3.50 – 4.00
Fat	0.04 – 0.50
Lactose	82.00 – 88.00
Ash	8.50 – 9.20
Moisture	2.73 – 4.80
Sodium	0.70 – 0.89
Calcium	0.36 – 0.62
Magnesium	0.10 – 0.13
Potasium	2.18 – 5.36

In this frame, whey permeate represents a cheap and higly avaiable substrate for circular economy activities. In fact, permeate market volume is increasing as reported in Tab. 3.2, with a Compound Annual Growth Rate (CAGR) of 4 % for period between 2017-2027. This market volume has an economical relevance, for example in Italy in 2017 it was 26.38 MT having an economical impact of 21.2 US$ Mn. Note that the permeate volume is composed by whey permeate and milk permeate and the first represents more than 80% of the total.

The possible use of this raw material by microbial transformation can represent a suitable solution for recycling a residue of the dairy industry into added-value products with industrial application.

Table 3.2: Western Europe and Italy permeate market volume (MT) analysis (2013-2015) and forecast (2017-2027).

Year	2013	2015	2017	2019	2021	2023	2025	2027	CAGR 2017-2027
Europe	162.43	174.68	188.94	205.48	224.34	244.46	266.36	288.25	+ 4.4 %
Italy	22.86	24.51	26.39	28.55	30.96	33.52	36.22	38.97	+ 4.0 %

Beside bacteriocins production, their purification at the industrial level is the main bottleneck since their purification protocol is generally very expensive and time-consuming. Crude bacteriocins form contains undesirable residual components coming from the initial culture formulation; moreover, purification methods are based on laborious series of steps to concentrate and purify these compounds from the culture supernatant. Usually the most applied protocols exploit ammonium sulphate precipitation, ion-exchange, hydrophobic interaction, gel-filtration and reverse-phase high-pressure liquid chromatography (Garsa et al., 2014). In addition, protocols often involve the use of centrifugation to obtain cell-free supernatant and for protein concentration: in the frame of an industrial scale, this is considered one of the major weakness for bacteriocins application. However, to improve production cost and bacteriocin yield, it is necessary to optimize the final production yield.

Bacteriocins are considered primary metabolites, being produced during the exponential growth phase; their production stops as cells enter the stationary phase, with subsequent loss of activity; for these reasons apart from medium composition and purification process, also pH, temperature and growth kinetics of microorganism influence the final yield and bacteriocins production (Messens et al., 2003; Bharti et al., 2015; Sidooski et al., 2018).

The pH has a significant effect because it affects the enzymatic activity of microorganisms and consequently the production of metabolites, but also regulates the mechanism of adsorption of bacteriocin on producing cells, that occurs in acidic conditions; the optimal pH range for bacteriocin production is generally correlated to the optimum for cell growth.

Bacteriocins production is often described as a temperature-sensitive process: as the pH, temperature has a direct effect on cell growth rate and on the enzymatic activity of the microorganism. Differently from the pH item, the optimum temperature for the production seems to be below the optimum for cell growth. For examples, Aasen et al. (2000) observed that the production of sakacin P by *Lactobacillus sakei* CCUG 42687 was seven times higher at 20 °C rather than 30 °C, that is considered the optimum temperature for the growth of LAB.

The objective of the present research was to develop and optimize a medium alternative to MRS, employing whey permeate, that could be used for *Lactobacillus sakei* growth and production of sakacin A. The aim of developing a low cost culture medium for bacteriocin production came from the need of producing high amount of sakacin A for food industrial application, as for example for incorporation in packaging materials; in this frame, production and purification conditions were also optimized in order to improve final yield. Classical purification techniques employing ammonium sulphate was used in the first part of the research and compared with a new protocol employing a reverse phase chromatography with a PoraPack TM RXN RP cartridge.

3.3. Materials and methods

3.3.1. Microorganism and standard culture conditions

Lactobacillus sakei DSMZ 6333 (Lb706) was chosen as sakacin A-producing strain (DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and maintained on MRS broth (DeMan-Rogosa-Sharpe, Merck K GaA, Darmstadt, Germany). The medium was inoculated (10% v/v) with a pre-grown culture (overnight, 30°C) and incubated overnight in stationary condition at 30 °C.
Stock cultures of the microorganism were stored at -80 °C in their proper liquid medium added with 20% (v/v) glycerol (VWR International, Leuven, Belgium). Cultures were propagated twice before use.

3.3.2. Bacteriocin activity units

Sakacin A antimicrobial activity was evaluated by agar diffusion assays using *Listeria innocua* DSMZ 20649 as indicator strain, maintained on TSB broth (Tryptic Soy Broth, VWR International) and incubated overnight at 37 °C. An aliquot of 300 µL overnight *Listeria* culture was added to 30 mL soft TSA (8 g/L agar) in a Petri dish, cell-free supernatant of *L. sakei* culture was serially diluted in distilled water and poured in wells made on the plate. Samples were incubated overnight at 37 °C. Bacteriocin production was quantified as the reciprocal of the highest dilution that exhibited a clear halo of inhibition and expressed as Activity Units per mL of culture medium (AU/mL) (Trinetta et al., 2008). Antimicrobial activity was also considered in terms of diameter of inhibition halo (cm).

Proteolytic activity of Pronase-E protease (Merck K GaA, 70000 PUK/g) was exploited in the agar diffusion assay to ensure that antimicrobial activity was due to a peptide (i.e., sakacin). 10 µL of Pronase were added in 500 µL of samples containing bacteriocin, activity of samples was evaluated by agar diffusion assay as reported above.

3.3.3. *L. sakei* growth evaluation

Sakacin A production was initially determined in MRS medium as benchmark: *L. sakei* growth and sakacin A production profile was studied, culture turbidity (OD) was measured spectrophotometrically at 600 nm every 15 min in a PowerWave™ XS2 Microplate Spectrophotometer (BioTek, USA); lag phase (min) and maximum growth rate (OD/h) were determined by fitting data through Baranyi and Roberts model on DMFit 3.5 software. Time course of L. sakei growth and bacteriocin production were also investigated in the final optimized medium.

3.3.4. HPLC determinations

Lactic acid production and sugar consumption were determined using an HPLC system (Merck-Hitachi L-7000 System); equipped with RI and UV (210 nm) detectors serially connected, using a (300 - 8 mm) SH1821 column (Shodex, München, Germany), maintained at 50 °C and eluted with 5 mM H_2SO_4 at 0.5 mL/min, and in terms of diameter of inhibition halo (cm) referred to the activity of the bacteriocin produced.

Free aminoacids were analysed in a culture samples formulated with L-arginine (0.5 g/L) or without, by ion-exchange chromatography using a Biochrom 30 + chromatograph automatic aminoacid analyser (Biochrom Ltd., Cambridge, UK) and elution conditions recommended by the manufacturer. A 14-step elution program with six lithium citrate buffers of increasing pH and ionic strength was adopted, with post-column derivatization with ninhydrin and detection at 440 and 570 nm (D'Incecco et al., 2016).

3.3.5. Statistical analysis

Data were submitted to one-way analysis of variance ANOVA performed using GraphPad Prism software (version 8.0.1, San Diego, CA, USA). When the effect was significant ($p < 0.05$), differences between means were assessed by Tukey test of multiple comparison.

3.3.6. Optimization of medium formulation

Several culture media alternative to MRS were formulated with industrial ingredients suitable for large-scale processes, in particular: Yeast extract (YE) (Costantino, Torino, Italy), Meat extract (ME) (Merck K GaA) and the addition of L-arginine (Merck K GaA). All ingredients were dissolved in deproteinized whey permeate, kindly supplied by Latteria Soresina (Soresina, Italy) at the concentrations reported below (Tab. 1).

Tween-80 (Merck K GaA) and a vitamin mix were added in each samples at the concentration of 0.5 g/L and 1 mL/L respectively; the mix had the following composition (g/50 mL): $MgSO_4$ (Sigma-Aldrich, Missouri, USA) 10, $MnSO_4$ (Sigma-Aldrich) 1.9, tiamin (Merck K GaA) 0.01, niacin (Merck K GaA) 0.01, folic acid (Carlo Erba, Cornaredo, Italy) 0.01, pyridoxal (Sigma-Aldrich) 0.01, pantothenic acid (BDH Chemicals, London, England) 0.01, cobalamin (Carlo Erba) 0.01.

- **Design of Experiments**

A general full factorial design with 3 variables tested at 3 levels with two replicates was set-up, for a total of 54 trials, to identify components and/or interactions having significant effect on bacteriocin production. The levels were as follows: A- meat extract 0-4-8 g/L, B- yeast extract 0-4-8 g/L, C- arginine 0-0.5-1 g/L.
All the media were inoculated (0.1% v/v) with a pre-grown culture of *L. sakei* and incubated in stationary condition at 26 °C to promote bacteriocin production.

Multiple factors interactions were determined employing Design Expert® v. 7.0 software (Stat-Ease Inc., Minneapolis, USA), combined with the Student test to evaluate if given terms had significant effect ($p \leq 0.05$). Results are expressed in terms of pH, measured with a pH-meter (FiveEasy™, Mettler Toledo, Schwerzenbach, Swiss), as well as lactic acid produced during the growth of the microorganism, determined through HPLC.
Quality of fitted model equation was expressed by the coefficient of correlation R^2. Statistical parameters and interactions significance were verified by the analysis of variance (ANOVA). Parametric estimations, calculated from the results, were carried out by minimization of the sum of quadratic differences between observed and model-predicted values, using the non-linear least-squares procedure.

Table 3.3: Scheme of the experimental factorial design: arrangement of the experiments (A: Meat Extract; B: yeast Extract; C: Arginine).

Trial	A: ME (g/L)	B: YE (g/L)	C: Arg (g/L)
1	0	0	0.5
2	0	4	0.5
3	0	8	0.5
4	4	0	0.5
5	4	4	0.5
6	4	8	0.5
7	8	0	0.5
8	8	4	0.5
9	8	8	0.5
10	0	0	1
11	0	4	1
12	0	8	1
13	4	0	1
14	4	4	1
15	4	8	1
16	8	0	1
17	8	4	1
18	8	8	1
19	0	0	0
20	0	4	0
21	0	8	0
22	4	0	0
23	4	4	0
24	4	8	0
25	8	0	0
26	8	4	0
27	8	8	0

3.3.7. *L. sakei* immobilization

Performance of immobilized L. *sakei* cells were compared with a cells free classical fermentation. *L. sakei* overnight culture was harvested by centrifugation and supernatant discharged; cells were recovered in order to obtain the same cell concentration of that used to inoculate trials with free cell; they were washed twice with saline solution (9 g/L NaCl), and suspended in MRS medium. Cells were entrapped in calcium alginate gels applying the following procedure: 1 volume of cell suspension was mixed with 1 volume of 4% (w/v) alginate (Alginic acid, Sodium salt, medium viscosity, Sigma-Aldrich). The cells-alginate suspension was then dripped in 0.1 M $CaCl_2$ (Merck K GaA), leaving at 4 °C for 1 h. Beads were finally washed with sterile water and inserted in fresh medium (MRS or alternative medium with whey permeate). Fermentation was carried out for 16 h. Glucose consumption (g/L) and acid lactic formation (g/L) were detected through an HPLC systems in the same conditions reported above (Merck-Hitachi L-7000 System) while bacteriocin activity was determined in terms of AU/mL.

3.3.8. Sakacin A production in bioreactor

A masterbatch of sakacin A was produced growing *L. sakei* in liquid batch cultures employing the low-cost medium formulation which gave the best performance in the DoE trials previously reported (g/L): yeast extract 8, meat extract 8, Tween-80 0.5, L-arginine 0.5; all ingredients were dissolved in deproteinized whey permeate. After medium sterilization, 1 mL/L of minerals and vitamins mix (sterilized by filtration) was added.

Liquid cultures were carried out in a 14 L fermenter (Omnitec Bio, Sedriano, Milano) (7 L volume) applying the following conditions: temperature 26 °C, 9 h incubation, no aeration, agitation speed 150 rpm, inoculum 5% (v/v) of a pre-grown culture in MRS medium. Supernatant was recovered after culture centrifugation at 8000 rpm for 40 min at 4 °C (Beckman Coulter, Brea, California, USA) and then purified.

3.3.9. Purification strategy

- **Precipitation with ammonium sulphate**

A classic purification strategy was employed to concentrate sakacin A produced during *L. sakei* fermentation through the application of salting-out methods. Cell-free supernatant was precipitated with ammonium sulfate 400 g/L in order to recover an enriched bacteriocin extract (Holck et el., 1992); the compound was added slowly to the sample until the desired saturation percentage is reached; after 1 h at 4 °C, salted out proteins were precipitated by centrifugation at 8000 rpm for 40 min at 4 °C (Beckman Coulter); precipitate was dissolved (10 X) in deionized water and subsequently freeze-dried overnight (Edwards Minifast MFD 01 lyophilizer, UK). The lyophilized protein extract containing sakacin A, once suspended in sterile water, was characterized in terms of antimicrobial activity.

- **Dialysis**

The enriched protein extract was subjected to dialysis to remove all remaining ammonium salt, using a Spectra/por 6 pre-wetted RC dialysis tubing (Spectrum, Repligen, Massachusetts, USA) with a 1 kD cut-off. Sample was loaded into the dialysis tube and inserted in an external chamber containing distilled water; dialysis was carried out overnight at 4 °C with gentle stirring, replacing the distilled water every 2 h and before the night. The remaining sample was freeze-dried overnight and characterized.

- **Chromatographic extraction**

Alternatively, a different purification strategy was applied employing a gravity reverse phase chromatography extraction column with a PoraPack TM RXN RP cartridge (2 g). Five PoraPack^{TM} cartridge were assembled to create a bigger column to scale-up the process. Batch of 750 mL of cells free supernatant was loaded in the column; a first washing of the column has been done with 100 mL of water added of 0.1 % of trifluoroacetic acid (TFA) (Sigma-Aldrich), after that three more washes were performed progressively with acetonitrile (ACN) (Sigma-Aldrich):

- 100 mL acetonitrile 25 % with 0.1 % of TFA,
- 100 mL acetonitrile 40 % with 0.1 % of TFA,
- 100 mL acetonitrile 100 % with 0.1 % of TFA.

Sakacin A resulted in the fraction eluted with acetonitrile 40 %; this aliquot was evaporated in rotavapor (Eppendorf, Concentrator plus/Vacufuge plus) to remove acetonitrile and finally freeze-dried overnight. The obtained protein extract was dissolved in water to be tested for antimicrobial activity.

- **Protein content**

The method of Lowry was used for proteins quantification since it is suitable for hydrophobic peptides. This is a colorimetric assays based on the use of bovine serum albumin (BSA) as standard, five different solutions were employed:

A) 10 g Na_2CO_3 in distilled water + 0.25 g Na-tartrate + 2 g NaOH in 250 mL H_2O (Sigma-Aldrich)
B) 0.2 g $CuSO_4$ * 5 H_2O in 100 mL H_2O (Sigma-Aldrich)
C) 2.0 g SDS in 100 mL H_2O (Sigma-Aldrich)
D) 9 part of A + 1 part of B
E) Folin-Ciocalteu reagent use at 1 N concentration.

Last two reagents are to be prepared fresh. 100 µl of samples or standard were added with 0.7 mL of regent D and 0.4 mL of reagent C, then vortexed and incubated at room temperature for 15 min; 0.4 mL of reagent E was then added and absorbance at 700 nm was read (Perkin Elmer UV/VIS Spectrometer Lambda) after 30 min incubation. The standard curve was plotted by using BSA ranging from 10 to 100 µg.

- **Reverse Phase HPLC**

Revers phase high pressure liquid chromatography was employed to characterize the samples obtained after reverse-phase purification process, using an HPLC systems (Waters, Milford, Massachusetts, USA) equipped with a 996 Photodiode Array Detector (Waters) and a Symmetry 300™ C18 column (4.6 × 250 mm, 5 µm) with a non-linear gradient of 0.1% aqueous TFA (A) and acetonitrile with 0.1% TFA (B). Samples were loaded and the following protocol was used:

Table 3.4: Reverse phase HPLC protocol.

Flow (mL/min)	% A	% B	Time (min)
0.8	100	0	0-10
0.8	40	60	10-40
0.8	0	100	40-41
0.8	0	100	41-47
0.8	100	0	47-48
0.8	100	0	48-55

3.4. Results

3.4.1. *L. sakei* growth and sakacin A production in MRS

L. sakei growth in MRS medium was monitored at 26 °C for 24 h through a Microplate Spectrophotometer, media were inoculated with different percentage of inoculum in order to observe the different time course of the curve (Fig. 3.2). Analyses of the growth curves allowed to identify the beginning of the stationary phase in order to understand the best combination between fermentation time and percentage of inoculum to have a high sakacin A concentration in culture supernatant.

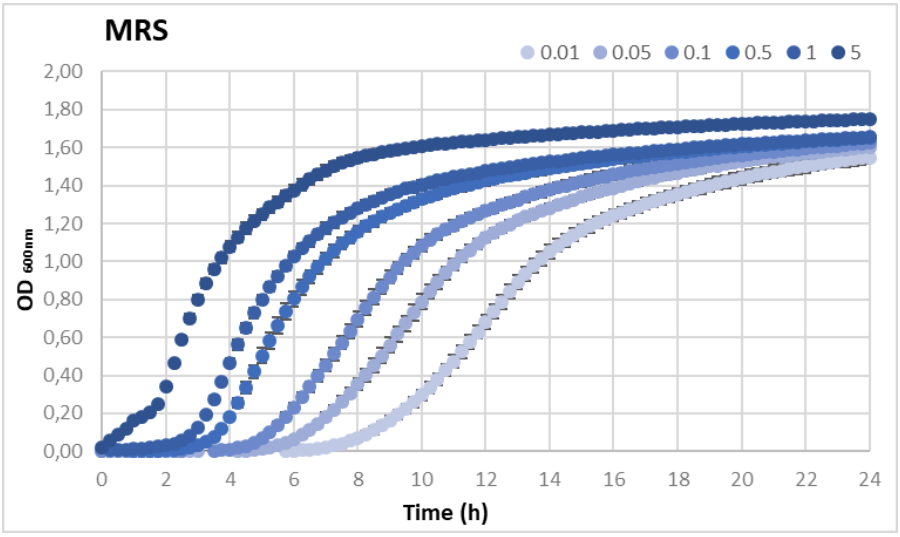

Figure 3.2: Time course of *L. sakei* 6333 growth in MRS culture medium at 26 °C. Different curves corresponding to different starting inocula from 0.01% to 5% v/v.

Data were fitted through the DMFit software employing the Baranyi and Roberts model, that includes three stages of bacterial growth (lag, exponential and stationary); the obtained parameters for each inoculum are reported in Table 3.3. Growth rate increased for percentage of inoculum higher than 0.5% (v/v), as expected and observed in Fig. 3.2. Lag phase increased with the decrease of inoculum while with maximum inoculum the lag was not detected. Significant differences were found between final OD values, nevertheless, for experimental purposes these can be considered negligible.

Table 3.5: Growth curves parameters fitted by Baranyi and Roberts model (DMFit software), (p-value < 0.0001).

Inoculum (% v/v)	**0.01**		**0.05**		**0.1**		**0.5**		**1**		**5**	
	Mean	± std.dev	Mean	± std.dev	Mean	± std.dev	Mean	± std.dev	Mean	± std.dev	Mean	± std.dev
R-square:	0.993		0.989		0.985		0.981		0.980		0.979	
SE of Fit:	0.047		0.059		0.066		0.077		0.077		0.069	
Lag/shoulder (h)	7.745	0.101	4.853	0.597	3.276	0.156	1.834	0.206	1.225	0.138	ND	
Maximum Rate (OD/h)	0.166^{a}	0.006	0.169^{a}	0.004	0.176^{a}	0.006	0.201^{b}	0.007	0.216^{b}	0.009	0.244^{c}	0.011
Final Value (OD)	1.466^{p}	0.033	1.516^{pq}	0.024	1.533^{q}	0.027	1.558^{q}	0.023	1.563^{q}	0.015	1.618^{r}	0.015

The quantification of the antimicrobial activity was initially carried out in trials set-up with a 5% (v/v) percentage of inoculum: samples of the supernatant were withdrawn a t 0 and after 2-4-6-8-16-24 h of incubation and tested. Time course of the antimicrobial activity in terms of AU/mL is reported together with the correspondent growth curve in Fig. 3.3.

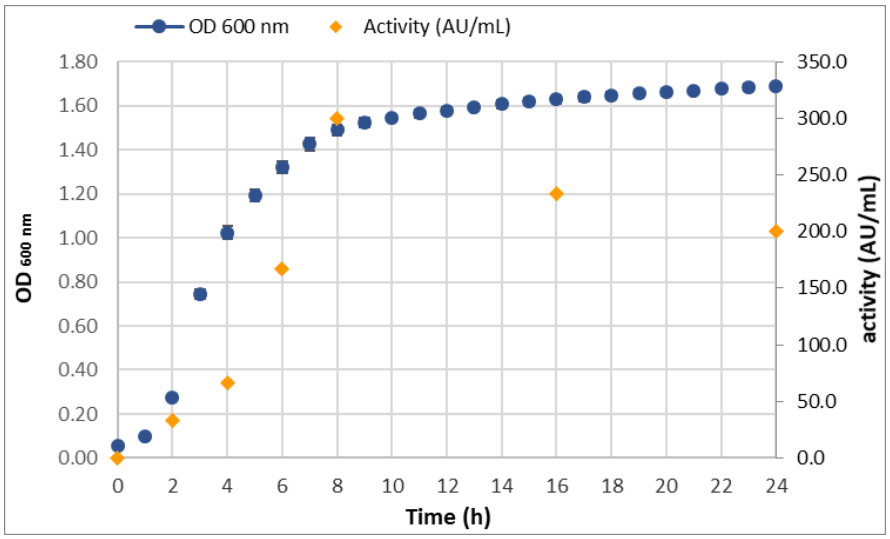

Figure 3.3: *L. sakei* growth (OD 600nm) in MRS at 26 °C and sakacin A production (AU/mL), inoculum 5% (v/v).

The maximum sakacin A production in MRS culture medium, 300 AU/mL, was achieved at the beginning of the stationary phase, around 8 h incubation, confirming that bacteriocin production takes place during the exponential phase of the microbial growth, as reported in the literature, and decreases afterwards; this decrease was reported in other studies and attributed mainly to bacteriocin adsorption on producer cells (Holck et al., 1992; Leroy and de Vuyst, 1999).

Based on these results, a point corresponding to the end of the exponential and the beginning of the stationary phase was identified for each curve. As noted in literature and observed in the previous antimicrobial trials, this time corresponds to the highest sakacin A concentration. Sakacin quantification (AU/mL) at 8, 16 or 24 h was thus carried out (Tab. 3.4) depending on the optimal point defined by the time course of microbial growth.

Table 3.6: Antimicrobial activity (AU/mL) of cultures supernatants set-up in MRS medium with different percentages of inoculum.

Inoculum (% v/v)	Sakacin A (AU/mL)		
	8 h	16 h	24 h
0.01	-	100	233
0.05	-	200	-
0.1	-	267	-
0.5	67	267	-
1	200	267	-
5	300	233	200

As expected, the highest activity was obtained when the growth was at the end of the exponential phase, i.e. 8 h for cultures inoculated at 5% (v/v),16 h for those inoculated between 0.05 and 1 % (v/v) and 24 h for trials at 0.01 % (v/v).

The parametrization of *L. sakei* growth and sakacin A production allowed to choose the optimal conditions for the prosecution of the results.

3.4.2. Optimization of medium formulation

Different media were formulated in order to replace MRS, all inoculated (0.1 % v/v) with a *L. sakei* pre-growth culture; samples were incubated at 26 °C for 16 h, time corresponding to the highest sakacin A production with this percentage of inoculum. Meat extract (ME), yeast extract (YE) as well as the other ingredients, dissolved in whey permeate, have been chosen on the basis of previously published data (Trinetta et al., 2008). The presence of arginine was also evaluated to investigate the possibility of promoting the arginine deaminase pathway, thus obtaining ATP for cell growth and also NH_3 to avoid pH decrease due to the production of lactic acid (Fig. 1.9). Ingredients were evaluated for their influence on microbial growth and bacteriocin production; *L. sakei* growth was evaluated in terms of lactic acid production, due to the fact that it is a primary metabolite of *L. sakei* fermentation, and thus can be correlated to the production of sakacin A, as discussed before.

As reported in Tab. 3.7, pH values ranged from 6.32 to 4.85; although cannot be considered totally representative of sakacin A production (the activation of the arginine deaminase pathway could limit pH decrease), these values were found interesting indicators for bacterial growth. In fact, higher pH values in samples containing arginine confirm the activation of the pathway with NH_3production.
Culture supernatants were all analysed to quantify the content of free amino acids; as example, in Tab. 3.6 the complete obtained data are reported for the trials containing L-arginine (Table 3.5 Run 9) and the correspondent trials without (Table 3.5 Run 27): arginine was totally consumed in both samples as expected, and ornithine (Orn) increased. No other lack of aminoacids was observed.
High concentrations of lactic acid were found when YE concentration was set at its maximum level with a slight increase in samples with arginine. The best productions were around 4.5 g/L (4.31 ± 0.70, 4.61 ± 0.11, 4.38 ± 0.04) corresponding to samples having the maximum level of ingredients. However, despite all arginine was used by the microorganism, this aminoacid seems to not be correlated with microbial growth and lactic acid production.

Table 3.7: Media composition and observed responses of the experimental design: culture pH after 16 h incubation at 26 °C, lactic acid production and sakacin A activity in terms of inhibition halo (cm) (dilution 1:10) vs. *L. innocua*.

Trial	A- ME (g/L)	B- YE (g/L)	C: Arg (g/L)	Culture pH 16 h		Lactic acid (g/L) 16 h		Sakacin A (Halo cm) 16 h	
				Mean	Std.dev	Mean	Std.dev	Mean	Std.dev
1	0	0	0.5	6.32	± 0.04	0.08	± 0.04	0.00	± 0.00
2	0	4	0.5	5.15	± 0.07	1.36	± 0.01	0.00	± 0.00
3	0	8	0.5	5.02	± 0.09	2.31	± 0.87	0.85	± 0.07
4	4	0	0.5	6.18	± 0.02	1.07	± 0.03	0.00	± 0.00
5	4	4	0.5	5.64	± 0.00	1.80	± 0.32	1.20	± 0.00
6	4	8	0.5	5.14	± 0.16	3.33	± 0.17	1.25	± 0.07
7	8	0	0.5	6.18	± 0.04	1.93	± 0.54	1.05	± 0.07
8	8	4	0.5	5.72	± 0.00	2.95	± 0.77	1.40	± 0.00
9	8	8	0.5	5.35	± 0.03	4.31	± 0.70	**1.50**	± 0.00
10	0	0	1	6.49	± 0.02	0.13	± 0.01	0.00	± 0.00
11	0	4	1	5.23	± 0.19	1.35	± 0.39	0.00	± 0.00
12	0	8	1	5.16	± 0.11	2.80	± 0.45	1.05	± 0.21
13	4	0	1	6.45	± 0.05	1.43	± 0.01	0.00	± 0.00
14	4	4	1	5.97	± 0.10	2.17	± 0.33	0.95	± 0.07
15	4	8	1	5.44	± 0.18	2.99	± 0.70	1.35	± 0.07
16	8	0	1	6.49	± 0.04	2.06	± 0.42	1.20	± 0.14
17	8	4	1	6.06	± 0.01	2.19	± 0.40	1.35	± 0.07
18	8	8	1	5.63	± 0.15	4.61	± 0.11	**1.60**	± 0.14
19	0	0	0	6.10	± 0.01	0.20	± 0.12	0.00	± 0.00
20	0	4	0	4.99	± 0.06	1.33	± 0.10	0.00	± 0.00
21	0	8	0	4.85	± 0.05	2.15	± 0.30	1.10	± 0.14
22	4	0	0	5.54	± 0.07	1.18	± 0.14	0.00	± 0.00
23	4	4	0	5.13	± 0.11	1.45	± 0.07	0.95	± 0.07
24	4	8	0	4.90	± 0.07	3.03	± 0.23	1.35	± 0.07
25	8	0	0	5.63	± 0.04	1.77	± 0.57	0.00	± 0.00
26	8	4	0	5.24	± 0.04	2.29	± 0.02	1.35	± 0.07
27	8	8	0	5.05	± 0.04	4.38	± 0.04	**1.50**	± 0.00

Table 3.8: Free aminoacidic profile (mg/L) present at t0 and after 16 h incubation in cultures formulated in absence or in presence of 0.5 g/L L-arginine.

AA	YE 8 – ME 8		YE 8 – ME 8 – ARG 0.5	
(mg/L)	t0	16h	t0	16h
ASP	138	128	137	132
THR	89	125	89	118
SER	98	11	94	5
ASN	78	66	76	57
GLU	403	430	392	424
GLN	0	0	0	0
GLY	70	89	69	90
ALA	171	184	169	183
CIT	26	24	26	25
VAL	177	221	177	221
CYS2	0	0	0	0
MET	50	52	48	53
ILE	132	175	130	176
LEU	254	296	251	293
TYR	96	141	139	181
PHE	180	201	181	202
GABA	10	10	10	10
ORN	**16**	**142**	**15**	**234**
LYS	189	217	186	215
HIS	44	49	46	51
TRP	40	54	40	54
ARG	**194**	**0**	**526**	**0**
PRO	53	70	52	71

Statistical analysis was first performed on **pH values**; with confidence intervals set at 95 %, Anova analysis identified as significant terms either the single parameter as well as their interaction as follows: A-B-C-AB-AC (p-value <0.0001), BC (p-value 0.0093).

The software estimated model adaptation goodness (R^2: 0.9828, Predicted R^2: 0.9591, Adjusted R^2: 0.9740), measures of variability (standard deviation: 0.086 % Confidence Variation: 1.53) and noise ratio (Adequate Precision: 32.449). Proceeding to diagnostics, attention was focused on Normal Probability plot of studentized residuals for normality of residuals. Model acceptability was evaluated using Studentized Residuals versus Predicted Values plot to evidence constant error. An externally studentized residual plot

was used to identify outliers and Box-Cox plots were used to power transformation and model acceptability.
The normal probability plot exhibited a “S” shape, while the Residual versus Predicted plot exhibited a “>” (inverted megaphone) shape. No points were considered as outliers. Data were not transformed to obtain more satisfactory fit with factorial model, as indicated by the Box-Cox plot.
Figure 3. 4 reports the interaction plots obtained with observed-predicted pH values obtained at 16 h incubation in trials performed as follows: i) 0 g/L arginine, ii) 0.5 g/L arginine, and iii) 1 g/L arginine.
In absence of arginine (3.4i), the lowest pH levels (i.e. the highest cell growth and hence the supposed highest sakacin A production) were associated with the highest concentration of Yeast Extract (blue line), but independently from the concentration of meat extract. In presence of 0.5 and 1 g/L arginine (Fig. 3.4ii), pH levels were higher, thus confirming the activation of the arginine deaminase pathway. In both these trials, lower levels were found in media formulated with the highest concentration of yeast extract; note that, contrarily to what highlighted without arginine, here the concentration of meat extract significantly increased culture pH.

Statistical analysis performed on **lactic acid** concentration levels evidenced that with confidence intervals set at 95 %, Anova analysis identified as significant terms only the parameter A- Meat Extract, and B- Yeast extract (p-value <0.0001).
Model adaptation goodness (R^2: 0.8925, Predicted R^2: 0.8695, Adjusted R^2: 0.8838), measures of variability (standard deviation: 0.40 % Confidence Variation: 19.49) and noise ratio (Adequate Precision: 30.622) were calculated. The normal probability plot exhibited a “S” shape, while the Residual versus Predicted plot exhibited a “>” (megaphone) shape. No points were considered as outliers, and again data were not transformed.
In Figure 3.5 is reported the 3-D graph related to lactic acid concentration values (predicted in bars and observed in points) in culture samples obtained when arginine was set at 0.5 g/L; this choice has been driven by the fact that even if the presence of this aminoacid did not produce any significant increase in sakacin A production, it helps keeping the culture pH buffered at around 5 without activation of the pH-stat control in the fermenter.
Results highlight that when no yeast and malt extracts were added, lactic acid production is at its lowest level (i.e. microbial growth is limited); a direct proportionality is instead evident between the presence of these two ingredients and lactic acid production. The highest concentration, meaning the most efficient *L. sakei* growth was achieved employing yeast and meat extract at 8 g/L.

Similar results were obtained for **sakacin A** production, evaluated as the diameter of growth inhibition halo against *Listeria* produced by supernatant samples diluted 1:10 v/v (Fig. 3.6): the highest bacteriocin levels were obtained in media formulated with 8 g/l of both yeast and meat extracts.

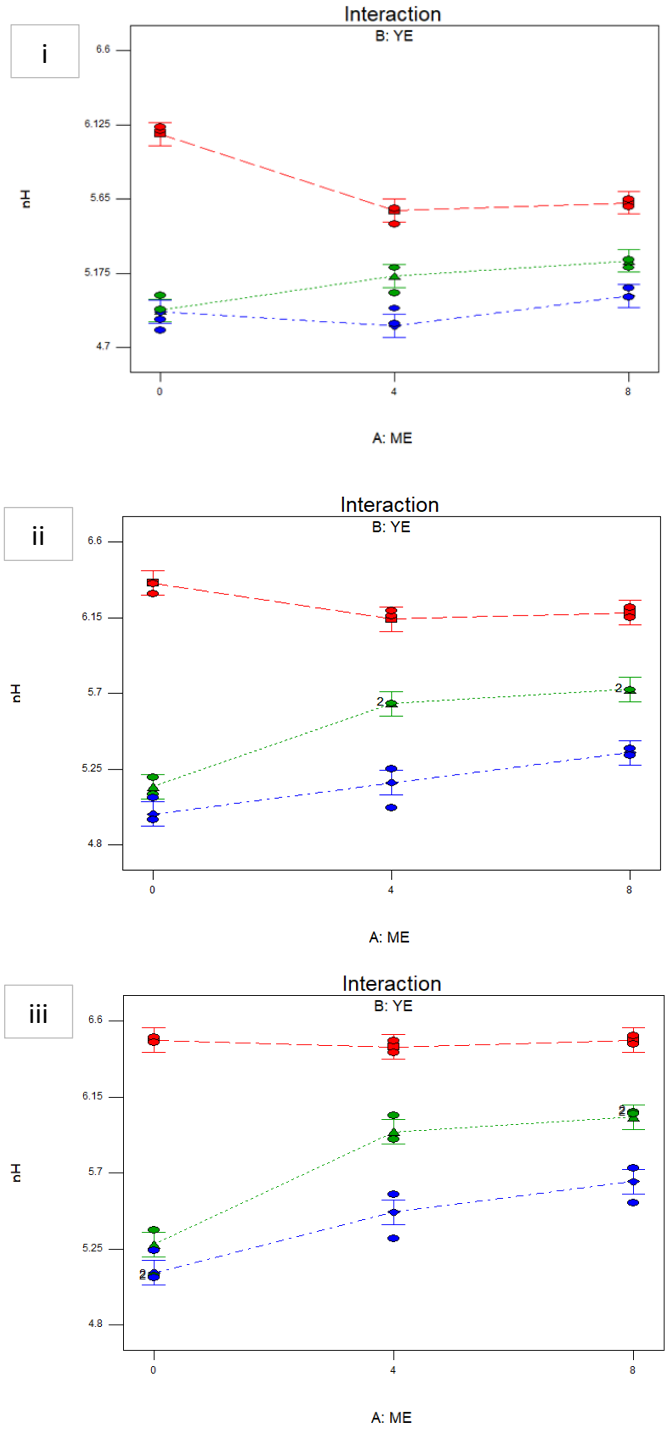

Figure 3.4: pH response obtained after 16 h of *L. sakei* incubation related to ingredients amounts and interaction between factors ME and YE with: i) 0 g/L arginine, ii) 0.5 g/L arginine and iii) 1 g/L arginine. pH value is reported on Y axis while ME levels on X-axis. Red line corresponds to 0 g/L YE, green line to 4 g/L YE and blue line to 8 g/L YE (R^2 = 0.9828).

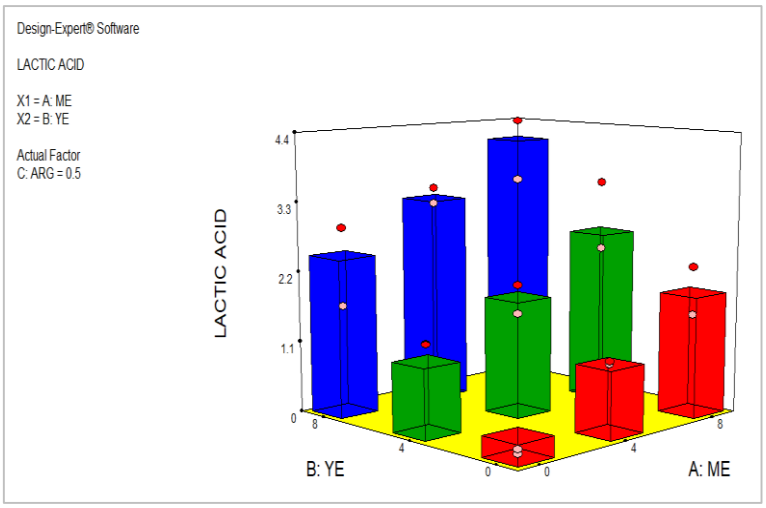

Figure 3.5: Lactic acid response obtained after 16 h of *L. sakei* incubation related to ingredients amounts and interaction between factors ME and YE. Arginine was fixed at 0.5 g/L.

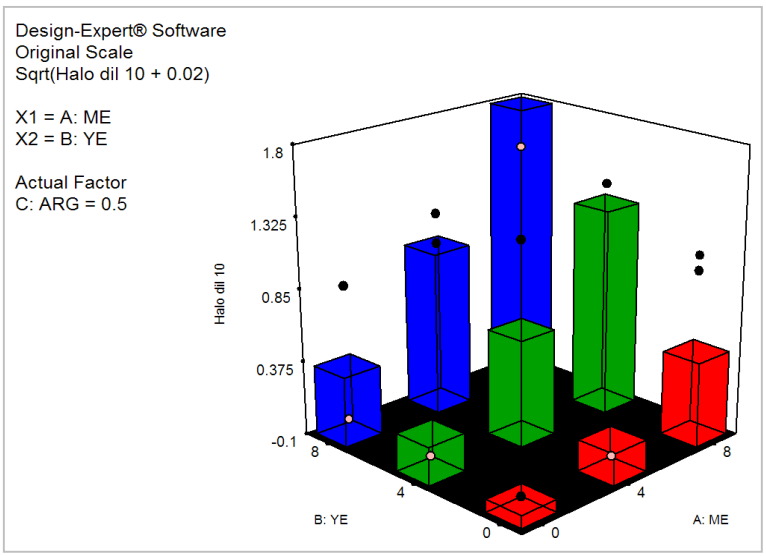

Figure 3.6: Sakacin A production response (halo cm) obtained after 16 h of *L. sakei* incubation related to ingredients amounts and interaction between factors ME and YE. Arginine was fixed at 0.5 g/L.

From an overall look at the obtained results, the formulation chosen for the prosecution of the research was that composed by meat extract 8 g/L, yeast extract 8 g/L, arginine 0.5 g/L, tween and vitamix dissolved in permeate whey; this formulation, named SAK, was then used throughout the thesis.

3.4.3. *L. sakei* growth and sakacin A production in SAK

Time course of *L. sakei* growth curves were also obtained in new formulated medium SAK, changing the percentage of inoculum as analyzed before in MRS medium. *L. sakei* growth was monitored at 26 °C for 24 h, and results reported in Fig. 3.7.

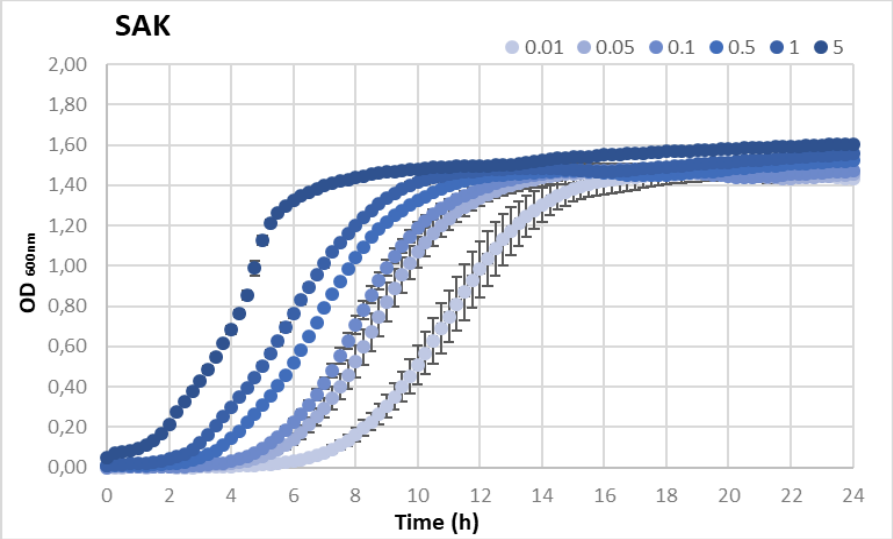

Figure 3.7: Time course of *L. sakei* 6333 growth in new formulated culture medium SAK at 26 °C. Different curves corresponding to different starting inocula from 0.01% to 5% v/v.

Also these data were fitted through the DMFit software employing the Baranyi and Roberts model and the obtained parameters for each inoculum are reported in Table 3.9. As observed in MRS medium, also when employing SAK the Lag phase increased with the decrease of the percentage of inoculum; differences were also found in terms of the maximum rate, however only sample inoculated with 5% of cells presents a rate value very different from those evidenced in other samples (0.308 *vs.* 0.227-0.255 OD/h). Also employing this medium, differences in the final OD among the percentage of inoculum were found significant from a statistical point of view but can be considered negligible considering the experimental deviations on obtained data. These results compared with those obtained growing *L. sakei* in MRS medium, showed that in this new medium the Lag-phase was longer while growth rate results were higher for all the tested percentages of inoculum. Final values were analogous for all the tested conditions in the two media.

Table 3.9*:* Growth curves parameters fitted by Baranyi and Roberts model (DMFit software), (p-value < 0.0001).

Inoculum (% v/v)	**0.01**		**0.05**		**0.1**		**0.5**		**1**		**5**	
	Mean	± std.dev	Mean	± std.dev	Mean	± std.dev	Mean	± std.dev	Mean	± std.dev	Mean	± std.dev
R-square:	0.999		0.999		0.999		0.998		0.998		0.991	
SE of Fit:	0.018		0.022		0.023		0.026		0.027		0.047	
Lag/shoulder (h)	7.743	0.307	5.914	0.278	5.284	0.116	3.743	0.084	2.764	0.070	1.847	0.049
Maximum Rate (OD/h)	0.227^{a}	0.020	0.251^{bc}	0.007	0.255^{b}	0.001	0.235^{ac}	0.001	0.232^{ac}	0.001	0.308^{d}	0.003
Final Value (OD)	1.448^{p}	0.033	1.450^{p}	0.030	1.462^{pq}	0.010	1.473^{pq}	0.011	1.497^{qr}	0.018	1.534^{r}	0.004

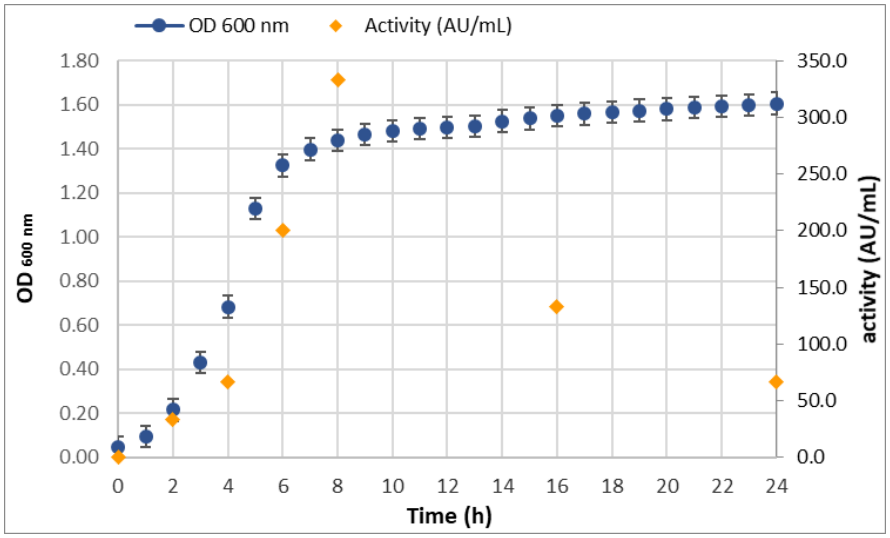

Figure 3.8*: L. sakei* growth (OD600nm) in SAK medium at 26 °C and bacteriocin production (AU/mL), inoculum 5% (v/v).

Following the procedure applied with the reference medium MRS, determination of sakacin A antimicrobial activity in SAK supernatants obtained with a 5% v/v inoculum was again measured at different incubation times, while for the other percentages of inoculum only at the most interesting times. The highest antimicrobial activity (333 AU/mL) was found in supernatants after 8 h fermentation, correspondent to the end of *L. sakei* exponential phase, to decrease progressively during the stationary phase (Fig. 3.8 and Tab. 3.8).

Table 3.10: Antimicrobial activity (AU/mL) of cultures supernatants in the new formulated medium.

Inoculum (%)	Sakacin A (AU/mL)		
	8 h	16 h	8 h
0.01	-	67	100
0.05	-	133	-
0.1	-	233	-
0.5	7	233	-
1	100	267	-
5	333	133	-

3.4.4. Performance with immobilized cells

Immobilization procedure (Fig. 3.9) was applied to simplify the recovery of a cell-free supernatant, containing sakacin A, avoiding the centrifugation step (alginate beads can in fact be easily separated employing strainers or cotton filters); *L. sakei* ability of producing sakacin A when confined in alginate was first evaluated in the reference MRS medium. Cells entrapped in alginate beads were incubated at 26 °C for 16 h. Microbial growth was compared with a standard culture (free cells) in terms of lactic acid production, while bacteriocin production in terms of AU/mL was also considered to understand the feasibility of the applied procedure.

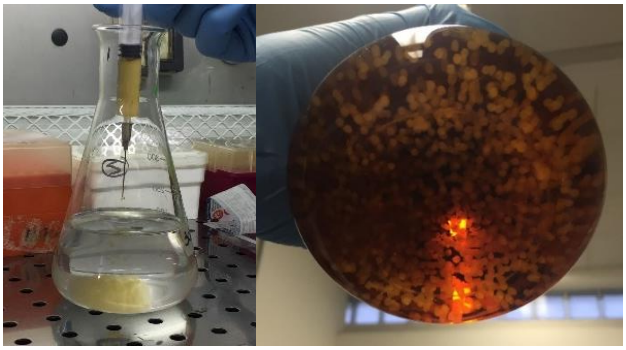

Figure 3.9: Immobilization techniques (left) and MRS medium containing alginate beads (right).

Table 3.11: Lactic acid production (g/L), pH and antimicrobial activity (AU/mL) of cultures with free and immobilized cells.

MRS 16 h	Lactic acid (g/L)	pH	Sakacin A (AU/mL)
Free cells	6.89 ± 0.42	4.54 ± 0.05	**300 ± 46**
Immobilized cells	6.09 ± 0.44	4.46 ± 0.02	**238 ± 14**

No significantly differences were found in fermentation parameters between the two samples, while the bacteriocin production was found higher in the standard culture 300 ± 46 vs. 238 ± 14 AU/mL (Tab. 3.11). At the end of the fermentation, alginate beads were separated with a strainer and re-inoculated in fresh MRS medium, analogously to the first step, in order to evaluate cells ability to maintain bacteriocin production. Unfortunately, after few hours of the second fermentation, beads started to break allowing the cells to leak out. The second fermentation was not considered efficient and the recovery-re-use was stopped.

This experiment was also performed employing the new formulated SAK medium: in this case cells were immediately released in the medium (Fig. 3.10), highlighting that the immobilization procedure as such cannot be applied with satisfying results to facilitate cells separation. The analyses carried out in alginate beads after re-use evidenced an increase of beads diameter, presumably due to the increased cell population during culture incubation (Fig. 3.11).

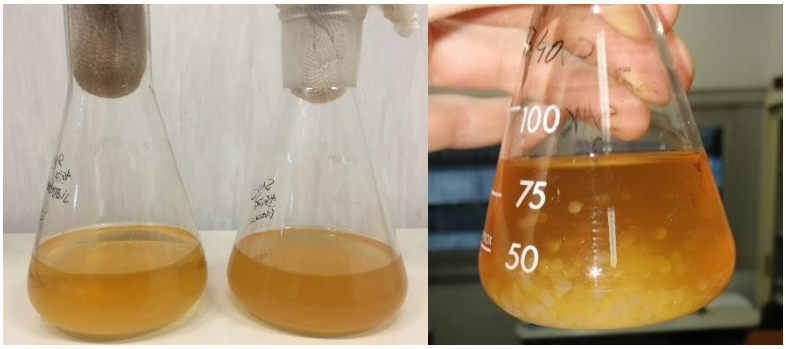

Figure 3.10: Cell immobilization in SAK medium, cells were released in medium (right).

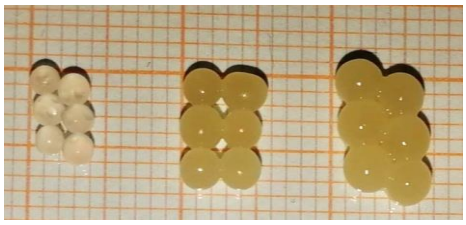

Figure 3.11: Alginate beds in MRS: from left to fight the t0, after 24 and 48 h of culture fermentation.

3.4.5. Sakacin A masterbatch production and downstream

Sakacin A was produced growing *L. sakei* for 9 h at 26 °C in stationary liquid cultures. The flow-sheet of the upstream and downstream processes is reported in Fig. 3.12.

A bioreactor with a total capacity of 10 L was used for sakacin A production. Cell free supernatant was recovered and mixed with ammonium sulphate in order to precipitate the contained protein fraction. Precipitate containing sakacin A was suspended in water and free-dried. A dialysis procedure was also applied to further purify the precipitate.

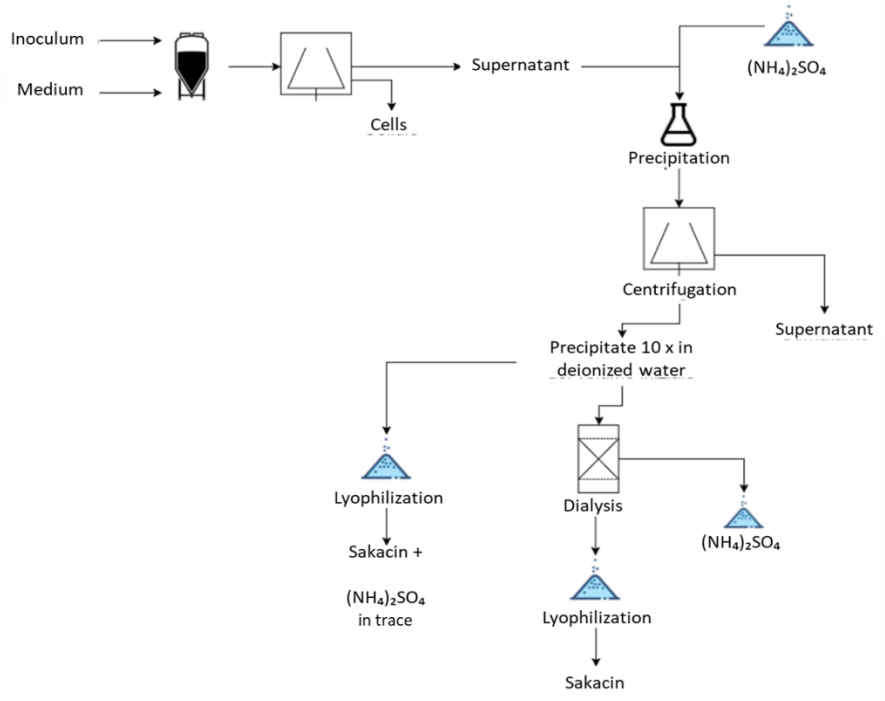

Figure 3.12: Sakacin A production, from the microbial culture to downstream process.

Several productions (F1-F7) were carried out to obtain high amount of sakacin A to use in the production of active materials. All obtained yields are reported in Tab. 3.12: results highlight that differences in the final amount of sakacin obtained exist among fermentations, as well as in terms of the relative antimicrobial activity: the high standard deviation values confirm that reproducibility represents up to now one of the biggest weaknesses of the protocol.

Table 3.12: Data related to 7 fermentation trials at pilot scale.

TRIALS	VOLUME (L)	SAKACIN A extract (g)	SAKACIN A extract (g/L)	SAKACIN A (AU/mg)
F1	6.60	16.00	2.42	20.00
F2	6.40	13.40	2.00	13.30
F3	4.00	11.38	2.85	10.00
F4	6.45	7.90	1.22	20.00
F5	8.15	16.90	2.07	13.30
F6	6.63	8.20	1.23	39.00
F7	8.00	17.32	2.16	20.00
		Mean	1.99 ± 0.59	19.37 ± 9.56

By examining the production process, 1 L of *L. sakei* culture allowed to obtain 0.9 L supernatant containing sakacin A at a concentration of 333333 AU/L; after precipitation, 93333 AU/L were recovered, with a 73% loss; other 1000 AU were lost after the lyophilisation step, with a final recovery of 25 % of the initial AU. Dialysis was performed to remove ammonium sulpahte from the enriched sakacin A extract, with a final recovery maintained at 25% of the initial AU: no sakacin A loss was thus evidenced during dialysis through the membrane.

The new purification protocol was also applied using a single step gravity RP-chromatography to compare production yields: the *L. sakei* culture supernatant was loaded directly into the column. Elution was performed using progressively different washing solutions, as follows:

1. water + 0.1 % of TFA
2. acetonitrile (25 %) + 0.1 % of TFA,
3. acetonitrile (40 %) + 0.1 % of TFA,
4. acetonitrile (100 %) + 0.1 % of TFA.

Washing steps were used to remove the unbound sakacin fraction and most of the non-active peptides. After evaporation of the solvent, the eluted sample was dissolved in water. Antimicrobial assay was performed and proteic content of each eluted samples was determined to prove the effective presence of sakacin A (Tab. 3.11). Sakacin A was found in the fraction obtained after the third washing, correspondent to 40% ACN and 0.1 % TFA as shown in Fig. 3.13: the presence of a *Listeria* growth inhibition halo in presence of the sample C (third washing) is clear, while the first two washings (A-B) correspondent to the elution of the polar proteic part of cell free supernatant did not show any antimicrobial activity.

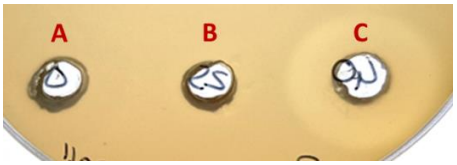

Figure 3.13: Result of antimicrobial test carried out on the eluted fraction corresponding to A: 0% ACN elution, B: 25% ACN elution and C: 40% ACN elution.

Table 3.13: Sakacin A purification yield

Fraction	Volume (mL)	Protein concentration (mg/mL)	Total protein (mg)	Protein recovery (%)	Specific activity (AU/ mL)	Total activity (AU)	Yield (%)	Specific activity (AU/PROTEIN mg)
L. SAKEI GROWTH MEDIUM	755	5.98	4515	100	167	126085	100	27.90
NOT BOUND + FIRST WASH	850	3.18	2706	59.90	0	0	0	0
25% ACN ELUTION	97	14.73	1428	31.60	0	0	0	0
40% ACN SAK A ELUTION	95	2.57	244	5.40	700	66500	53	272.30

With this purification protocol almost 53% of total initial AU were recovered.
RP-HPLC profiles of the three fractions were collected (Fig. 3.14): the first two washing fractions, with 0% and 25% of acetonitrile eluted the polar proteic part, while in the third wash a specific peak with Rt 37.2 min was highlighted; this peak, collected and analysed in antimicrobial assay, was found to correspond to sakacin A. the presence of the peak only in the third fraction confirmed the efficacy of the set up protocol for sakacin purification.

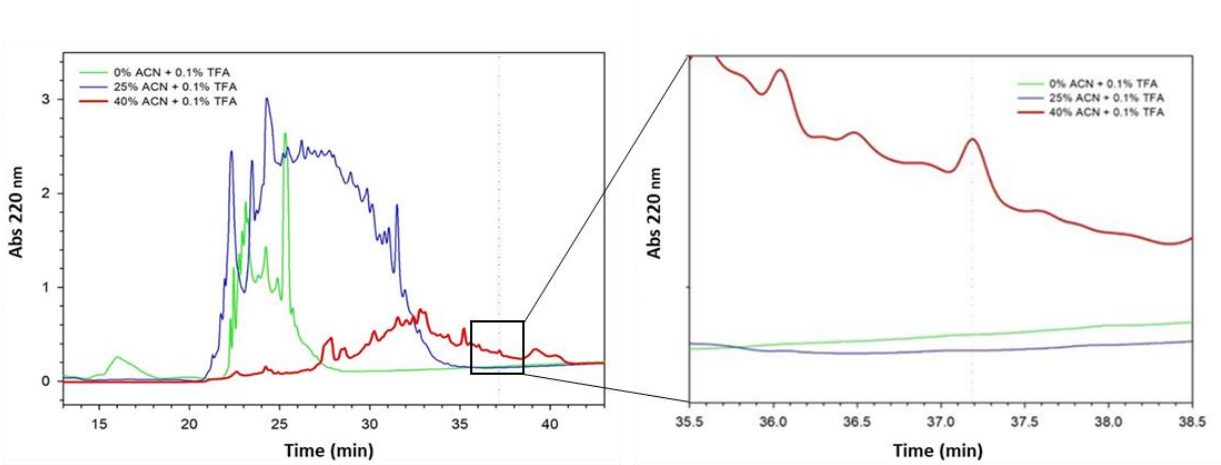

Figure 3.14: RP-HPLC profile of the three eluted fractions.

3.4.6. Cost evaluation

To better compare all the tested culture media and sakacin A production downstream processes, it is essential to calculate and compare yields and production costs of each step.
As regards sakacin A production, the reference medium taken in consideration in this study has been MRS, characterized by a cost formulation of around 5.40 €/L and by a sakacin A fermentation yield of 300 AU/mL in the tested conditions. Sakacin A was subsequently produced in the complex SAK medium containing ingredients generally used at industrial scale; this medium, formulated with yeast extract, meat extract, tween-80 and a vitamin mix, costs approximately 2.30 €/L and allows a sakacin A fermentation yield of 333

AU/mL. So far, the estimation cost for 1000 AU results in 0.018 € for production in MRS and 0.007 € in SAK; so far, approximately, sakacin A production in the new SAK medium results almost three times more convenient.

Table 3.14: Ingredient cost at lab scale.

Medium	Ingredient	Cost €/Kg	g/L used	€/L
MRS		104	52.2	**5.40**
SAK	Yeast Extract	60	8	0.48
	Meat Extract	149	8	1.19
	Arginine	415	0.5	0.20
	Tween-80	83.70	0.5	0.04
	VitMix	0.37 €/mL	1 mL	0.37
			total	**2.30**

As regards the two developed purification strategies, the traditional precipitation with ammonium sulphate costs around 24 €/L at lab scale and 500 €/ton at industrial scale; considering the 25% recovery of the initial AU, the estimate cost for purification is 0.002 €/1000 AU (industrial scale) or 0.115 €/1000 AU for lab scale; which means, with this process at lab scale 1 € allows to produce approximately 8300 AU sakacin A. With the new purification protocol, the cost evaluation is more complex: although chromatographic cartridges are considered a disposable material (use-and-discharge), trials performed collaterally to this research have proved their efficient reuse. A column composed by 5 cartridges costs around 60 € and can be repeatedly employed. The use of acetonitrile produces a cost of 10.6 €/L of cell-free supernatant, purified at lab scale. Not considering the initial cost for the column (we believe in re-use), this new purification method allows to obtain a higher purification yield than the traditional protocol with ammonium sulphate, with a purification cost of about 0.06 €/1000 AU. So in this condition, 1 € allows to obtain approximately 15000 AU sakacin A.
The estimated costs for production and purification indicated that at lab scale the new downstream protocol allows to reduce the purification costs to 0.06 €/1000 AU *vs.* 0.115 €/1000 AU also avoiding the time-consuming centrifugation step needed to separate the ammonium sulphate precipitate.

3.5. Discussion

In this chapter the bacteriocin sakacin A was produced in liquid cultures employing a low cost medium SAK formulated with whey permeate, a by-product obtained by extraction of whey proteins from cheese whey by ultrafiltration. The possibilities to use this by-product is noteworthy, because the major portion of world cheese whey is actually discarded as effluent, causing severe pollution problems for the environment. The best chance of this research is to obtain high added value products by the re-use of whey and whey by-products. Although statistical data on production and market value are available both for whey protein and cheese whey, Eurostat for EU and Clal for Italy, no data can be found for whey permeate. Even if such lack of data witnesses its low value for standard economic activities, whey permeate production is pulled by the highly profitable activity of whey protein extraction. In 2017 liquid cheese whey available was 48 million of tonnes for EU-28, with an increase of 12% over the last 10 years. In this frame, whey permeate may represent a cheap and highly available substrate for circular economy activities (Rollini et al., 2018).

About whey valorization Panesar et al. (2007) reviewed the possible upgrade of this effluent as a substrate for fermentation, and employed it for lactic acid production through fermentation of different LAB species. Media composition as well as growth condition directly influenced bacteriocins production, being these primary metabolites. Several media have been evaluated by numerous authors to improve bacteriocins synthesis: generally, LABs require complex media for growth such as MRS, that however cannot be classified as food grade and for its complex proteic nature complicates the subsequent purification steps.
In this study an alternative food-grade and low-cost medium has been formulated starting from whey permeate use, named SAK. Yeast extract and Meat extract were added to whey permeate as main ingredients, while arginine was added to help microorganism in increasing pH level during fermentation without the use of a pH-stat control system. Arginine utilization by microorganism was also demonstrated analyzing the aminoacidic profile of media containing or not this aminoacid.
Sakacin A production, evaluated in terms of its antimicrobial activity against *L. innocua* (AU/mL) was compared in MRS and SAK: 300 AU/mL were detected in cell-free supernatants after 8 h *L. sakei* fermentation in MRS while 333 AU/mL in SAK, evidencing that sakacin A yields are comparable between the two media. So far, this new medium represents an effective alternative to the more expensive MRS (cost for sakacin A production is reduced of about three times than in MRS). It has to be remembered that this kind of indirect bacteriocin quantification has several pitfalls, above all reproducibility. For what concerns sakacin production, a comparison between differents studies results quite complex due to the fact that different methods are used to determine bacteriocin production. Despite several studies report bacteriocins activity in terms of AU/mL, each research group differs in the way of considering the term “arbitrary unit”: in most cases activity has been determined by spot-on-the-lawn method or by microtitre assay and the obtained AU are difficultly comparable with the activity measured in this study. In addition, the absence of a sakacin A standard does not allow to standardize results. Examples of different quantification methods employed in the literature related to sakacin A activity are reported in Tab. 3.15.

Table 3.15: Literature data related to sakacin A quantification.

Reference	Medium	Quantification method	Definition of Unit	Activity *vs.* *Listeria spp.*
Holk et al. 1992	MRS	Microtitre assay	Bacteriocin Unit: amount of bacteriocin which inhibited growth of the indicator organism by 50%	250 BU/mg protein
Yang et al. 1992	MRS	Spot onto a lawn	Arbitrary Units: reciprocal of the highest dilution showing a clear zone of growth inhibition	31000 AU/mL
Guyonnet et al. 2000	MRS	Well diffusion method	MIC: lower concentration inducing a detectable zone of inhibition	0.065 µg/mL
Katla et al. 2003	MRS	Microtitre plate assay	IC_{50}: concentrations of a bacteriocin needed to inhibit growth by 50%	0.16-44.2 ng/mL
Trinetta et al. 2008	MRS	Well agar diffusion assay	Arbitrary Units: reciprocal of the highest dilution exhibiting a clear zone of inhibition	180 AU/mL
Trinetta et al. 2008	Bactopeptone, Meat extract, Yeast extract, glucose, $CaCO_3$	Well agar diffusion assay	Arbitrary Units: reciprocal of the highest dilution exhibiting a clear zone of inhibition	480 AU/mL
Trinetta et al. 2010	Bactopeptone, Meat extract, Yeast extract, glucose, $CaCO_3$	Well agar diffusion assay	Arbitrary Units: reciprocal of the highest dilution exhibiting a clear zone of inhibition	1600 AU/mL
Barbosa et al. 2014	MRS	Spot onto a lawn	Arbitrary Units: the reciprocal of the highest dilution that resulted in production of a clear zone of inhibition	1600 AU/mL
Barbosa et al. 2015	MRS	Spot onto a lawn	Arbitrary Units: the reciprocal of the highest dilution that resulted in production of a clear zone of inhibition	12000 AU/mL
Barbiroli et al. 2017	Bactopeptone, Meat extract, Yeast extract, glucose, $CaCO_3$	Well agar diffusion assay	Arbitrary Units: reciprocal of the highest dilution exhibiting a clear zone of inhibition	106 AU/mL

The cells immobilization technique was also investigated to evaluate the possibility of facilitating the production and recovery systems and to exploit a possible cell re-use - recycle also to reduce process cost. Results were not found satisfactory: the first fermentation allows to obtain almost the same sakacin A amount than a standard cell-free culture in MRS, but in the first recovery and re-use, cells leaked out from alginate beads in the medium. Despite this expected result, being sakacin a primary metabolite produced during cells growth, this technique was taken into account to facilitate the downstream steps thus reducing the subsequent sakacin A purification.

As regards sakacin A purification, in the present study an ammonium sulphate precipitation was applied to the culture supernatant, allowing to obtain an enriched bacteriocin extract containing 19 ± 9 AU/mg of the active compound, with a total activity yield of about 25%. Purification is an essential step for bacteriocin applications but also represents a time-consuming and a low-yield step of the entire

production process (Barbiroli et al., 2017). Barbiroli et al. (2017) reported on sakacin A purification from a food-grade medium by one-step diafiltration, giving a freeze-dried enriched sakacin A with an antimicrobial titer of 1.36 AU/mg and a total activity yield of 20%. Trinetta et al. (2010) recovered concentrated sakacin A by ultra-filtration through a 3 kDa mol exclusion membrane, lyophilized overnight and stored at 4 °C for further experiments; one milli- gram of the lyophilized powder was resuspended in 1 mL of sterile water and showed an antimicrobial titre of 1600 AU/mL; however, no data about process yields were reported. Guyonnet et al. (2000) carried out an alternative bacteriocin purification procedure based on cation exchange chromatography with a yield of 10% of pure bacteriocin, probably due to the hydrophobic characteristics of this molecule. In general, the low bacteriocin production yields and the necessity to combine several purification procedures such as extraction, precipitation, ultrafiltration, chromatographic and molecular methods represent the limiting step for bacteriocin application at an industrial scale (Kaskoniene et al., 2017; Garsa et al., 2014). Up to now there is no single purification technique suitable for all classes of bacteriocins. All purification possibilities depend on the nature of bacteriocin and differ by the duration of purification process, the recovery and yield of bacteriocin and the target of the analysis, so a general method or protocol is not available (Kaskoniene et al., 2017; Vera Pingitore et al., 2007). Different purification ways are run over and most commonly strategy are reported in Fig. 3.15.

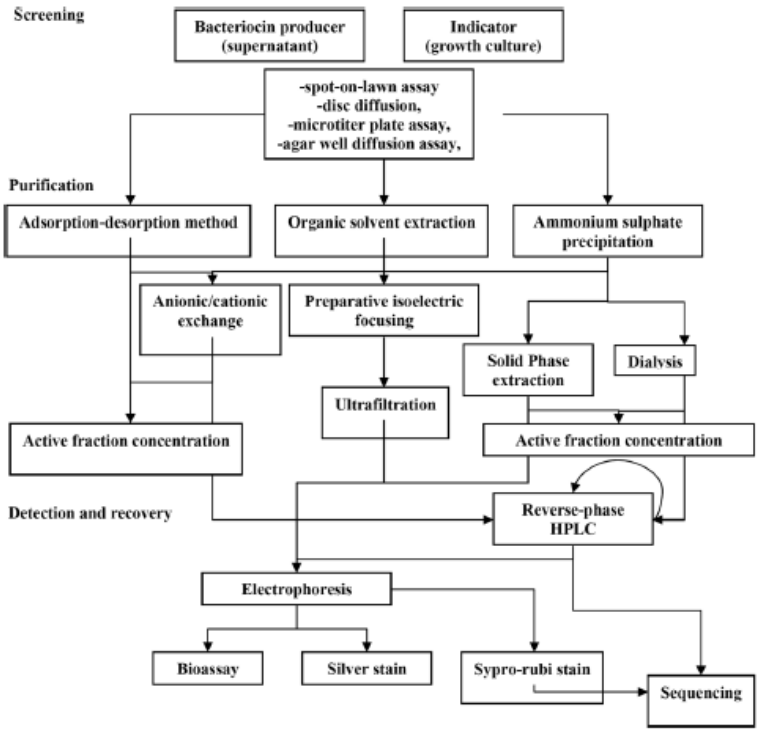

Figure 3.15: Possible ways of bacteriocins purification (Pingitore et al., 2007).

An alternative purification method has been applied in the last part of this study, using a single step gravity RP-chromatography. This technique allowed to increase sakacin A production yield and to reduce the number of centrifuges simplyifing downstream steps; using the same fermentation conditions almost 53% of the initial concentration of sakacin A was recovered and freeze-dried, while it was less than 30% with ammonium sulphate precipitation.

The result obtained in this study allowed to achieve sakacin A production at interesting levels and with a food-grade process considering that all the ingredients, reagents and solvents used are food-grade (note that acetonitrile is completely removed through evaporation). These results could be considered a good starting point for further improvements in terms of bacteriocins recovery yields, thus opening new applications of bacteriocins as natural antimicrobials.

Conclusion to chapter 3

This work aimed at developing a food-grade medium alternative to MRS for sakacin A production, demonstrated the possibility to use a food waste, whey permeate, as cheap substrate for *L. sakei* growth and sakacin A production. High amounts of this bacteriocin were produced in the study, increasing production and purification yields and scale-up the process in 20 L bioreactor. The new purification protocol set up allowed to obtain 53% of the total sakacin A present in cell free supernatant. Sakacin A produced was subsequently used for preparation of active solutions for food packaging purposes that are presented the next chapter.

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Chapter 4:

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4.1. Sakacin A application in the production of antimicrobial packaging materials

Abstract

In the present chapter, protein extract containing the bacteriocin sakacin A, produced by *Lactobacillus sakei* Lb 706 in a low-cost culture medium containing deproteinized cheese whey, was combined with cellulose nanofibers (CNF) applying several strategies to produce active materials. The applied fermentation conditions allowed to obtain 4.51 g/L of freeze dried protein extract, characterized by a total antimicrobial activity of near 16700 AU/mg, that was used for active materials preparation. The active materials were then characterized by infrared spectra and thermo-gravimetric analyses. Antimicrobial trials were carried out *in vitro* using *Listeria innocua* as indicator strain; results were also confirmed *in vivo,* employing smoked salmon fillets intentionally inoculated with *L. innocua*; in presence of the best active mat, *Listeria* population was reduced of about 2.5-3 Log cycles after 28 d storage at 6 °C, compared with negative control produced without the bacteriocin extract.

4.2. Introduction

In last decades, bacteriocins produced by Lactic Acid Bacteria (LAB) have received great attention due to their safe origin, since the producer organisms are GRAS, and to the fact that, being degraded by proteolytic enzymes in human intestinal tract, they can be considered harmless (Cleveland et al., 2001; Campos et al., 2013; Garsa et al., 2014). Therefore, the use of bacteriocins in food preservation may offer several benefits such as safety and their use can reduce the need of chemical preservatives and thermal treatments on food products, thus meeting the consumers request for minimally-processed food containing natural additives (Campos et al., 2013).

There are two main methods of using bacteriocins into food: 1) *in situ*, by adding bacteriocins-producing bacteria, or 2) *ex situ* with the addition of purified or semi purified peptides (Balciunas et al., 2013; Campos et al., 2013; Mapelli et al., 2018). However, one of the main concerns on the application of these molecules is related to their low production yields and high purification costs.

An interesting solution for bacteriocins application to food can be represented by their delivery through active packaging materials. One of the potential and innovative ways to inhibit, reduce or retard microbial growth in food may derive from food packaging; this generation of food packaging include materials with antimicrobial proprieties able to prevent surface growth of microorganisms in foods (Appendini et al., 2002).

Sakacin A is a class IIa bacteriocin produced by the LAB *Lactobacillus sakei*; it is 41 amino acids peptide with a molecular mass of 4308 Da; as all the bacteriocins belonging to class IIa, it exerts an antimicrobial effectiveness against the causative microorganism of listeriosis, a highly fatal opportunistic foodborne infection (Ennhar et al., 2000; Trinetta et al., 2012). In this frame, sakacin A can focus significant interest for their potential use as bio preservatives in the food area (Cleveland et al., 2001; Etayash et al., 2016; Barbiroli et al., 2017; Mapelli et al., 2018). In particular, sakacin A, due to its specific antimicrobial activity, may represent a good candidate to develop active packaging solutions for food potentially contaminated with *Listeria* such as Ready-to-Eat fishery, meat products and fresh cheese (Trinetta et al., 2008; Mapelli et al.,2018).

A variety of antimicrobial packaging systems has been studied; some films have been produced incorporating the antimicrobial agent into the polymer, others have used biopolymers as effective carriers of antimicrobial agent (Padgett et al., 1998; Scannell et al., 2000; Coma et al., 2001; Mauriello et al., 2004; Imran et al., 2010; Saini et al., 2016). Incorporation can be carried out through the coating technique, applying a layer of material much thinner than the underlying substrate; in this case the polymer systems embed the active molecules to be released into the food, possibly in a controlled manner (Barbiroli et al., 2017). Examples of active package solutions in which bacteriocins are applied by coating have been reported by Ming et al. (1997), Mauriello et al. (2004), Ercolini et al. (2006), La Storia et al. (2008). Another alternative to produce active packages is the incorporation of the antimicrobial compound directly into the polymeric matrix; however, this procedure cannot be considered feasible becuase high processing pressure and temperature or incompatibility with the packaging material can inactivate the antimicrobial agents (Perez-Perez et al., 2006; Barbiroli et al., 2017). In parallel to continuous film, active mats have also been proposed, made up of materials not feasible as primary packaging, but with promising properties in terms of a rational application of the active substance.

Some of polymeric films used to produce active devices are cellulose-based, with interesting properties like renewability, biodegradability, biocompatibility and being cost-effective materials. Barbiroli et al. (2017) reported the possibility of incorporating lysozyme and lactoferrin into paper containing carboxymethyl cellulose, that allowed non-covalent binding of the positively charged proteins to the paper

matrix; tests on thin cuts of raw meat also confirmed their antimicrobial effect. Saini et al. (2016) developed a novel antimicrobial film with covalently linked nisin on surface of TEMPO oxidized cellulose nanofibers for food packaging. Espitia et al. (2013) studied the effects of pediocin incorporation into a cellulosic packaging produced with cellulose acetate resin, determining the tensile strength at break (MPa), load at break (N) and elongation at break (%), water vapor permeability and structure. Santiago-Silva et al. (2009) developed and evaluated the antimicrobial effect of cellulose acetate matrix films incorporated with pediocin on the preservation of samples of sliced ham.

In the past decade, a nanomaterial of cellulose, named cellulose nanofibers (CNF), was developed, with diameter between 10-50 nm and length of several millimetres. In addition to cellulose, this novel material can enhance mechanical and barrier properties when applied in packaging materials (Khan et al., 2014; Saini et al., 2016). The use of cellulose nanocomposites in food packaging can help extending shelf-life and enhancing food quality, since they serve not only as barrier to moisture, water vapour, gases, and solutes but they can also be considered carriers of active substances such as antimicrobials (Khan et al., 2014).

In the present study, a protein extract containing the bacteriocin sakacin A, produced by *L. sakei* Lb 706 in a low-cost culture medium, was combined with CNF to obtain active materials. The produced mats were then characterized to analyse the antimicrobial efficacy of surface modification of sakacin with the CNF. The efficacy of the resulting active package was assessed performing either *in vitro* antimicrobial tests and confirmed by *in vivo* storage trials of Ready-To-Eat smoked salmon fillets intentionally inoculated with *Listeria*.

4.3. Materials and methods

4.3.1. Microorganisms and maintenance

The sakacin A-producing strain used in this study was *Lactobacillus sakei* DSMZ 6333 (Lb706) (DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) while *Listeria innocua* DSMZ 20649 was used as target strain. *L. sakei* was maintained on MRS broth (DeMan-Rogosa-Sharpe, Merck K GaA, Darmstadt, Germany) while *L. innocua* on TSB (Tryptic Soy Broth; Merck K GaA). Media were inoculated (10% v/v) with a pre-grown culture and incubated in stationary condition at 30 °C for *L. sakei* and 37 °C for *L. innocua* for 16-24 h. Stock cultures of both microorganisms were stored at -80 °C in their appropriate liquid medium added with 20% (v/v) glycerol (VWR International, Leuven, Belgium). Cultures were propagated twice before use.

4.3.2. Sakacin A production and purification

Sakacin A was produced growing *L. sakei* in liquid batch cultures employing a low-cost medium formulation containing (g/L): yeast extract (Costantino, Torino, Italy) 8, meat extract (Merck K GaA) 8, Tween-80 (Merck K GaA) 0.5, L-arginine (Merck K GaA) 0.5; all ingredients were dissolved in deproteinized cheese whey, kindly supplied by Latteria Soresina (Soresina, Italy). After medium sterilization, 1 mL/L of minerals and vitamins mix (sterilized by filtration) was added. The mix had the following composition (g/50 mL): $MgSO_4$ (Sigma Aldrich, Missouri, USA) 10, $MnSO_4$ (Sigma Aldrich) 1.9, tiamin (Merck K GaA) 0.01, niacin (Merck K GaA) 0.01, folic acid (Carlo Erba, Cornaredo, Italy) 0.01, pyridoxal (Sigma Aldrich) 0.01, pantothenic acid (BDH Chemicals, London, England) 0.01, cobalamin (Carlo Erba) 0.01.

In order to have a masterbatch of sakacin A to incorporate into the active materials, *L. sakei* liquid cultures were carried out in a 14 L fermenter (Omnitec Bio, Sedriano, Milano) (7 L volume) applying the following conditions: 26 °C, 9 h incubation, no aeration, agitation speed 150 rpm, pH-stat 4,5, inoculum 5% (v/v) of

a pre-grown culture in MRS medium. Cell-free supernatant containing sakacin A was obtained at the end of incubation by centrifuging the culture broth at 8000 rpm for 40 min at 4 °C (Beckman Coulter, Brea, California, USA).
Sakacin A was precipitated from the obtained supernatant employing ammonium sulfate (400 g/L) (Holck et el., 1992); after 1 h at 4 °C, the sample was centrifuged at 8000 rpm for 40 min at 4 °C; the precipitate was dissolved (10 X) in deionized water and subsequently freeze-dried overnight (Edwards Minifast MFD 01 lyophilizer, UK).
The protein extract containing sakacin-A (hereafter sakacin-A extract), was characterized in terms of antimicrobial activity, evaluated against *L. innocua* by agar diffusion assay as reported below, and in terms of protein content, determined by Lowry assay.

4.3.3. Sakacin A determination

Sakacin A extract antimicrobial activity was determined employing the agar diffusion assay: aliquots of culture broth were centrifuged at 8000 rpm for 20 min; serial dilutions were then prepared in distilled sterile H_2O and 150 µL of each dilution were poured in wells made on a Petri dish containing 30 mL of soft (8 g/L agar) TSA (Tryptic Soy Broth; Merck K GaA) inoculated with *L. innocua* (0.1 % v/v of a pre-grown culture in TSB). Plates were incubated overnight at 37 °C. Bacteriocin activity (AU/mL) was quantified as the reciprocal of the highest dilution exhibiting a clear zone of inhibition, per mL, as reported elsewhere (Trinetta et al., 2008). Sakacin A concentration was also expressed in terms of AU/mg of freeze dried crude extract.

4.3.4. Preparation of active materials

Cellulose nanofibers (CNF) were acquired by Exilva Borregaard (Sarpsborg, Norway). To prepare sakacin A active materials, different preparation strategies were taken in consideration, as follows:

- **Incorporation**

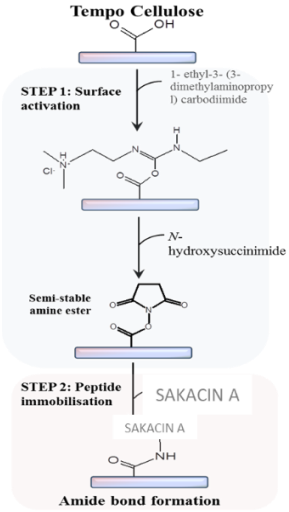
Active mats were prepared by mixing 0.2 g of the liophilized extract containing sakacin A with 0.2 g of CNF (10 g commercial 2% CNF suspension), adding 20 mL of deionized water. After magnetic stirring for 30 min, the suspension was laid out in Petri dish and dried at 60 °C to remove water and obtain mats. Mats without sakacin A (negative controls) were also prepared by suspending 0.2 g CNF in 20 mL of deionized water.

- **Thermal grafting**

Mats were prepared by mixing 0.2 g of the liophilized extract containing sakacin A with 0.2 g of CNF, as previously described. Samples were then thermally treated at 70 °C x 12 h in a vacuum dessicator in order to achieve the chemical reaction of carboxylix groups with CNF.
After the grafting, a Soxhlet extraction with 30% ethanol (VWR) was performed for 16 h to remove all the unbound sakacin and the impurity formed during the reaction. Briefly, one active film (68 cm^2) was inserted in a Soxhlet extractor connected with a condenser; the extractor was placed onto a flask containing the extraction solvent (around 500 mL).

- **Chemical grafting**

In this case, carboxylate cellulose nanofibers (TEMPO CNF: 2, 2, 6, 6- tetramethylpiperidine-1-oxyl, Betulium) with 4,8 % of solid content were used.
1 g of dry TEMPO CNF was diluted with 160 mL 2-(N-morpholino) ethanesulfonic acid (MES) buffer (10 mM, pH 4.5) (Sigma Aldrich). The CNF suspension was stirred for 30 min, and then the required quantity



of sakacin A (0.2 g) was added. Two solutions were also prepared: i) 0.84 g of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, Sigma Aldrich) in 10 mL of MES buffer, and ii) 0.5 g of NHS (N-hydroxy- succinimide) (Sigma Aldrich in 10 mL of MES buffer. The two solutions were then added to the mixture (Fig. 4.1), and the mixture stirred for 24 h at room temperature, laid out and dried to obtain mats.

Figure 4.1: Schematic representation of the main steps of the chemical grafting strategy using TEMPO-CNF cellulose.

- **Coating**

Sakacin A was also applied to films by the coating strategy. Three different supports were used: paper, PP/EVOH (Polypropylene / Ethylene Vinyl Alcohol copolymer) and PP/EVOH with caseinate in coating solution (Sigma Aldrich, 8% casein sodium salt in water pH 9.6 + 10% glycerol); the last two supports were subjected to a Corona discharge treatment generating ozone from an electrical discharge, to create a superficial oxygenation to increase the surface energy and improve wettability and coating adhesion. The sakacin A extract was mixed with 1% CNF in distilled water and then coated onto the supports (10 µL/cm^2): three different amounts of extract were initially applied on PP/EVOH and correspondent to 1, 2 and 5 AU/cm^2. The suspension containing the desired concentration was then applied to all the supports and spread through a Baker Universal Film Applicator (0 to 250 µm, width 80mm) (Neurtek S. A., Eibar, Spain).

4.3.5. *In vitro* antimicrobial activity of active materials

Antimicrobial efficacy of the produced materials was confirmed by two *in vitro* tests. Qualitative and quantitative assessments of antimicrobial activity were carried out using *L. innocua* as indicator strain.

- **Qualitative assessment**

Listeria pre-inoculated agar plates were prepared as follows: an aliquot of 300 µL overnight *Listeria* culture was added to 30 mL soft TSA (8 g/L agar) in a Petri dish. Circular film portions of 20 mm diameter were then placed onto agar medium and then incubated for 16 h at 37 °C. The leaching ability of the CNF only (negative control) and of the active CNF-sakacin A materials were determined by the formation of a clear halo of *Listeria* growth inhibition under and around the deposited samples. All experiments were repeated three times.

- **Quantitative assessment**

Quantitative assessment of the antimicrobial activity was performed under static conditions. A previously weighed and dry sterilized film portion (0.05 g, around 2 x 2 cm) was placed in a sterile Petri dish; on its surface, 200 µL of a *Listeria* cell suspension (around 5 x10^5 CFU/mL) prepared in diluted TSB (1 volume TSB added with 4 volumes of sterile isotonic solution) were added.

After 24 h incubation at 37 °C, bacteria were resuspended employing 50 mL of neutralizing solution having the following composition (g/L): lecithine (Carl Roth GmbH, Karlsruhe, Germany) 3, sodium thiosulphate (Carl Roth GmbH) 5, L-histidine (Carl Roth GmbH) 1, Tween-80 (Merck K GaA) 30, potassium dihydrogen phosphate buffer (Sigma Aldrich) 10 mL, pH 7.2 ± 0.2.

The numbers of colony forming units (CFU) within the resulting suspensions were then enumerated using the plate count method.
For each type of film, trials were repeated twice.

4.3.6. Materials characterization

Infrared spectra were recorded for neat and modified CNF in ATR mode using a Perkin Elmer Spectrum 65. All spectra were recorded between 4000 and 600 cm^{-1}, with a resolution of 4 cm^{-1} and 8 scans.
Thermo-gravimetric analysis was carried out using a Perkin Elmer simultaneous thermal analyzer (STA 6000). Samples of about 30 mg were placed in a pan and tested at a heating rate of 10 °C/min from ambient temperature to 900 °C under air. All experiments were repeated at least twice.

4.3.7. *In vivo* antimicrobial activity of active materials on smoked salmon

Smoked salmon fillets were purchased from a large-scale retail channel (Vega Salmon GmbH, ingredient: salmon (*Salmo salar*), salt 3% on salmon weight). Fillets were aseptically cut into sections of 9 cm diameter and an area of around 60 cm^2 and then inoculated with a *L. innocua* cells suspension to obtain a total microbial load of approximately 10^3 cells/cm. Inoculated samples were covered (top and bottom) with CNF-sakacin A films or CNF-only films (negative control) and then transferred in Petri dishes (Fig. 4.2).

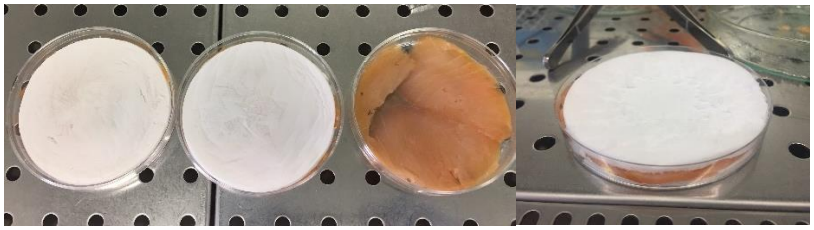


Figure 4.2: Samples preparation.

All samples were packed in PA/PE plastic food vacuum bags (Reber, Luzzara, Italy) under vacuum and then stored at 6 °C up to 28 d. Samples of salmon fillet stored un-covered under vacuum were also prepared. Starting from t0, every 7 d salmon fillets were transferred aseptically into Stomacher bags (VWR blender bag, Milano, Italy), filled with physiological solution (9 g/L NaCl, Merck K GaA, 9x sample weight) and blended in a Stomacher (Star Blender LB 400, VWR, Milano, Italy) for 3 min. Decimal dilutions series of the obtained suspension were then carried out for *Listeria* determination (CFU/mL). Selective *L. innocua* determination was performed employing ALOA culture medium (Agar *Listeria* Ottaviani Agosti added with enriched and selective supplements; Biolife, Milano, Italy) and plates incubated at 37 °C for 48 h. Experiments were replicated twice. Counts were reported as logarithm of the number of colony forming units (Log CFU/g salmon), and mean and standard deviation calculated.

4.3.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism software (version 8.0.1, San Diego, CA, USA), the effect of two factors (time and treatment) were investigated by ANOVA according to the general linear model. When the effect was significant ($p < 0.05$), differences between means were separated by Tukey test of multiple comparisons.

4.4. Results

4.4.1. Sakacin A production and purification

L. sakei in the applied culture conditions was found to grow with a lag phase of 98.29 ± 6.99 min, a final value of 1.61 OD and a max rate of 0.010 ± 0.001 OD/min (R^2= 0.987, SE of Fit = 0.056).

The highest sakacin A production (333 AU/mL) was achieved at the beginning of the stationary phase, around 8 h incubation as previously reported in Fig. 3.8.

Based on the obtained results, a masterbatch of 7 L of *L. sakei* liquid culture was produced. After centrifugation, supernatant was added with ammonium sulphate to obtain the sakacin A enriched precipitate. The process allowed to obtain 4.51 g/L of initial culture medium of freeze-dried protein extract containing sakacin A, characterized by a total antimicrobial activity of near 530000 AU (Tab. 4.1), that means 25% of initial activity. The wet precipitate was collected in distilled water (1/10 of supernatant volume) and then freeze-dried, obtaining 31.60 g of sakacin-A extract (4.51 g/L of initial culture medium). The total yield of the enrichment procedure set near to a total antimicrobial activity of 530000 AU (Tab. 4.1), that means 25% of initial activity. Although ammonium sulphate precipitation is causative of the main losses (and its implementation is a future desirable outcome), this step allows a straightforward purification of the bacteriocin, increasing the specific activity from 56 to 256 AU per mg of total protein.

Table 4.1: Sakacin A recovery yield.

Fraction	Volume (mL)	Specific Activity (AU/mL)	Total Activity (AU)	Protein content (mg/mL)	Specific activity (AU/mg protein)	Yield (%)
Culture medium	7000	337	2359000	n.d.*	n.d.	100
Supernatant	6300	333	2097900	6.0	56	90
After $(NH_4)_2SO_4$ precipitation, 10x re-dissolved	630	933	587790	3.2	291	28
Sakacin A extract	31.60 g	16.70 AU/mg	527720	63 mg/g	265	25

*: n.d.: not determinable

4.4.2. *In vitro* antimicrobial activity

Note that *L. innocua* was taken as surrogate of the food-borne pathogenic *L. monocytogenes*. A surrogate is a bacterium that has physiological characteristics nearly identical to a pathogenic bacterium of interest, and is used to give a margin of safety to the researchers and to prevent unnecessary exposure to pathogens (Friedly et al., 2008).

Qualitative assessment

As regards mats produced by incorporation of sakacin A in CNF, trials carried out *in vitro* with solid cultures of *L. innocua* confirmed that CNF-sakacin A film possessed antimicrobial activity, as was evidenced from the formation of a halo of growth inhibition around the area in which the film was deposited onto *Listeria-*

agar plate. In contrast, CNF alone mats prepared without the bacteriocin (negative control) did not produce any growth inhibition (Fig. 4.3).

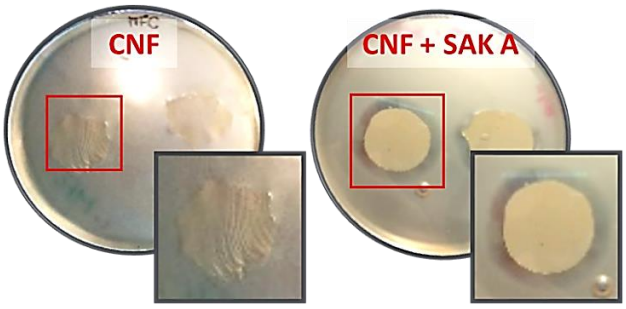


Figure 4.3: Solid TSA culture of *Listeria innocua* after incubation at 37°C in presence of CNF-alone (negative control, left) or with CNF- sakacin A (antimicrobial sample, right) mats.

Samples produced by grafting evidenced a small halo of *Listeria* growth inhibition, nevertheless, when mats were exposed to shoxhlet treatment to complete the grafting between CNF and sakacin A and remove the surplus, antimicrobial activity was lost, and inhibition halo was absent around the film (Fig. 4.4).
Also when TEMPO CNF was used and a chemical grafting was promoted through oxidation between sakacin A and cellulose, no inhibition halo was observed in plates, confirming that samples did not exert any antimicrobial activity (Fig. 4.4).

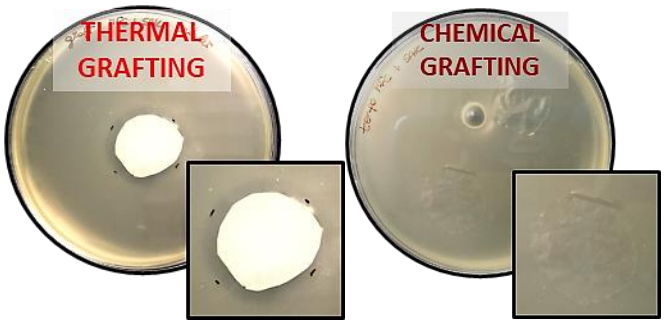


Figure 4.4: Solid TSA culture of *Listeria innocua* after incubation at 37°C in presence of CNF- sakacin A films obtained after: thermal grafting process (left) and chemical grafting (right).

As regards active films produced by the coating technique, qualitative *in vitro* tests were initially performed to determine the amount of sakacin A to add to samples. Sakacin A extract was added in order to obtain three specific sakacin A concentration, i.e. 1, 2 and 5 AU/cm^2; these solutions were layered on PP/EVOH and tested.

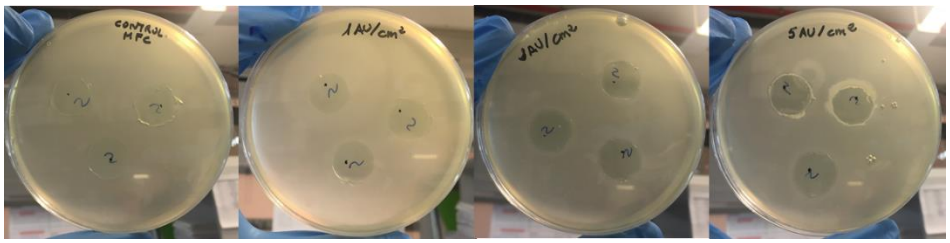


Figure 4.5: Solid TSA culture of *Listeria innocua* after incubation at 37°C in presence of samples obtained after coating on PP/EVOH, different amounts of sakacin A were tested: 0 (control), 1 AU/cm^2, 2 AU/cm^2 and 5 AU/cm^2.

All the three samples compared with the negative control showed a clear zone of *Listeria* growth inhibition; nevertheless, unlike the previous samples, the inhibition halo was found present only under and not around the films (Fig. 4.5).

Quantitative assessment

Quantitative trials were performed to determine the ability of the produced active materials to reduce the population present in a *Listeria* cell suspension.

As regards mats obtained by incorporation of sakacin A with CNF, a 2-log cycle reduction (from 6 to 4 Log CFU/mL) was evidenced when the active material was kept in contact with the cell suspension for 24 h (Fig. 4.6).

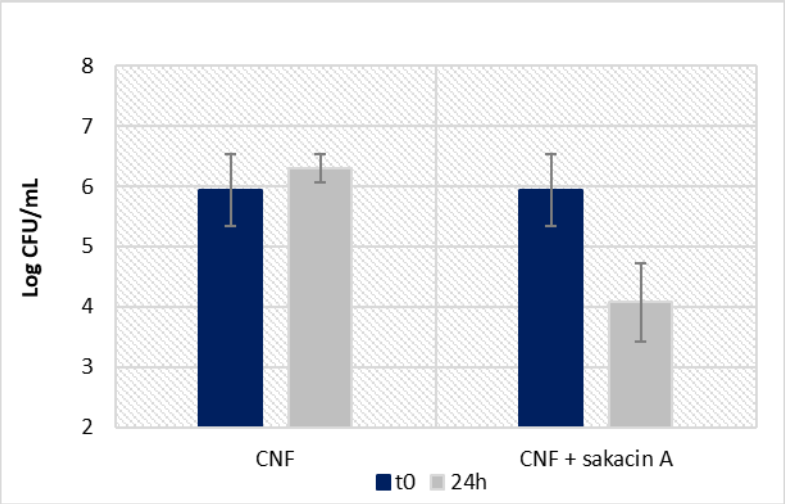


Figure 4.6: Cell concentration (in terms of Log CFU/mL) of *L. innocua* cultures incubated at 37°C for 24 h in presence of CNF-alone (negative control, left) or with CNF- sakacin A (antimicrobial sample, right) mats. Means with different superscript letters are different ($p < 0.05$).

As regards the materials produced applying the coating techniques and containing 5 AU/cm^2 sakacin, results are reported in Fig. 4.7.

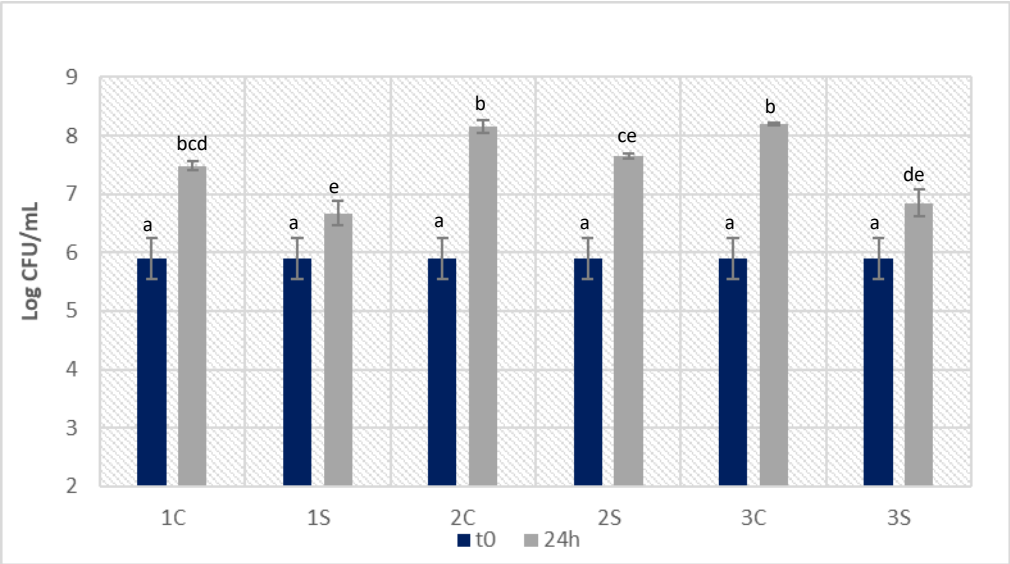


Figure 4.7: Cell concentration (in terms of Log CFU/mL) of *L. innocua* cultures incubated at 37°C for 24 h in presence of films obtained with coating techniques. 1- paper, 2- PP/EVOH and 3- PP/EVOH with caseinate. C represents the negative control without sakacin A while S the active film with 5 AU/cm^2. Means with different superscript letters are different ($p < 0.05$).

Unfortunately, *Listeria* was found to grow in presence of all the active materials; all the active samples were found different from each respective negative control; moreover, note that the active material 1S was found statistically different from all the other negative controls; (Fig. 4.7). From the overall results, these materials did not evidence satisfactory results as no inhibition, even at low levels, was proved.

4.4.3. Materials characterization

Characterization was performed only on materials produced by incorporation with CNF with the sakacin A enriched extract, the only active materials against *Listeria*. FTIR analysis was carried out on pure sakacin A, CNF film alone (negative control) and sakacin A incorporated CNF mats (Figure 4.8).

For the pure sakacin A, a strong bong for the alkyl group can be observed at 2903 cm^{-1} and at 1600 cm^{-1} for carboxyl group. The peak beween 1500 and 1300 cm^{-1} represents the presence of the nitrogen groups. Well known conventional bands for cellulose were observed at 3300 cm^{-1}, 1250–1460 cm^{-1}, 2850–2980 cm^{-1} and 1170–1150 cm^{-1} corresponding to the stretching vibrations of hydroxyl groups (OH), alkyl groups (CH and CH_2) and C–O–C bonds from glycosidic bridges respectively. Sakacin A incorporated CNF mats present both prime peaks for the sakacin A and CNF. With the addition of sakacin A, there is a significant increase in the peak for the alkyl; moreover, the appearance of a small peak at 1600 cm^{-1} for carboxyl group confirmed the presence of sakacin A incorporation.

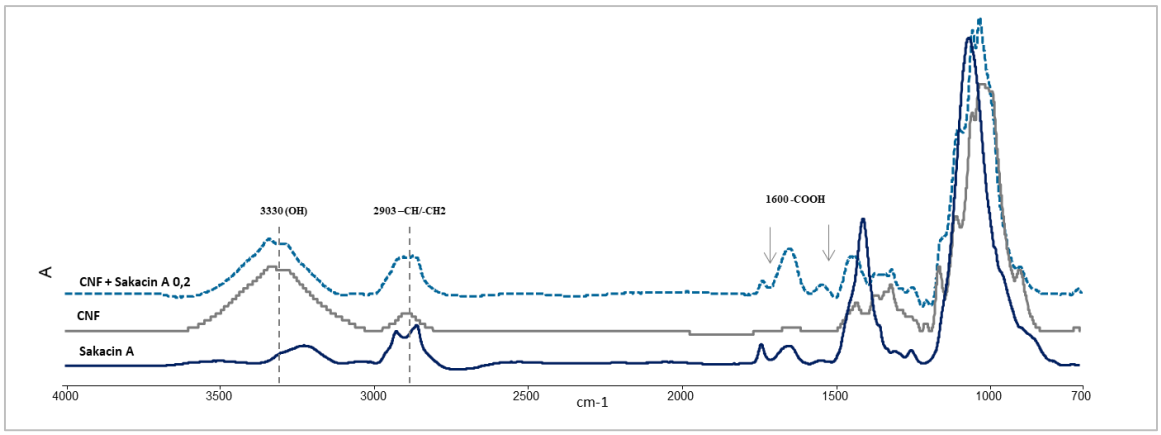


Figure 4.8: FTIR spectra of mats prepared employing CNF only and CNF-sakacin A; spectrum of the sakacin A enriched extract in the same condition is also reported.

The thermal properties analysis of the fibers provides valuable information about the physical and chemical characteristics after incorporation of sakacin A. The thermograms (weight loss) of the sakacin A, neat cellulose nanofibers and sakacin A incorporated CNF are presented in Fig. 4.9.

The neat cellulose showed a conventional thermogram with a maximum degradation temperature at around 350 °C (Alemdar and Sain, 2008). The results indicated a weight loss of 5–7 % below 150 °C due to the residual moisture stored in the neat cellulose nanofiber films. The pure sakacin A was also examined and demonstrated a double step degradation profile at 200 °C (a steep decrease) and 385 °C (a linear decrease). With the incorporation of the sakacin A in CNF, even if thermograms showed degradation profiles similar to the CNF-only sample, dTGA profiles evidenced the presence of the sakacin A signal.

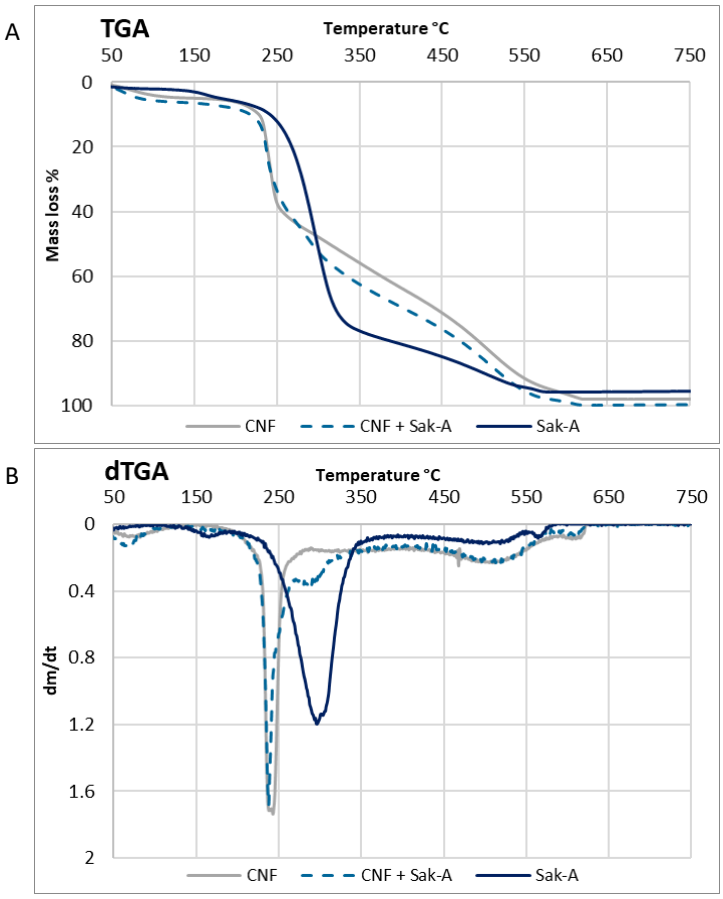


Figure 4.9: Thermogravimetric analysis of materials prepared employing CNF only and CNF-sakacin A. A: original unit, B: derivative. The behavior of an enriched sakacin A solution at the same concentration employed in film preparation is also reported.

4.4.4. In vivo antimicrobial activity

CNF-sakacin A active materials were evaluated for their antimicrobial effectiveness on food samples. *Listeria* population was determined in samples of smoked salmon purposely contaminated with *L. innocua* and then stored at 6 °C for up to 28 d (Table 4.2).

Results evidenced a 2-Log cycles reduction of *Listeria* population in salmon samples stored in presence of CNF sak A mats, respect to the population inoculated at t0 (6.08 *vs.* 3.99 Log CFU/g). Moreover, when comparing results with the other trials performed without or with CNF-only film, final population was reduced of about 2.5-3 Log cycles (8.70 - 8.90 Log CFU/g) ($p = 0.0007$).
These experiments demonstrate the antimicrobial activity of the CNF-sakacin A active mats and their effectiveness against *L. innocua* in storage trials of a RTE food product.

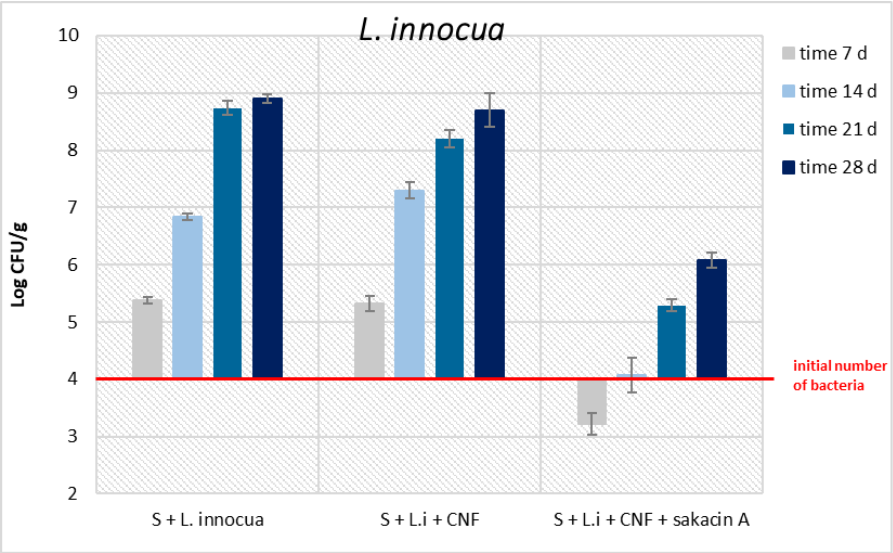


Figure 4.10: Log reduction of *Listeria innocua* population inoculated in salmon stored in absence or presence of the active materials.

Table 4.2: *Listeria* population (Log cfu/g) in samples of smoked salmon intentionally inoculated and then stored for up to 28 d at 6 ± 1 °C in absence and in presence of the CNF-only or the active CNF- sakacin A mats. Means with different superscript letters are different ($p < 0.05$).

SAMPLE / Days	Salmon + *L. innocua*			Salmon + *L. innocua* + CNF mat			Salmon + *L. innocua* + CNF sak A mat		
	Log CFU/g	±	Growth value	Log CFU/g	±	Growth value	Log CFU/g	±	Growth value
0	3.99 ^{b}	0.08	0.00	3.99 ^{b}	0.08	0.00	3.99 ^{b}	0.08	0.00
7	5.38 ^{c}	0.06	+1.39	5.32 ^{c}	0.13	+1.34	3.22 ^{a}	0.19	-0.76
14	6.84 ^{e}	0.06	+2.85	7.29 ^{e}	0.14	+3.30	4.08 ^{b}	0.30	+0.09
21	8.74 ^{f}	0.12	+4.75	8.20 ^{f}	0.14	+4.21	5.29 ^{c}	0.11	+1.91
28	8.90 ^{f}	0.08	+4.91	8.70 ^{f}	0.29	+4.71	6.08 ^{d}	0.13	+2.09

Active materials produced by the coatings technique and containing 5 AU/cm^2 were also tested in *in vivo* trials: smoked salmon intentionally contaminated was stored at 6 °C and *Listeria* population was detected at t0 and after 10 days of storage (Tab. 4.3). All active materials were found effective in *Listeria* reduction (around 1 Log cycle) even though Listeria grew till around 5 Log (starting from 3.8 Log).

Table 4.3: *Listeria* population (Log cfu/g) in samples of smoked salmon intentionally inoculated and then stored for 10 d at 6 ± 1 °C in absence and in presence of sakacin A active materials. Films were obtained by coating (sakacin present at 5 AU/cm^2) onto respectively: 1- paper, 2- PP/EVOH and 3- PP/EVOH with caseinate.

Sample		Log CFU/g	± std. dev	Growth value	Antimicrobial activity
Salmon + *L. innocua* t0		3.79 [a]	0.02	0.00	
Salmon + *L. innocua* 10 d		5.41 [b]	0.01	+1.64	
1C	**(Negative control)**	6.69 [c]	0.02	+2.91	
1S	**(+ SAKACIN)**	5.71 [d]	0.13	+1.94	-0.97
2C	**(Negative control)**	6.10 [e]	0.04	+2.33	
2S	**(+ SAKACIN)**	5.40 [b]	0.00	+1.63	-0.71
3C	**(Negative control)**	5.99 [e]	0.01	+2.22	
3S	**(+ SAKACIN)**	5.02 [f]	0.09	+1.25	-0.97

4.5. Discussion

The present research was focused on developing and characterizing a novel antimicrobial material employing a freeze-dried enriched preparation of the bacteriocin sakacin A. In the first step, the bacteriocin was produced in liquid culture employing a low cost medium formulated with cheese whey permeate and purified using a classic procedure involving ammonium sulphate precipitation; a total activity yield of 25% was obtained, with an enriched sakacin A extract containing 16.70 AU/mg.

Sakacin A extract was combined with CNF to produce antimicrobial cellulosic materials. Cellulose shows interesting properties to produce biodegradable films as well as for its application as a carrier of antimicrobial molecules. Different strategies for the conjugation or grafting of antimicrobial compounds on CNF have been described in literature but procedures result closely connected to the active molecule (aminosilanes, antibiotics, bacteriocins etc.) (Espitia et al., 2013; Saini et al., 2016; Saini et al., 2017). To the best of our knowledge, this is the first study related to sakacin A and CNF combination for the formation of antimicrobial materials.

Different methods are commonly used to prepare antimicrobial cellulose-based food packaging: one is the addition of the antimicrobial compound into the polymer matrix by directly incorporation or chemical immobilization into the packaging matrix by ion or covalent linkage, while the other relates to coating the substance onto the surface of packaging matrix.

Several strategies to conjugate cellulose and bacteriocins were considered in this study, taking cues from the literature; nevertheless, data reported in most of the research are referred to the bacteriocin nisin, due to the fact that to date it is the only bacteriocin approved by FDA and by the EU. Generally, nisin is incorporated into the polymer via direct and simple blending, as reported Coma et al. (2001) and also Imran et al. (2010) that combined nisin and hydroxypropylmethylcellulose by incorporating the bacteriocin into the film-forming solution prior to film formation, however this strategy presents limitation and shows disadvantages in maintaining the antimicrobial activity for long terms.

Different possible solutions were showed by Saini et al. (2016) that covalently linked nisin on the surface of TEMPO oxidized CNF for food packaging application, and by Wu et al. (2018) that developed a green

process of anchoring nisin onto oxidized cellulose, exploiting the interaction between the amino group of nisin and the aldehyde group present on oxidized cellulose; both demonstrated the antimicrobial activity of the produced films for long-term usage.

Some studies investigated the use of pediocin as antimicrobial agent, as for example Ming et al. (1997): they produced pediocin and nisin by growing the respective microorganisms in skim milk based medium and then applied the pediocin powder into inner surfaces of plastic packaging bags at a concentration of 7.75 μg/cm^2, demonstrating the antimicrobial efficacy on meat sample inoculated with *L. monocytogenes.* Santiago-Silva et al. (2009) evaluated the antimicrobial activity of films produced incorporating pediocin (ALTA 2351) in a cellulose base emulsion and tested against *Listeria innocua* and *Salmonella sp*. on samples of sliced ham.

Nevertheless, studies related to use of bacteriocin in packaging solutions have already highlighted that main encountered difficulty is to set-up an appropriate protocol for the use of the bacteriocin sakacin A. Results obtained in these trials showed that when this bacteriocin was involved in a chemical bond, as in the grafting, the antimicrobial activity disappeared and the obtained films were found inefficacy. On the contrary, the production of CNF-sakacin A active mats by incorporation were found effective in *in vitro* tests and also in reducing *Listeria* population in samples of smoked salmon of about 3 Log cycles respect to the values reached without or with CNF-only film after 28 d of storage. In the applied conditions, the bacteriocin concentration in mats was around 50 AU/cm^2. Other researchers have already proved sakacin A effectiveness in food packaging: Barbiroli et al. (2017) reported on the development of a sakacin A active paper (0.63 mg protein/cm^2) produced by coating a polyethylene-coated paper sheets employing a crude sakacin A extract to obtain an active antimicrobial package. Storage trials of thin-cut veal meat slices inoculated with *Listeria* laid on active paper sheets evidenced a 1.5 Log units reduction of *Listeria* population respect to control after 48 h at 4 °C. Trinetta et al. (2010) demonstrated the efficacy of the bacteriocin against different epidemic clones of *L. monocytogenes* and the effectiveness of sakacin A pullulan films, experimentally inoculated surfaces of turkey breast were covered with a section of sakacin A-containing (1 mg/cm^2) pullulan films. The results showed reduction of up to 3 Log CFU/g after 3 weeks under refrigerated storage. Comparison among these results on sakacin application was found complex since sakacin A was differently produced and differently purified as well as the amount of bacteriocin to use for creating packaging solution estimation.

As regards the coatings solutions tested, results were considered up to now not totally satisfactory even if preliminary: although a slight difference in *Listeria* population was found comparing active samples with the negative controls in *in vivo* trials, the amount of sakacin used in coatings preparation resulted too low to produce an antimicrobial activity lasting over time. The choice of using such a low amount of sakacin was suggested by preliminary qualitative trials that showed a clear inhibition halo under the sample at the applied concentration. As previously evidenced, however, the halo was present but not extended over the films; the positive results obtained in qualitative trials were not enough to exert a strong *Listeria* inhibition in quantitative *in vitro* and *in vivo* samples.

The coating at 5 AU/cm^2 was also initially chosen in order to obtain a cost-effective solution; further trials on the coating application employing higher amount of sakacin A are now under investigation.

From an overall look at the obtained results, the developed package, not intended as a way to “clean” a contaminated food product, can significantly contribute to reduce the risk of *L. monocytogenes* outbreaks in ready-to-eat food products.

Conclusions to chapter 4

This work aimed at creating a sustainable and active food package, in which a protein extract containing the bacteriocin sakacin A was combined with cellulose nanofibers (CNF). Antimicrobial materials were prepared applying incorporation, grafting or coating techniques, comparatively. Neat incorporation of the antimicrobial produced the best antimicrobial performances. Application of sakacin A-CNF films on smoked salmon fillets under conditions similar to those foreseeable for a future practical use proved the antimicrobial activity against *Listeria*. Future trials will be aimed at investigating whether these antimicrobial films may find practical uses (e.g., paper liners or wraps) and their effectiveness when in contact with other food products, taking into account that antimicrobial release and activity may vary depending on the nature, the composition and humidity of food items.

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Chapter 5:
Conclusions & Perspectives

5. Conclusions

This work has been fostered by the willingness to find viable solutions to control the food-associated pathogen *L. monocytogenes* known as the causative agent of listeriosis, a highly fatal opportunistic foodborne infection. Although listeriosis is rare, it has high social and economic impact representing the third-leading cause of death among foodborne bacterial pathogens.
The EU monitoring programme confirmed 2536 invasive human cases of listeriosis for the year 2016, with a statistically significant increasing outbreaks trend if compared with the 1720 cases in 2012 (+47%). Listeriosis is predominately associated with RTE products since they are characterized by an extended shelf-life at refrigeration temperature and they are in general consumed in their raw state without further cooking or processing.
In this frame, the application of bacteriocins can represent a natural biopreservation strategy that may be effectively exploited to control *L. monocytogenes* in RTE products, meeting the request of consumers for food containing only natural additives. An interesting solution for applying bacteriocins to food is to deliver them as part of an active packaging; this mechanism may represent an efficient solution to control the release of the antimicrobial agent during food storage, maintaining its antimicrobial activity over time.
In this research Sakacin A, a class IIa bacteriocin produced by the LAB *Lactobacillus sakei*, was taken into consideration due to its antilisterial action, to its ability to resist to high temperature and acid conditions and to its GRAS classification. Production and applications of this bacteriocin in food systems have been investigated and optimized in the chapter of thesis:

In chapter 3:

- A new food grade medium was developed for sakacin A production employing whey permeate, a dairy waste, in order to scale up production at industrial scale with competitive results.
- Sakacin A production was satisfactorily scaled up from flask to 20 L bio-reactor and a protein extract containing the bacteriocin sakacin A was produced in high quantity.
- New purification protocol was set up and applied to increase sakacin A yield; this protocol allowed to recover 53% of the initial antimicrobial Units, significantly increasing the process yield.

In chapter 4:

- Antimicrobial materials were produced using a protein extract containing sakacin A and cellulose nanofibers.
- All the materials produced trough incorporation and coating were found active against *L. innocua* in *in vivo* trails.
- The most effective antimicrobial mat was found active also in *in vivo* tests on salmon fillets, in which *Listeria* population was reduced of about 2.5-3 Log cycles after 28 d storage at 6 °C, compared with negative controls set-up without the bacteriocin extract.

The present research, focused on developing a novel antimicrobial material employing a freeze-dried enriched preparation of the bacteriocin sakacin A, proved the possibility to use a food-waste as whey permeate, suitable for the industrial up-scaling of a natural antimicrobial molecule, as sakacin A. Improvements in antimicrobial unit recovery were obtained increasing bacteriocin yield form 25%, with classical purification, to 53% employing a single step gravity RP-chromatography. The produced protein extract containing sakacin A proved active also when applied in antimicrobial packaging solutions and a remarkable action against *L. innocua* was demonstrated also in food when the extract was incorporated in

nanofibers for mats preparation. It is possible to conclude that the present work demonstrates the possibility of producing a nanocellulose-based active packaging employing a protein extract enriched in sakacin A, starting from a food industry waste.
Although this work should be considered a preliminary investigation, it will pave the way into the potential applicability of sakacin A in bio-based active packaging solutions.

Nevethless, some critical points related to sakacin production and application were highlighted during the present experimental work, such as the difficulties in comparing results in terms of antimicrobial activity due to the absence of a sakacin A standard and to the subjectivity of the antimicrobial tests employed.
To sort this trouble out, different strategies to produce a pure extract of sakacin A are actually on the way: obtaining sakacin A by a recombinant microorganism such as *Escherichia coli*, applying more efficient purification techniques, and synthetizing a sakacin A standard trought chemical synthesis.
As regards the difficulty encountered when dealing with the antimicrobial test and sakacin production, different methods were taken into consideration with the aim of replacing agar diffusion assays, nevertheless results were not satisfactory and resulted hardly applicable. Others critical issues were found in sakacin A production and recovery due to the limited reproducibility among fermentation trials in terms of grams of protein extract obtained and relative activity.
As regards the development of the antimicrobials materials, the greatest difficulty was to find the best way to combine sakacin A with nanocellulose: results evidenced that sakacin antimicrobial activity was lost when the molecule is involved in a linkage; at the moment the best solution seems a simple mixing of the two substances for the production of antimicrobial mats. Sakacin A conjugation with nanocellulose crystals is now under investigation as a part of the Nanosak project.
Future trials will also be aimed at investigating whether these antimicrobial materials may find practical uses (e.g., paper liners or wraps) and their effectiveness when in contact with other food products, taking into account that antimicrobial release and activity may vary depending on the nature, the composition and humidity of food items.

Chapter 6: Products

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6.1. Dissemination of results

Mapelli C., Musatti A., Barbiroli A., Saini S., Bras J., Cavicchioli D. & Rollini M. (2019). Cellulose nanofibers (CNF) - Sakacin-A active material: production, characterization and application in storage trials of smoked salmon. *Journal of the Science of Food and Agriculture.* (DOI 10.1002/jsfa.9715)

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Cellulose nanofiber (CNF)–sakacin-A active material: production, characterization and application in storage trials of smoked salmon

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ABSTRACT

BACKGROUND: Sakacin-A due to its specific antimicrobial activity may represent a good candidate to develop active packaging solutions for food items supporting *Listeria* growth. In the present study a protein extract containing the bacteriocin sakacin-A, produced by *Lactobacillus sakei* Lb 706 in a low-cost culture medium containing deproteinized cheese whey, was adsorbed onto cellulose nanofibers (CNFs) to obtain an active material to be used as a mat (or a separator) in direct contact with foods.

RESULTS: The applied fermentation conditions allowed 4.51 g L^{-1} of freeze-dried protein extract to be obtained, characterized by an antimicrobial activity of near 16 700 AU g^{-1}, that was used for the preparation of the active material by casting. The active material was then characterized by infrared spectra and thermogravimetric analyses. Antimicrobial trials were carried out *in vitro* using *Listeria innocua* as indicator strain; results were also confirmed *in vivo*, employing smoked salmon fillets intentionally inoculated with *Listeria innocua*: its final population was reduced to about 2.5 – 3 Log cycles after 28 days of storage at 6 °C in presence of sakacin-A, compared with negative control mats produced without the bacteriocin extract.

CONCLUSION: This study demonstrates the possibility of producing an antimicrobial active material containing sakacin-A absorbed onto CNFs to decrease *Listeria* population in smoked salmon, a ready-to eat-food product.

Mapelli C., Barbiroli A., De Benedetti S., Musatti A. & Rollini M. (2019). Antilisterial bacteriocins for food security: the case of sakacin A. In: Ferranti, P., Berry, E.M., Anderson, J.R. (Eds.), *Encyclopaedia of Food Security and Sustainability*, vol. 2, pp. 385–392. Elsevier.

Antilisterial Bacteriocins for Food Security: The Case of Sakacin A

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Abstract

Listeriosis is a rare but one among foodborne diseases with the highest social and economic impact. The cases of listeriosis are most frequently associated to Ready to Eat (RTE) products characterized by an extended shelf life at refrigeration temperature and generally consumed without further cooking. Bacteriocins are antimicrobial compounds produced mainly by Lactic Acid Bacteria; among them, sakacin A, a class IIa bacteriocin produced by *L. sakei*, is a 41 amino acids peptide possessing a strong antimicrobial activity against *Listeria*, and may focus great potential as food biopreservative. The source of sakacin A for food security application can be either the purified compound, the crude grown culture, as well as the bacteriocin-producing organism itself. We present here an overview of bacteriocins applications to improve food security with a particular focus on sakacin A, taking in consideration also its release through the use of active packaging systems.

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Submitted for Poster presentation

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Cellulose Nano Fibers (CNF) - Sakacin-A active package to reduce *Listeria* population in smoked salmon

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Background: Sakacin-A is a class IIa bacteriocin produced by the LAB *Lactobacillus sakei*; like all bacteriocins belonging to this class, it exerts an antimicrobial effect against the causative microorganism of listeriosis, a highly fatal opportunistic foodborne infection. An interesting feature of this bacteriocin is its potential to be delivered through active packaging materials for food which could support *Listeria monocytogenes* growth.
Objectives: High yield production of a protein extract enriched in sakacin-A, employing a low-cost medium formulated with cheese whey permeate; the extract will be used to produce active films, whose antimicrobial activity will be tested *in vitro* and *in vivo* trials.
Methods: *L. sakei* DSMZ 6333 was grown in a 14 L fermenter. Sakacin-A was purified from the cell-free supernatant by ammonium sulphate precipitation and lyophilization. The produced sakacin-A extract was adsorbed onto cellulose nanofibers (CNF) to obtain active films that were characterized by infrared spectra and thermo-gravimetric analyses. Antimicrobial trials were carried out *in vitro* using *Listeria innocua* as an indicator strain; results were also confirmed *in vivo,* employing intentionally inoculated smoked salmon (10^3 cells/g).
Results: 4.51 g of freeze dried protein extract were obtained from 1 L supernatant, containing about 75400 AU. A clear halo of inhibition was observed in *in vitro* tests around *L. innocua* solid cultures; *in vivo* tests yielded a similar outcome*:* in smoked salmon, *L. innocua* population was reduced by about 2.5-3 Log cycles after 28 days at 6°C in presence of the active films, compared with negative control without the bacteriocin extract.

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From Cheese Whey Permeate To An Anti-Listeria Food Packaging Device: Bacterial Cellulose Nanocrystals/Sakacin-A Conjugates (Nanosak)

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In the present project cheese whey permeate (CWP), the residual by-product obtained by extraction of whey proteins from cheese whey, was used as substrate for the growth of bacterial species that produce two appealing molecules: the anti-listerial bacteriocin sakacin-A and bacterial cellulose (BC). BC is then turned into nanocrystals (BCNCs) that are finally conjugated with sakacin-A to obtain an innovative antimicrobial device for food which could support Listeria monocytogenes growth. Sakacin-A was produced by Lactobacillus sakei DSMZ 6333 in liquid cultures. The highest bacteriocin production (around 300 AU/mL) was achieved after 9 h at 26°C; a food-grade, salt-free enriched sakacin-A extract was obtained by using a gravity reverse phase chromatography. BC was produced by Komagataeibacter xylinus DSMZ 2325 by static fermentation of CWP in presence of 0.5 U/mL of β-galactosidase at 30°C; after 7 days, BC yield was around 7 g/L. BCNCs were then obtained by acid hydrolysis mediated by sulfuric acid, with the goal of removing the amorphous regions of BC and introduce a net negative charge by esterification on the hydroxyl group on C6. BCNCs/sakacin-A conjugates were prepared by exploiting their opposite charge: enriched sakacinA extract was mixed with BCNCs and, after incubation, conjugates collected by centrifugation have a specific activity of 100 AU/mg BCNCs. Among all peptides present in the enriched sample, sakacin-A appears to preferentially absorb onto BCNCs, thus allowing its further purification. Sakacin-A as well its BCNCs conjugates were then included in a hydroxypropil-cellulose coating spread onto paper sheets at a concentration of 5 and 25 AU/cm2. The addition of the coating did not bring any significant change in the oxygen barrier properties of the cellulosic substrate. In a similar way, the static contact angle of both uncoated and coated substrate was of approximately 130°. However, the presence of BCNCs seemed to increase the swelling phenomenon of the coating. Sakacin A was also included in whey, caseine and cellulose derived matrices to prepare films and coatings with diverse results. The kinetics of Sakacin-A released from active films to aqueous food was analyzed by immersion of samples in water (as simulant) and measuring the anti-Listeria activity of the simulant after increasing times of exposure. In vitro and in vivo antimicrobial trials were carried out on real food products demonstrated their anti-listerial effectiveness, proving that the developed devices can contribute to increase shelf life, quality and safety of perishable foods.

Keywords: sakacin-A, bacterial cellulose, cellulose nanocrystals, active packaging, *Listeria*
Acknowledgement: Work supported by funds of the Fondazione Cariplo 2015-0464 NANOSAK - Nanocellulose–sakacin A conjugates for food packaging purposes.

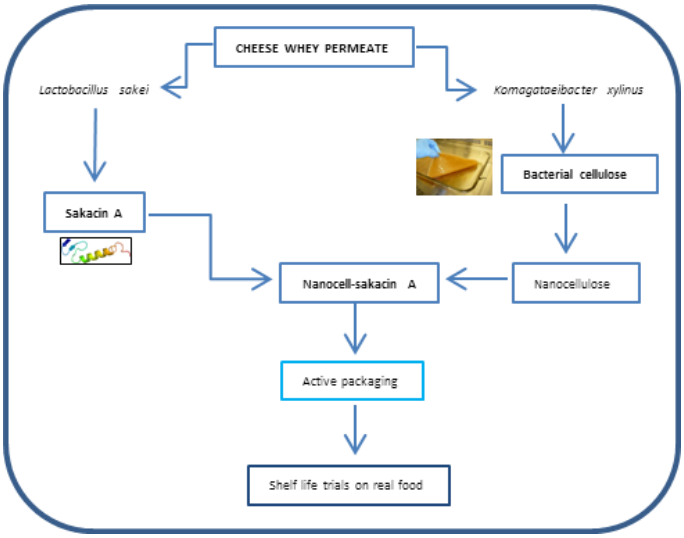
MATBIM – 5th International Meeting on Material/Bioproduct interactions - Milan (Italy), May 8-10, 2019

Submitted for Oral presentation

Authors: M. Rollini, A. Musatti, A. Barbiroli, **C. Mapelli**, P. Motta, S. Farris, D. Romano, R. Gavara, P. Hernandez Munoz, L. Higueras, D. Bussini, D. Cavicchioli, D. Bertoni

Conjugates of bacterial cellulose nanocrystals and sakacin-A in an anti-listeria food packaging material

Affiliation: University of Milan, Department of Food, Environmental and Nutritional Science, Milan, Italy.
INNOVHUB, Stazione sperimentale carta, cartoni e paste per carta, Milan, Italy.
Instituto de Agroquimica y Tecnologia de Alimentos, IATA-CSIC, Valencia, Spain.



In the present project, financed by Fondazione Cariplo, cheese whey permeate, the residual by-product obtained by extraction of whey proteins from cheese whey, is used as substrate for the growth of bacterial species that produce the anti-listerial bacteriocin sakacin-A from *Lactobacillus sakei* and bacterial cellulose from *Komagataeibacter xylinus*. BC is then turned into nanocrystals that are finally conjugated with sakacin-A to obtain an innovative antimicrobial device for food which could support *Listeria monocytogenes* growth.

Antimicrobial materials were prepared from sakacin A and/or conjugates applying incorporation, grafting or coating techniques, comparatively. An economic analysis of cheese whey market at Europe level was also carried out as well as the estimation of the production cost of the most performing material developed, even if at preliminary lab-scale level.

Antimicrobial activity of the obtained materials against *Listeria* was finally confirmed either by *in vitro* and *in vivo* trials on real food products, demonstrating that the developed device can contribute to increase shelf life and quality of perishable foods, thus decreasing the loss of food resources.

Advanced school on Food Protein - Bergamo, (Italy). May 2-4, 2018

Oral presentation

Authors: **C. Mapelli***, A. Musatti, A. Barbiroli, M. Rollini

Sakacin A: an anti-*Listeria* peptide with food applications

Affiliation: University of Milan, Department of Food, Environmental and Nutritional Science, Milan, Italy.

Sakacin A is a ribosomally synthesized peptide belonging to class IIa bacteriocin, produced by the GRAS bacterium *Lactobacillus sakei* Lb706, with a specific antimicrobial action against the foodborne pathogen *Listeria monocytogenes*. It consists of 41 amino acids with a molecular mass of 4.31 kDa; charged amino acids are located in the N-terminal hydrophilic region while C-terminal consists of pair of hydrophobic residues that leads to the formation of pores into bacterial membrane. Due to sakacin A strong antimicrobial activity, the interest in using this peptide for food preservation has recently been increasing. Here sakacin A was produced growing *L. sakei* for 9 h at 26 °C in stationary liquid cultures with a low cost medium formulation containing deproteinized cheese whey and arginine (0.5 g/L). At the end of incubation, cultures were centrifuged and sakacin A precipitated with ammonium sulphate; after lyophilization, antimicrobial activity of the protein extract (expressed in terms of AU/mL) was evaluated against *L. innocua* DSM 20649 by diffusion assay; a correlation curve of antimicrobial activity *vs.* peak area was also built through RP-HPLC.
This process allowed to obtain 5.02 g freeze-dried sakacin A extract/L supernatant, characterized by an antimicrobial activity of 16.7 AU/mg and a protein content around 5%.
The obtained sakacin A was used for the preparation of active papers, prepared by incorporating the peptide in cellulose nanofibers (0.2 g sakacin A extract, 0.2 g CNF). Films produced clear halos of *Listeria* growth inhibition in *in-vitro* tests, confirming its antimicrobial efficacy. Films were also put in contact to smoked salmon fillets inoculated with *L. innocua* and incubated at 8 °C for 7 days to test the *in vivo* antilisterial effectiveness. Results evidenced that antimicrobial films let to reach 1 Logarithmic reduction of *Listeria* contamination compared to control films prepared without sakacin A.

IFIB 2018 - International Forum on Industrial Biotechnology and Bioeconomy. *Turin, (Italy), Sept 27-28, 2018.*

FROM CHEESE WHEY PERMEATE TO AN INNOVATIVE ANTI-*Listeria* FOOD PACKAGING DEVICE: BACTERIAL CELLULOSE NANOCRYSTALS/SAKACIN-A CONJUGATES (NANOSAK)

Manuela Rollini[1], Alberto Barbiroli[1], Danilo Bertoni[2], Francesco Bonomi[1], Daniele Cavicchioli[2], Concetta Compagno[1], Stefano Debenedetti[1], Valerio De Vitis[1], Stefano Farris[1], Stefania Iametti[1], Chiara Mapelli[1], Francesco Molinari[1], Paolo Motta[1], Alida Musatti[1], Luisa Pellegrino[1], Luciano Piergiovanni[1], Roberto Pretolani[2], Diego Romano[1], Cesare Rovera[1], Immacolata Serra[1]

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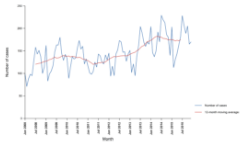
LISTERIOSIS

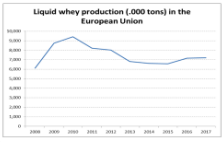
Invasive listeriosis is a foodborne infection caused by the Gram-positive bacterium *Listeria monocytogenes:* it occurs after two to three weeks incubation in which, from the intestinal tissue, bacteria arrives to nervous system or blood causing primarily septicaemia, meningitis and meningoencephalitis, with fatality rates of 20% to 30% among hospitalized patients.



Trend in reported confirmed human cases of listeriosis in the EU/EEA, by month, 2008–2015. Data source: ECDC (European Centre for Disease Prevention and Control).

CHEESE WHEY PERMEATE (CWP)

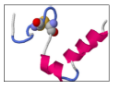
Cheese whey permeate (CWP) is a residual by-product obtained by extraction of whey proteins (WP) from cheese whey (CW).
While for both WP and CW official statistical data on production and market value are available - Eurostat for EU and Clal for Italy - no official figures can be found for CWP. Even if such lack of data witnesses its low value for standard economic activities, CWP production is pulled by the highly profitable activity of WP extraction.



In 2017 liquid CW available was 7.2 millions of tons for EU-28, with an increase of 18% over the last 10 years. In this frame, CWP may represent a cheap and highly available substrate for circular economy activities.

SAKACIN-A PRODUCTION AND OVEREXPRESSION

Fermentation parameters:
Inoculum (5%) *Lactobacillus sakei* DSM 6333
26 °C, 9 h incubation, no aeration, agitation speed 150 rpm, pH-stat 4.5. Batch culture.
Culture medium SAK (g/L):
Meat Extract 8, Yeast Extract 8, Arginine 0.5, Tween 0.5, Vitamin Mix 1, CWP to 1 L, pH 6.5.



A food-grade enriched sakacin-A extract was obtained by using a single step gravity RP-chromatography. *L. sakei* culture medium was loaded directly into the column.
Washing steps have been used to remove the not bound fraction and most of the non-active peptides. After evaporation of the solvent, the eluted sample was dissolved in water .

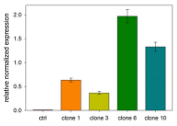
Fraction	Volume (ml)	Total protein concentration (mg/ml)	Total protein (mg)	Activity (AU/ml)	Specific activity (AU/mg)	Total activity (AU)	Yield (%)	Specific activity (Purity)
L. sakei growth medium	755	5.98	4515	100	167	126085	100	27.9
Not bound + first wash	850	3.18	2706	59.9	0	0	0	0
Second wash (low solvent)	97	14.73	1428	31.6	0	0	0	0
eSakA elution (medium solvent)	95	2.57	244	5.4	700	66500	52.7	272.3

Purification table

Overexpression was attempted in the GRAS yeasts *Kluyveromyces lactis* and *S. cerevisiae*. Although the gene expression was confirmed by RT-PCR (Figure X), no activity and any band of the correct size (6 kDa) after SDS-PAGE analysis (6 kDa) could be detected.

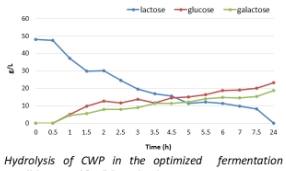
The sakacin-A gene was also cloned in frame with the gluthation-S-transferase gene into an *E. coli* expression plasmid. The crude extract of *E. coli* Origami 2(DE3) showed *anti-Listeria* activity: the formation of the disulfide bridge, which is enhanced in the Origami 2 (DE3) strain, is of striking importance for sakacin-A activity. The purification of GST-sakacin-A by affinity chromatography is currently ongoing.

Sakacin-A production costs: 2.16 €/L - 7.2 €/10^6 AU



Transcription of Sak gene in K. lactis *clones evaluated by RT-PCR*

BACTERIAL CELLULOSE (BC) PRODUCTION AND MODIFICATION INTO NANOCRYSTALS (BCNCs)

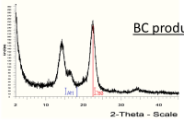


Hydrolysis of CWP in the optimized fermentation conditions used for BC production

After treatment at 90°C/NaOH 0.5 M for 1 h and washing, 6.78 g/L of BC were obtained

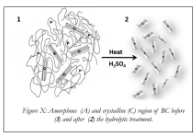
Fermentation parameters:
Inoculum (10%): *Komagataeibacter xylinus* DSMZ 2325 grown in CPW added with 0.5 U/mL of β-galactosidase pH 6 in agitated culture (150 rpm, 30°C, 48h)
Initial pH 6, static culture at 30°C for 7 days.

BC production costs: 0.18 €/L - 0.025 €/g

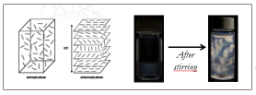


Cristallinity index of the purified BC is 82% (Segal method, XRD analysis)

BCNCs were obtained using a top-down approach by mean acid hydrolysis mediated by sulfuric acid, with the goal of removing the amorphous regions of BC and introduce a net negative charge by esterification.



Amorphous (A) and crystalline (C) regions of BC before (1) and after (2) the hydrolytic treatment

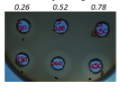


An indirect indication related to the presence of BCNCs inside the aqueous solution: the birefringence phenomena

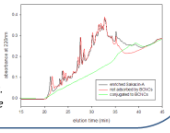
BCNCs/ SAKACIN-A CONJUGATES

Enriched sakacin-A extract was mixed with BCNCs and, after incubation, conjugates collected by centrifugation have a specific activity of 100 AU/mg BCNCs. In food applications, such as incorporation in active packaging, BCNCs may carry the bacteriocin, thus modulating its release.

35 AU eSakA mixed with the following amount of BCNCs (mg)



Among all peptides present in the enriched sample, Sakacin-A appears to preferentially adsorbs onto BCNCs. Conjugation procedure allows a further purification of the bacteriocin.

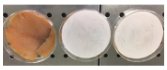


RP-HPLC chromatograms of peptides adsorbed, or not, by BCNCs. Peptides conjugated to BCNCs were released at alkaline pH to be analyzed in HPLC.

ACTIVE PACKAGING AND SHELF LIFE TRIALS

Antimicrobial activity of the obtained conjugates was finally confirmed either by *in vitro* and *in vivo* trials on real food products, demonstrating that the developed device can contribute to increase shelf life and quality of perishable foods, thus decreasing the loss of food resources.

Cellulose-based active films confirmed in vitro antimicrobial efficacy against L. innocua



Listeria population (Log cfu/g) in samples of smoked salmon intentionally inoculated and then stored for up to 28 d at 6 ± 1 °

Sample	Salmon + *L. innocua*		Salmon + *L. innocua* + film		Salmon + *L. innocua* + sak A film	
Days	log CFU/g	Decimal reduction	log CFU/g	Decimal reduction	log CFU/g	Decimal reduction
0	3.99 ± 0.08	0.00	3.99 ± 0.08	0.00	3.99 ± 0.08	0.00
7	5.38 ± 0.06	+1.39	5.32 ± 0.13	+1.34	3.22 ± 0.19	-0.76
14	6.84 ± 0.06	+2.85	7.29 ± 0.14	+3.30	4.08 ± 0.30	+0.09
21	8.74 ± 0.12	+4.75	8.20 ± 0.14	+4.21	5.90 ± 0.11	+1.31
28	8.90 ± 0.08	+4.91	8.70 ± 0.29	+4.71	6.08 ± 0.13	+2.09

UNIVERSITÀ DEGLI STUDI DI MILANO

Fondazione CARIPLO

2nd Innovation in Food Packaging, Shelf Life and Food Safety Conference. *Munich (Germany), Oct 3-6, 2017.*

UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE PER GLI ALIMENTI, LA NUTRIZIONE E L'AMBIENTE

Nanocellulose-Sakacin A: conjugates for food packaging purposes

Mapelli C.[1], *Musatti A.*[1], *Barbiroli A.*[1], *Heras Mozos R.*[1], *Saini S.*[2], *Bras J.*[2] *and Rollini M.*[1]

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Intro

Cases of listeriosis, a serious food-borne infection, increased sharply between 2002 and 2006, and remained constant afterward, with severe illness and a 20% mortality reported. Food exceeding the safety criterion for ***Listeria monocytogenes*** was highest in Ready-to-Eat fishery products, followed by meat and fresh cheese. Among antimicrobial agents that can be used to reduce food-related risks, bacteriocins are a class of small bacterial peptides with a great potential.

Aims of the project

Exploring the use of cheese whey and/or their derivatives as cheap substrates for growth of bacterial species that produce two families of molecules, a specific bacteriocin **sakacin A**, and **bacterial cellulose**, that will be turned into nanocellulose (BNC) using sustainable procedures.

The focus of this project is to optimize sakacin A production and its food-grade isolation. On a subsequent step this bacteriocin will be incorporated in BNC films and coatings.

The *in vitro* antimicrobial activity of these films will be evaluated, and the efficacy of the developed active packaging solutions will also be validated on real food, i.e. chicken meat and fresh dairy product. The results will contribute to increase shelf life and quality of perishable food.

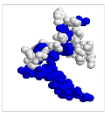
Sakacin A

- class IIa bacteriocins
- produced by ***Lactobacillus sakei***
- 41 aminoacids
- one disulfide bridge
- amphipathic
- molecular mass: 4308 Da
- **Listeria-targeted**

Mechanisms of actions

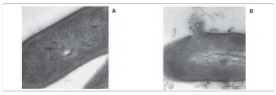
Sakacin A is able to kill *Listeria* cells by making their membranes permeable.

It possesses also a slow hydrolytic action towards *Listeria* cell walls, suggesting that this molecule can break specific bonds in the peptoglycan structure.
(Trinetta et. al., 2012)



Spacefill representation. Hydrophobic and hydrophilic residues are in blue and gray, respectively

Cartoons representation. Alpha-helixes are in magenta. Cysteines are as spacefill and CPK coloured



Trasmission electron micrographs of ultrathin section of *Listeria innocua* grown for 8 h without (control sample A) or in presence of sakacin A (1600 AU mL^{-1}, B)

Adapted from Trinetta et al., (2008) Influence of temperature and sakacin A concentration on survival of Listeria innocua *cultures. Annals of Microbiology, 58 (4) 633-639*

Sakacin A production and characterization

Production

Low cost medium formulation (g/L):
yeast extract 8,
meat extract 8,
tween 80 0.5,
arginine 0.5,
minerals and vitamins mix 1 mL
in deproteinized cheese whey (Soresina, Italy).

Lb. sakei

Fermentation parameters:
26 °C, 9 h incubation, no aeration, agitation speed 150 rpm, inoculum 5% (v/v).

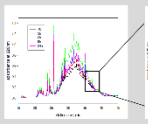
Culture filtrate purification

Precipitation with ammonium sulphate 400 g/L → Liophylization

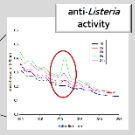
Yield: 2.5 g freeze-dried sakacin A extract /L supernatant
Activity: 10 AU/mg

Characterization

RP-HPLC chromatograms 220 nm



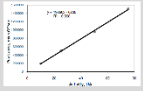
anti-Listeria activity



Sakacin A antimicrobial activity

Listeria innocua

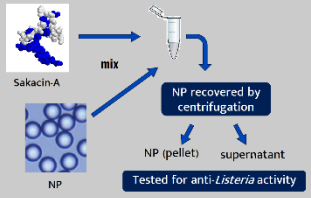
Correlation between sakacin A antimicrobial activity (x) and HPLC peak area (y)



Hydrophobic and negatively charged nanoparticles + Sakacin A

NP binding

200 nm polystyrene nanoparticles (NP) are being used as a model of non-covalent interaction



Activity test

Binding of sakacin A onto nanoparticles was evaluated using *Listeria innocua* DSMZ 20649 as indicator strain

	NP resuspended in		supernatant	NP alone (control)
	water	NaCl 0.5M	(water)	(water)
+ Tween 80	++	++	-	-
- Tween 80	+	+	-	-

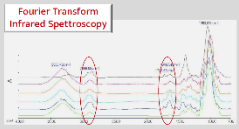
- Sakacin-A shows a good adhesiveness
- Non-covalent interaction does not prevent anti-*Listeria* activity

Incorporation on nanocellulose films

Film preparation

Active films were made by mixing
0.2 g of NanoFibrillated Cellulose (CNF Exilva, Borregaard, Sarpsborg, Norway)
0.2 g of enriched sakacin A
applying different strategies.

Fourier Transform Infrared Spettroscopy

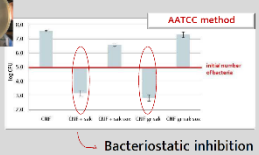


Antimicrobial activity

- qualitative standard method (AFNOR NF EN 1104)
- quantitative standard (AATCC Test Method 100-1998)

AFNOR NF EN 1104

AATCC method



Post soxhlet extraction → Loss of activity

Bacteriostatic inhibition

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Lee K.Y., Buldum G., Mantalaris A. and Bismarck A. (2013). **More than meets the eye in bacterial cellulose: biosynthesis, bioprocessing, and applications in advanced fiber composites.** *Macromol. Biosci.* 14: 10–32.
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Trinetta V., Rollini M. and Manzoni M. (2008). **Development of a low cost culture medium for sakacin A production by *L. sakei*.** *Process. Biochem.* 43: 1275–1281.

Perspectives

- *In VIVO* trials with sakacin-CNF conjugates on real food (i.e. carpaccio, cheese);
- optimization of the interaction between sakacin A and nanostructures;
- analysis of sakacin A kinetic release from a food package.

Supported by: fondazione cariplo

XXII Workshop on the Developments in the Italian PhD research on Food Science Technology and Biotechnology.
Bolzano (Italy), Sept 20-22, 2017.

Nanocellulose-Sakacin A: conjugates for food packaging purposes

Chiara Mapelli (chiara.mapelli1@unimi.it)
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Tutor: Dott. Manuela Rollini

1 Aims

Exploring the use of cheese whey and/or their derivatives as cheap substrates for growth of bacterial species that produce two families of molecules: **sakacin A** and **bacterial cellulose**, that will be turned into nanocellulose using sustainable procedures.

The focus of this project is to optimize sakacin A production and its food-grade isolation. On a subsequent step this bacteriocin will be incorporated in BNC films and coatings.

The *in vitro* antimicrobial activity of these films will be evaluated, and the efficacy of the developed active packaging solutions will also be validated on real food, i.e. chicken meat and fresh dairy product. The results will contribute to increase shelf life and quality of perishable food.

2 Materials & Methods

Lactobacillus sakei DSMZ 6333 was used as sakacin A producer.

Sakacin A was produced in 14 L fermenter (Omnitec Bio, Sedriano, Milano) (7 L working culture) employing a low cost medium formulation containing deproteinized cheese whey.

Sakacin A antimicrobial activity was evaluated using *Listeria innocua* DSMZ 20649 as indicator strain (AU/mL), grown on TSA (Tryptic Soy Agar, Scharlab, Barcelona, Spain).

Active films were made by mixing 0.2 g of NanoFibrillated Cellulose (CNF Exilva, Borregaard, Sarpsborg, Norway) with 0.2 g of enriched sakacin A applying different strategies.

Antimicrobial activity of the neat and modified CNF films was assessed using the following protocols:

- qualitative standard method (AFNOR NF EN 1104)
- quantitative standard (AATCC Test Method 100-1998)

3 Results & Discussion

Sakacin A production and characterization

Production

Low cost medium formulation (g/L):
yeast extract 8,
meat extract 8,
tween 80 0.5,
arginine 0.5,
minerals and vitamins mix 1 mL
in deproteinized **cheese whey** (Soresina, Italy).

Lb. sakei

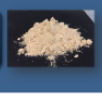
Fermentation parameters:
26 °C, 9 h incubation, no aeration, agitation speed 150 rpm, inoculum 5% (v/v).

Culture filtrate purification

Precipitation with ammonium sulphate 400 g/L

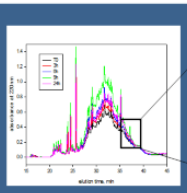
Liophylization

Yield: 2.5 g freeze-dried sakacin A extract /L supernatant
Activity: 10 AU/mg

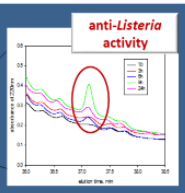


Characterization

RP-HPLC chromatograms 220 nm

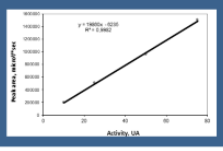


anti-*Listeria* activity



Sakacin A antimicrobial activity

Correlation between sakacin A antimicrobial activity (x) and HPLC peak area (y)



Incorporation on nanocellulose films and coatings

CNF + sakacin A

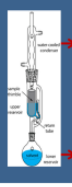
CNF

CNF + sak

Thermally grafted at 70 °C x 12 h in vacuum desiccator

CNF grafted sak

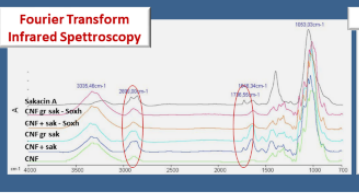
Soxhlet extraction (S)



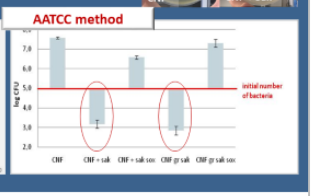
CNF + sak - S

CNF grafted sak - S

Fourier Transform Infrared Spettroscopy



AATCC method



AFNOR NF EN 1104

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Perspectives

- *In VIVO* trials with sakacin-CNF conjugates on real food (i.e. carpaccio, cheese);
- optimization of the interaction between sakacin A and nanostructures;
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XXII WORKSHOP
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BOLZANO, SEPTEMBER 20th-22nd, 2017

42nd FEBS Congress. *Jerusalem (Israel), Sept 10-14, 2017*.

ANTI-*LISTERIA* BACTERIOCIN SAKACIN-A: BIOTECHNOLOGICAL AND BIOCHEMICAL APPROACHES FOR HIGH-YIELD FOOD-GRADE PRODUCTION

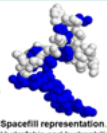
Alberto Barbiroli, Chiara Mapelli, Immacolata Serra, Stefano De Benedetti, Alida Musatti, Concetta Compagno, Manuela Rollini
DeFENS, University of Milan, Milan, Italy - alberto.barbiroli@unimi.it

Rational

Cases of listeriosis, a serious food-borne infection, increased sharply between 2002 and 2006, and remained constant afterward, with severe illness and a 20% mortality reported. Food exceeding the safety criterion for *Listeria monocytogenes* was highest in Ready-to-Eat fishery products, followed by meat and fresh cheese. Among antimicrobial agents that can be used to reduce food-related risks, bacteriocins are a class of small bacterial peptides with a great potential. Sakacin-A is a class IIa bacteriocin, produced by the GRAS bacterium *Lactobacillus sakei*, showing a specific anti-*Listeria* activity. Its application as food preservative in active packaging - through appropriate devices - may offer many advantages (Barbiroli et al., 2017), but requires low production cost and high conversion yield. Two biochemical approaches were followed: ❶ the investigation of *L. sakei* specific nutritional requirements as well as ❷ its overexpression and isolation in a food-grade form. Moreover, practical applications of sakacin-A need to control the diffusion rate from the packaging to the food. ❸ Hydrophobic (polystyrene) and negatively charged (cellulose) nanoparticles are being tested, taking advantage of the amphipathic nature of sakacin-A.

Sakacin-A

- 41aminoacids
- **amphipathic**
- ***Listeria*-targeted**
- one disulfide bridge



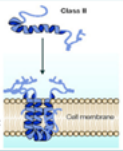
Spacefill representation. Hydrofobic and hydrophilic residues are in blue and gray, respectively

Cartoons representation. Alpha-helixes are in magenta. Cysteines are as spacefill and CPK coloured

Mechanisms of actions

Sakacin-A is able to impair **membranes permeability**, and possesses a **slow hydrolytic action** toward *Listeria* cell walls (Trinetta et al., 2012)

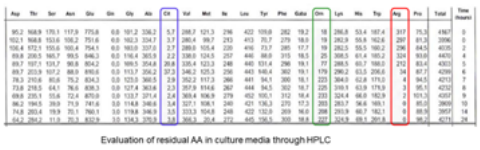
Adapted from Cotter P.D., Hill C., Ross R.P. (2005) Bacteriocins: developing innate immunity for food. Nat Rev Micro. 3.777-788



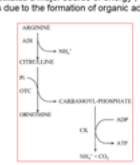
RESULTS

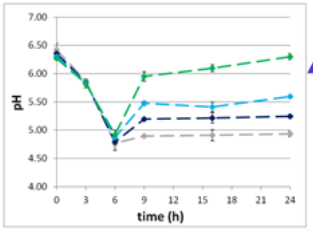
❶ HPLC analysis of free aminoacids present into culture supernatant showed **arginine** to be the only component **totally depleted** during *L. sakei* growth and sakacin-A production. **Arginine supplementation**, through the arginine deaminase pathway, **helps maintaining culture pH around 6**, thus allowing a significant **increase of *L. sakei* proliferation**.

Arginine deiminase (ADI) pathway in *Lactobacillus sakei*. The ADI pathway constitutes a major source of energy , while reducing the pH drops due to the formation of organic acids.



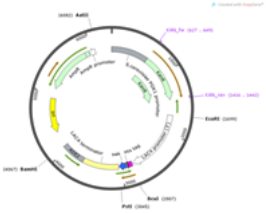
Evaluation of residual AA in culture media through HPLC



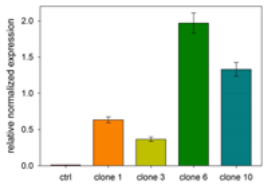


Time course of culture media pH after supplementation of different amount of arginine (from 0 to 2 mg/mL)

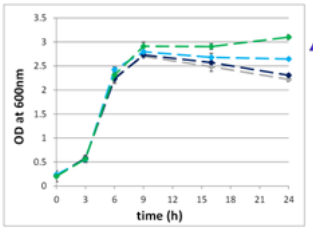
❷ **Overexpression of sakacin-A** under food-grade requirements was attempted in the **GRAS yeast *Kluyveromyces lactis***. Competent wild-type cells of *K. lactis* were transformed by employing an integrative plasmid carrying the his-tagged sakacin-A gene optimized for codon-usage under the control of the strong inducible promoter LAC4. The highest producer clone was selected by RT-PCR and isolation of sakacin-A from the culture media of this clone is currently underway.



Map of pYC030-Sak plasmid

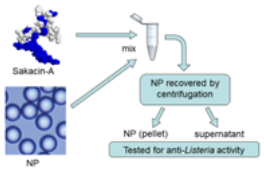


Transcription of *Sak* gene in *K. lactis* clones evaluated by RT-PCR



L. sakei proliferation after supplementation of different amount of arginine (from 0 to 2 mg/mL)

❸ Sakacin-A should be vehiculated into the active packaging, but must be released to allow the interaction of the bacteriocin with the *Listeria* cell membrane. Has absorption onto nanostructure an inhibitory effect? **200 nm polystyrene nanoparticles** (NP) are being used as a model of non-covalent interaction.



	NP resuspended in		supernatant	NP alone (control)
	water	NaCl 0.5M	(water)	(water)
+ Tween 80	++	++	-	-
- Tween 80	+	+	-	-

Binding of sakacin-A onto nanoparticles evaluated through anti-*Listeria* activity test

- Sakacin-A shows a **good adhesiveness** to hydrophobic nanoparticles;
- non-covalent interaction **does not prevent anti-*Listeria* activity**;
- surfactant (Tween 80) enhances the activity

CONCLUSIONS

Achieving an increase of a microbial metabolite production is generally a stressful goal, due to the complex roadmap of metabolic pathways and regulation mechanisms.

- Satisfaction of precise nutritional requirement (by arginine supplementation) results in a significant increase of *L. sakei* cells proliferation and, therefore, sakacin-A production.
- Overexpression in a food-grade system appears to be a promising strategy, virtually bypassing physiological feed-back mechanism governing sakacin-A accumulation.
- Non covalent vehiculation on nanostructure does not hinder anti-*Listeria* activity, thus paving the way to the application of this natural antimicrobial in food packaging.

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- Barbiroli, A., Musatti, A., Capretti, G., Iametti, S. and Rollini, M. (2017) Sakacin-A antimicrobial packaging for decreasing Listeria contamination in thin-cut meat: preliminary assessment. J. Sci. Food Agric., 97: 1042–1047
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Acknowledgement

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FACOLTÀ DI SCIENZE AGRARIE E ALIMENTARI

XXI WORKSHOP
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TECHNOLOGY AND BIOTECHNOLOGY
Portici (NA), September 14th-16th, 2016

Nanocellulose-sakacin A: conjugates for food packaging purposes

Chiara Mapelli (chiara.mapelli1@unimi.it)
Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, Italy Tutor: Dott. Manuela Rollini

AIMS Exploring the use of cheese whey and/or their derivatives as cheap substrates for growth of bacterial species that produce two families of molecules, a specific bacteriocin sakacin A, and bacterial cellulose, that will be turned into nanocellulose using sustainable procedures. The focus of this project is to optimize sakacin A production and its food-grade isolation. On a subsequent step this bacteriocin will be incorporated in BNC films and coatings. The *in vitro* antimicrobial activity of these films will be evaluated, and the efficacy of the developed active packaging solutions will also be validated on real food, i.e. chicken meat and fresh dairy product. The results will contribute to increase shelf life and quality of perishable food.

Liquid whey

ULTRAFILTRATION → **PERMEATE**

valuable raw material due to the high lactose, minerals and vitamins content

Sakacin A

Lactobacillus sakei

Class IIa **BACTERIOCIN**

1 Ala-Arg-Ser-Tyr-Gly-Asn-Gly-Val-Tyr-Cys-10
11 Asn-Asn-Lys-Lys-Cys-Trp-Val-Asn-Arg-Gly-20
21 Glu-Ala-Thr-Gln-Ser-Ile-Ile-Gly-Gly-Met-30
31 Ile-Ser-Gly-Trp-Ala-Ser-Gly-Leu-Ala-Gly-40
41 Met-***

Listeria-active peptide of 41 aminoacid residues with one disulfide bridge and a mass of 4308 daltons

Able to kill *Listeria* cells by making their membranes permeable, possesses also a slow hydrolytic action towards *Listeria* cell walls

Bacterial cellulose

Gluconoacetobacter xylinus

High crystallinity, tensile strength and water holding capacity

Turned into **bacterial nanocellulose** by chemical modification

Set-up of an innovative material that finds applications across several industrial sectors, including the use in the **food packaging sector**

BIOACTIVE PACKAGING ← **Nanocell-sakA**

Use of cellulose nanocomposites to extend shelf life and enhance the quality of perishable foods by serving as a carrier of active substances, such as antimicrobials

Milestones

Sakacin A production and characteriz.

- Formulation of the culture medium
- *Lb. sakei* cell immobilization in food grade matrices
- Possibility of obtaining sakacin A by employing engineered yeast strains
- Set up of a purification procedure for sakacin A suitable for food application

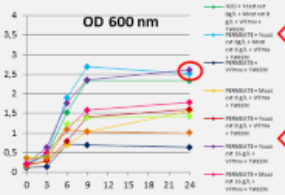
Nanocell. for packaging and active packaging

- Assessing the effects of incorporating sakacin A on the functional properties of the nanocellulose films and coatings
- Testing the kinetics of release/mode of action of sakacin A in model systems

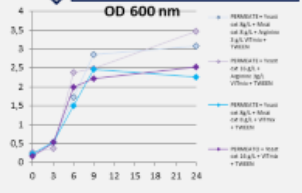
Shelf life trials on real food

- Shelf life tests on chicken meat
- Shelf life tests on fresh cheese

Influence of

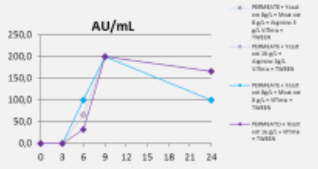


Investigation of the two better conditions with or without ARGININE 3 g/L



Fermentation trials with *Lb. sakei* in media containing cheese whey permeate

Detection of the amount of Sakacin A in terms of arbitrary unit (AU/mL)



EXPECTED RESULTS

The packaging materials developed in the project will be used for the preparation of active packaging which will rely on controlled release of sakacin A as an antimicrobial directed to the surface of spoilage-sensitive foods.

6.2. Collateral works

Musatti A., **Mapelli C.**, Rollini M., Foschino R., Picozzi C. (2018). Can *Zymomonas mobilis* substitute *Saccharomyces cerevisiae* in cereal dough leavening? *Foods,* 7, 61: 1-8. (DOI:10.3390/foods7040061).

foods

MDPI

Article

Can *Zymomonas mobilis* Substitute *Saccharomyces cerevisiae* in Cereal Dough Leavening?

Alida Musatti, Chiara Mapelli, Manuela Rollini, Roberto Foschino and Claudia Picozzi *

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check for updates

Abstract: Baker's yeast intolerance is rising among Western populations, where *Saccharomyces cerevisiae* is spread in fermented food and food components. *Zymomonas mobilis* is a bacterium commonly used in tropical areas to produce alcoholic beverages, and it has only rarely been considered for dough leavening probably because it only ferments glucose, fructose and sucrose, which are scarcely present in flour. However, through alcoholic fermentation, similarly to *S. cerevisiae*, it provides an equimolar mixture of ethanol and CO_2 that can rise a dough. Here, we propose *Z. mobilis* as a new leavening agent, as an alternative to *S. cerevisiae*, overcoming its technological limit with different strategies: (1) adding glucose to the dough formulation; and (2) exploiting the maltose hydrolytic activity of *Lactobacillus sanfranciscensis* associated with *Z. mobilis*. CO_2 production, dough volume increase, pH value, microbial counts, sugars consumption and ethanol production were monitored. Results suggest that glucose addition to the dough lets *Z. mobilis* efficiently leaven a dough, while glucose released by *L. sanfranciscensis* is not so well fermented by *Z. mobilis*, probably due to the strong acidification. Nevertheless, the use of *Z. mobilis* as a leavening agent could contribute to increasing the variety of baked goods alternative to those leavened by *S. cerevisiae*.

Musatti A., Ficara E., **Mapelli C**., Sambusiti C., Rollini M. (2017). Use of solid digestate for lignocellulolytic enzymes production through submerged fungal fermentation. *Journal of Environmental Management*, 199: 1-6 (DOI: 10.1016/j.jenvman.2017.05.022).

Journal of Environmental Management 199 (2017) 1–6

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Research article

Use of solid digestate for lignocellulolytic enzymes production through submerged fungal fermentation

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Fungi
Lignocellulolytic enzymes
Wheat straw
Submerged fermentation

ABSTRACT

Studies were performed on the use of the solid fraction of digestate (D) for the production of lignocellulolytic enzymes (endo- and exo-glucanase, xylanase, β-glucosidase and laccase) by fungi, in comparison with wheat straw (benchmark) (W). To date, this is the first report on the use of such an inexpensive substrate in a liquid environment. Submerged instead of solid state fermentation was applied to overcome pH inhibition and increase surface accessibility. A total of 21 fungal strains were tested: the most performing ones were *Irpex lacteus* DSM1183 for both β-glucosidase (52 IU/g with D, + 400% compared to W) and endo-glucanase (236 IU/g with D, + 470% compared to W), *Schizophyllum commune* CBS30132 for xylanase (715 IU/g with W, + 145% compared to D) and *Pleurotus ostreatus* ATCC96997 for laccase (124 IU/g with D, +230% compared to D).

Cultures from *S. commune* and *P. ostreatus* were analyzed at the beginning and at the end of the growth test to determine soluble COD, total (TS) and volatile (VS) solids. COD was always lower at the end of the test suggesting a faster uptake than hydrolysis. *P. ostreatus* evidenced a higher VS reduction (−11% rather than −32%), suggesting a more effective growth of this strain on D.

Results may open up new avenues for the utilization of solid digestate, an inexpensive agricultural by-product, for the production of value-added products as well as to increase biodegradation of lignocellulosic materials.

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Unconventional bacterial association for dough leavening

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ABSTRACT

The purpose of the research was to obtain innovative yeast-free doughs leavened by *Zymomonas mobilis* and *Lactobacillus sanfranciscensis*. *Z. mobilis*, as well as *Saccharomyces cerevisiae*, produces an equimolar mixture of ethanol and CO_2 through glucose, fructose or sucrose fermentation. In the present work, the inability of *Z. mobilis* to metabolize maltose has been circumvented by the addition of *L. sanfranciscensis* in the formulation. Indeed, *L. sanfranciscensis*, a heterofermentative lactic acid bacterium (LAB) typical of sourdough environment, hydrolyzes maltose releasing glucose which can be used by *Z. mobilis* for its metabolism.

Biomass samples of *Z. mobilis* subs. *mobilis* DSM 424 and *L. sanfranciscensis* DSM 20663 were grown separately in liquid media and then associated in a model dough. Leavening trials set up by using three different microbial combinations (*Lactobacillus*:*Zymomonas* 80 + 80 mg, 15 + 145 mg and 145 + 15 mg biomass, i.e. 1:1, 1:10 and 10:1 respectively on cell dry weight basis) evidenced CO_2 production levels (mL) higher than the mathematical sum of CO_2 produced by the single bacteria. In particular, when the biomass combination of *L. sanfranciscensis* and *Z. mobilis* was 1:1 (80 + 80 mg cdw) and 10:1 (145 + 15 mg cdw) a CO_2 production of 46.3–41.4 mL versus 26.7–28.5 mL was achieved. The calculated productivity gain showed positive performances of the microbial combination up to 180–240 min leavening. The subsequent efficiency loss may be due several factors, above all glucose shortage for *Z. mobilis*, as well as decrease of dough pH that can negatively affect both *Lactobacillus* and *Zymomonas* metabolism. As in traditional sourdoughs, *L. sanfranciscensis* was responsible for the souring activity with positive effects on both dough tasting and reduction of spoilage microbiota; *Z. mobilis* was instead responsible for most of the CO_2 production.

A bakery product leavened with the unconventional association *Z. mobilis* - *L. sanfranciscensis* will be addressed to people having adverse responses to the ingestion of bakery goods, thus providing innovation in the area of yeast-free leavened food.

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Submitted for Poster presentation

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***Zymomonas mobilis*: an alternative dough leavener for the production of yeast-free baked doughs**

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Background: adverse responses to *Saccharomyces cerevisiae* occur in particular in people with Inflammatory Bowel Disease and with Crohn's disease, in which anti-*S. cerevisiae* antibodies directed against the phosphopeptidomannan of the yeast cell wall have been identified. *Zymomonas mobilis*, a Gram-negative bacterium GRAS classified by the FDA, can represent an interesting candidate for producing yeast-free fermented goods.
Objectives: as *Z. mobilis* does not utilize maltose present in flour, different strategies were applied to improve its leavening ability in dough: glucose or sucrose addition, or exploitation of the maltose hydrolytic activity of *Lactobacillus sanfranciscensis*.
Methods: five *Z. mobilis* and one *L. sanfranciscensis* strains were used in dough leavening trials. Experiments were set up with different dough consistency and inoculum (10^7-10^9 UFC/g), leavening temperature (25 - 30 ± 0.5 °C) and time (6 to 24 h), with or without sugars (3-5%) and NaCl (1%). Dough leavening properties (e.g., CO_2 production, dough volume increase, lag leavening time and rates) as well as time course of microbial population, sugars consumption and ethanol production were evaluated.
Results: the addition of glucose or sucrose as well as the highest leavening temperature increased the gaseous production and retention and consequently the dough development. *L. sanfranciscensis* contribution was fickle: the productivity gain showed positive performance only at high inoculum and short leavening time (3-4 h). NaCl worsened *Z. mobilis* fermentation performance but this behavior seems to be strain-related. Results highlight that developing a dough leavened by *Z. mobilis* and thus suitable for yeast-sensitive people is possible.

32 nd EFFoST: International Conference– Nantes (France), Nov 6-8, 2018

Oral presentation

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Microbiological and technological aspects of bread doughs leavened by *Zymomonas mobilis*

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Zymomonas mobilis is a Gram negative bacterium with a fermentation metabolism similar to that of *Saccharomyces cerevisiae*. It has potential food applications in the development of leavened baked goods for people with adverse responses to the ingestion of baker's yeast.

The aim of this research was to assess the leavening performances (up to 24 h at 26°C) of *Z. mobilis* in bread doughs. Since it cannot ferment flour maltose, the association with *Lactobacillus sanfranciscensis*, possessing a constitutive maltose hydrolytic activity, or the direct addition of glucose to the dough (5 g/100 g flour) were investigated. The leavening performances of *S. cerevisiae* were chosen as benchmark. Preliminary baking trials were also conducted.

Limited bacterium grown (from 7.5 to 8 log cfu/g), low dough development and CO_2 production characterized samples leavened by *Z. mobilis* without glucose addition. Whit glucose*, Zymomonas* reached 9 log cfu/g and more than three times of ethanol was found. The addition of *L. sanfranciscensis*, slightly improved CO_2 production but reduced the relative gas retention ability of the dough. The rheofermentographic analyses pointed out that glucose addition in samples leavened by *Z. mobilis* alone or coupled with *L. sanfranciscensis* caused a double raising of the doughs, contrarily to what happened with *S. cerevisiae*. As regards the preliminary baking trials, doughs leavened by *Z. mobilis* with glucose and by *S. cerevisiae* were compared: promising results were obtained in terms of bread development and alveolar crumb structure.

In conclusion, results demonstrated that *Z. mobilis* in presence of glucose could efficiently leaven a bread dough, whereas the association with *L. sanfranciscensis* did not favour *Zymomonas* growth in a relevant manner: the pH dropped to values lower than 4, thus limiting *Zymomonas* leavening performances. The well-developed-breads obtained with *Z. mobilis* proved that this bacterium can be used to produce yeast-free leavened baked foods.

FoodInnova2017: International Conference on Food Innovation – Cesena (Italy), Jan 31-Feb 3, 2017

Oral presentation

Authors: C. Mapelli, A. Musatti; C. Picozzi, M. Rollini

Innovative use of *Zymomonas mobilis* in bread-making

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Keywords: *Zymomonas mobilis*, yeast intolerance, *Lactobacillus sanfranciscensis*, dough-model system, bacterial association, yeast free-products.

Introduction: The research is focused on the possibility of creating a new area of leavened bakery products avoiding the addition of baker's yeast (*Saccharomyces cerevisiae*). *Zymomonas mobilis* is an obligate fermentative GRAS bacterium. Its sugar catabolism proceeds through the ED pathway, with the latter step to ethanol + CO_2 proceeding similarly to *S. cerevisiae*. As *Zymomonas* does not convert maltose and starch present in flour, the effect of glucose addition to doughs, as well as the possibility of an unconventional microbial association with *Lactobacillus sanfranciscensis*, lactic acid bacterium (LAB) usually present in sourdoughs, was investigated. This LAB hydrolyzes maltose releasing one mole of glucose in the medium, potentially used by *Z. mobilis* to carry on the alcoholic fermentation.

Materials and Methods: Growth of *Z. mobilis* subs. *mobilis* DSM 424 and *L. sanfranciscensis* DSM 20663 were achieved in DSM liquid medium and MRS maltose media. Biomass were differently combined (1:1, 1:10 and 1:100 *Lb:Zym*) in a model dough containing 333 g flour type 0 Manitoba and 167 mL H_2O. Leavening trials were also performed by adding glucose (1 and 5 % w/w on flour) to dough. In the sourdough back-sloping procedure, 50 g of a 16 h-old leavened dough was added to a new freshly formulated dough for a total of 500 g. CO_2 production was monitored as reported in Musatti et al. (2015). Time course of ethanol production and sugars consumption were determined by RI- and UV-HPLC (column SH1821 Shodex, 210 nm).

Results: CO_2 production in doughs leavened by *Zymomonas* alone was limited (max 20 mL/g). Glucose addition allowed to increase dough leavening up to 250 mL/g after 6 h incubation at 26°C. As regards the microbial association, the best leavening performances were found employing the combination 1:100 *Lb:Zym*, in which the pH decrease due to LAB metabolism limited only marginally *Zymomonas* alcoholic fermentation.

Trials performed by applying the sourdough back-sloping procedure highlighted that *Zymomonas* was found to suffer the acidic environment created by the LAB, which has more sugar availability (i.e. maltose) in doughs.

Conclusions:

The research illustrates an innovative strategy to set-up yeast-free baked goods leavened by Z. mobilis alone or in combination with *L. sanfranciscensis*, in which LAB metabolism favors the set-up of a mutualistic association. Nevertheless, glucose released by the LAB is probably not enough to support *Z. mobilis* fermentative metabolism.

6th Sourdough Symposium – Nantes (France), Sept 30-Oct 2, 2015.

Oral presentation

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Unconventional bacterial association for sourdough: *Zymomonas mobilis* and *Lactobacillus sanfranciscensis*

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The development of a yeast-free bakery product leavened only with bacteria is a great challenge. *Zymomonas mobilis* ferments only glucose, fructose and sucrose, providing an equimolar mixture of ethanol and CO_2 and theoretically, the gas evolved can be used to leaven a dough (1). In this study, a new bacterial association between *Z. mobilis* and *Lactobacillus sanfranciscensis* has overcome the inability of *Zymomonas* in hydrolysing maltose. Indeed, *L. sanfranciscensis* hydrolysing maltose (2), releases glucose in the medium that might be used by *Z. mobilis*. During this study, different strains were screened, and their biomasses were first grown up separately and then combined with different ratio (i.e. 1:1; 10:1; 1:10 on dry cell weight) in leavening trials performed in a model system (flour, water and cell biomass, 29°C and 6 h). CO_2 production ability was determined volumetrically, while concentration of ethanol and other organic acids were verified through HPLC. Time course of CO_2 production was fitted (DMFit 3.0) to estimate lag leavening phase duration, gas production rate and total amount of gas evolved.
CO_2 produced by *Z. mobilis* alone leavens the dough only scarcely due to the limited amount of glucose in flour. The amount of CO_2 reached by the bacterial association during the first 2-3 hours is at least 50% higher than the sum of the CO_2 separately produced by the two bacteria.
Therefore, this unconventional microbial association could be addressed to people having adverse responses to the ingestion of baker's yeast, providing innovation in the area of yeast-free leavened baked goods.

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