Utilisation of whey fermentation products for antimicrobial modification of biodegradable polymers

Ing. Pavlína Holčapková, Ph.D.

Doctoral Thesis Summary

Tomas Bata University in Zlín

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Faculty of Technology

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Využití fermentačních produktů syrovátky pro antimikrobiální modifikace biorozložitelných polymerů

Utilisation of whey fermentation products for antimicrobial modification of biodegradable polymers

Author: **Ing. Pavlína Holčapková, Ph.D.**

Degree programme: P2808 Chemistry and Materials Technology

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Supervisor: prof. Ing. Vladimír Sedlařík, Ph.D.

External examiners: prof. Ing. Mohamed Bakar, Ph.D.
RNDr. Jiří Zedník, Ph.D.
doc. Ing. Michal Sedlačík, Ph.D.

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ABSTRACT

The doctoral thesis focuses on utilising whey fermentation products for antimicrobial modification of biodegradable polymers. The theoretical part describes the latest advances in biodegradable polymers, as well as potential application of whey as a fermentation medium for producing substances with antibacterial properties. Description is given on antimicrobial alteration of polymers and on modifying polymer systems with bacteriocins. The experimental part of work is dedicated to preparation of novel, biodegradable, PLA-based polymer systems – modified with bacteriocin nisin – in the form of blend films or microparticles. The author investigates the general characteristics of structural, thermal and mechanical properties, as well as the effect of the given polymer matrix on the release profile of nisin, through the employ of chromatographic separation techniques. As for the antibacterial properties of the materials, these are evaluated by three different testing methods. Finally, the activity exhibited by the systems containing nisin is gauged for stability over the long-term and stability under conditions of high temperature.

ABSTRAKT

Předložená disertační práce se zaměřuje na využití fermentačních produktů syrovátky pro antimikrobiální modifikace biorozložitelných polymerů. Teoretická část práce přináší přehled současného stavu poznání v oblasti biorozložitelných polymerů a potenciálu využití syrovátky jako fermentačního média pro získávání látek s antibakteriálními vlastnostmi. Dále jsou popsány antimikrobiální modifikace polymerů a především pak polymerní systémy modifikované bakteriocinem nisinem. Experimentální část práce je věnována přípravě nových biorozložitelných polymerních systémů na bázi polylaktidu modifikovaných nisinem ve formě filmů a mikročástic. Mimo obecné charakterizace strukturních, tepelných a mechanických vlastností byl účinnek dané polymerní matrice na profil uvolňování nisinu zkoumán pomocí chromatografických separačních technik. Hodnocení antibakteriálních vlastností bylo prováděno třemi rozdílnými testovacími metodami. V neposlední řadě byla testována také dlouhodobá a vysoko-tepelná stabilita aktivity připravených systémů s inkorporovaným nisinem.

INTRODUCTION

Nowadays, plastics are an indispensable part of modern life. They play an essential role in providing, protecting and delivering high-quality products in every market segment. However, the growing environmental awareness of consumers, as well as the knowledge about the finite nature of the planet's fossil resources, are the reasons for increased demand for bioplastic materials [1].

According to European Bioplastics, a plastic material is defined as a bioplastic if it is either bio-based, biodegradable or features both properties [1]. According to the European Standard EN 16575:2014, the term 'bio-based' means that the material or product is derived from biomass – material of biological origin (whole or parts of plants, trees, algae, marine organisms, micro-organisms, animals, etc.) [2]. On the other hand, biodegradable polymers are defined as 'degradable plastics in which the degradation results from the action of naturally occurring organisms, bacteria, fungi and algae', according to ASTM D6400-04 standard [3]. With this in mind, it is obvious that the property of biodegradation does not depend on the resource basis of a material but is rather linked to its chemical structure.

The development and use of biodegradable polymers have been significantly growing over recent years, especially in the environmental and medical fields [4–7]. The former mainly relates to disposable packaging, while the latter pertains to medical devices and drug-delivery systems. It is clear that capacity for degradation by living organisms cannot be the sole feature of these materials. Indeed, other 'added value' functionality is needed in order to increase their competitiveness on the market. An example of such added value in developing materials is enhancement with antibacterial activity, a desirable property to the packaging, textile, automotive and medical sectors, among others [8–10].

Although many different chemicals of organic and inorganic origin have been successfully used for antimicrobial modifications of polymers [11–13], there are also limitations to their use employing the safety aspects in connection with residual toxicity as well as potentially carcinogenic and teratogenic effects [14]. Therefore, in alignment with new trends, stress is increasingly being put on the need to apply naturally derived biocides [8]. As a consequence, the use of whey fermentation products as natural bioactive substances seems to be advantageous.

The presented thesis is devoted to the preparation of biodegradable polymer systems modified with products of processed whey, which is known as a waste from the dairy industry. The first part summarises the current state of advancement in this area. It deals with the following: introduction of biodegradable polymers, whey and its potential use, antimicrobial modifications to specimens and bioactive polymer systems modified by nisin. That builds a foundation for the experimental part, where attention is paid to the utilisation of nisin, a bacteriocin, for modification of biodegradable polymers in order to achieve the bioactive material with antibacterial properties.

1. THEORETICAL BACKGROUND

1.1 Biodegradable polymers

In polymer chemistry, biodegradation can be simply described as a process involving the cleavage of hydrolytically or enzymatically sensitive bonds in the polymer resulting in polymer erosion (Fig. 1) [6].

Exact terms and definitions of biodegradability are then specified in various standards (e.g. ASTM Standard test method D5488-94d or European Standards EN 13432:2000 and EN 14995:2006) [15,16].

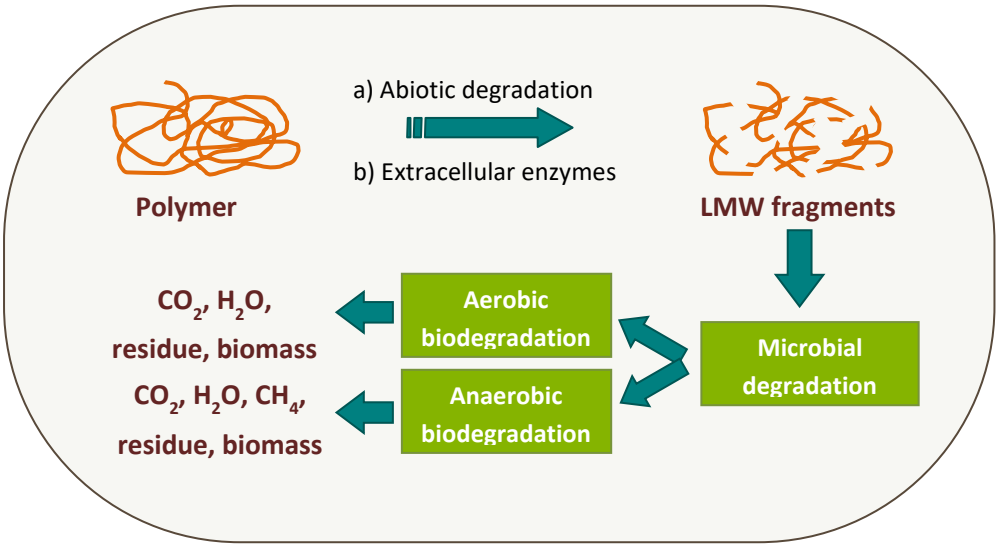

Figure 1. Illustration of the chemistry pertaining to biodegradation [5].

The biodegradable polymers can be classified based on various criteria, for example, according to their origin, chemical composition, synthesis method, processing method, application, economic importance, etc. [6,17]. In the present thesis, biodegradable polymers are divided, according to their origin, into two groups and four subgroups (Fig. 2). The main groups are a) natural polymers, which are directly obtained from natural resources, and b) synthetic polymers, which can be produced from bio-derived monomers or synthetic monomers from petrochemical products. Further subclassification is performed on the basis of the method by which are they obtained [17]:

- polymers directly extracted from biomass such as agro-polymers from agro-resources (e.g. starch, cellulose),
- polymers obtained by microbial production (e.g. polyhydroxy-alkanoates, bacterial cellulose),
- polymers conventionally and chemically synthesised from bio-derived monomers (e.g. polylactic acid),
- polymers synthesised from fossil resources.

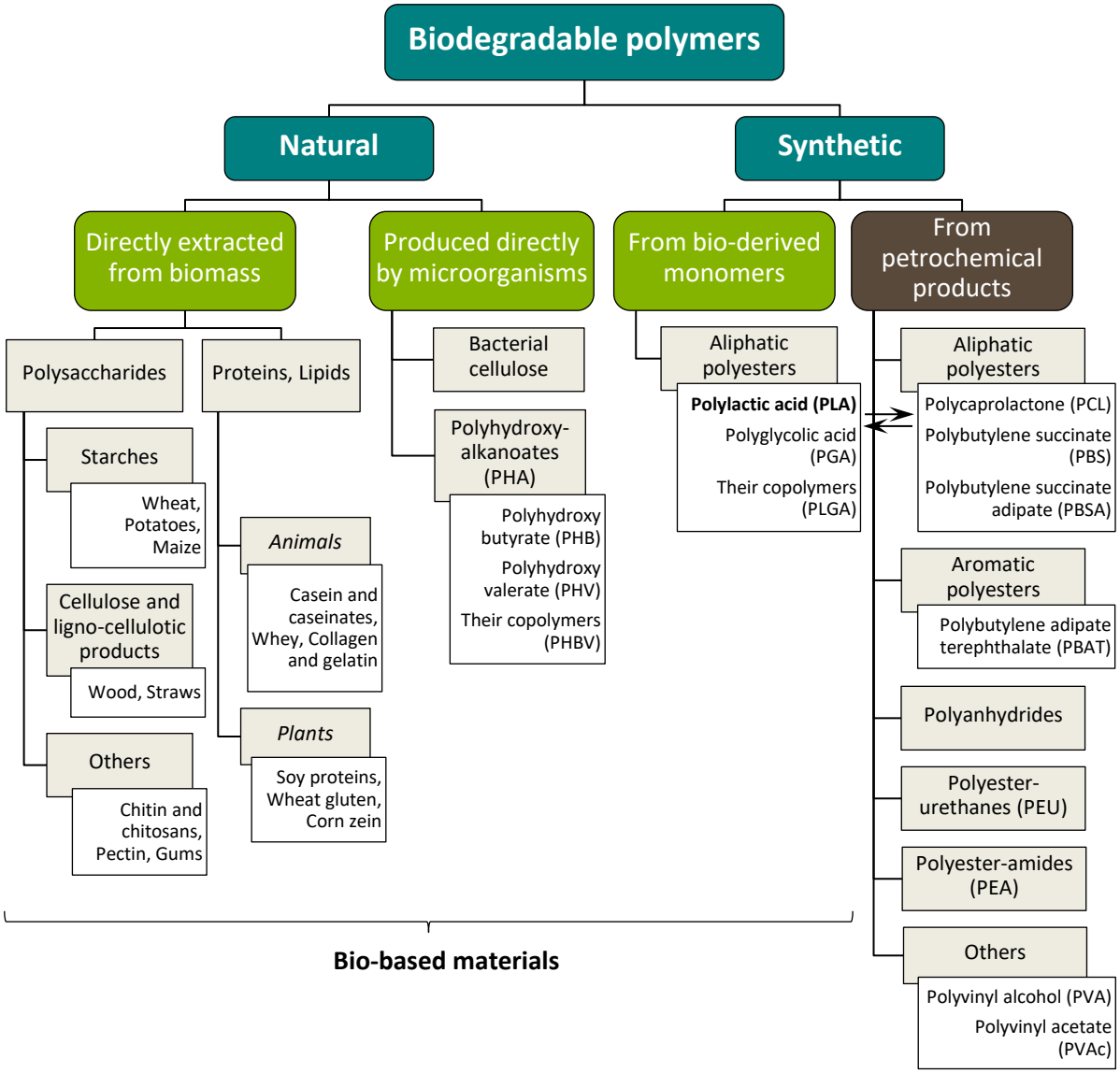

Figure 2. Chart with classifications of biodegradable polymers.

The application possibilities of biodegradable polymers are very high. As shown in Figure 3, they can be found in many market segments, from packaging, agriculture and horticulture, electronics, consumer goods, automotive to textile industry and a number of other segments. It is obvious that the largest field of applications of biodegradable polymers remains packaging (often associated with food applications), with almost 60% of the total biodegradable plastics market in 2017 [1].

Moreover, biodegradable polymers offer great potential in biomedical applications such as drug delivery, tissue engineering, gene therapy, regenerative medicine, temporary implantable devices, etc. [4,5,18].

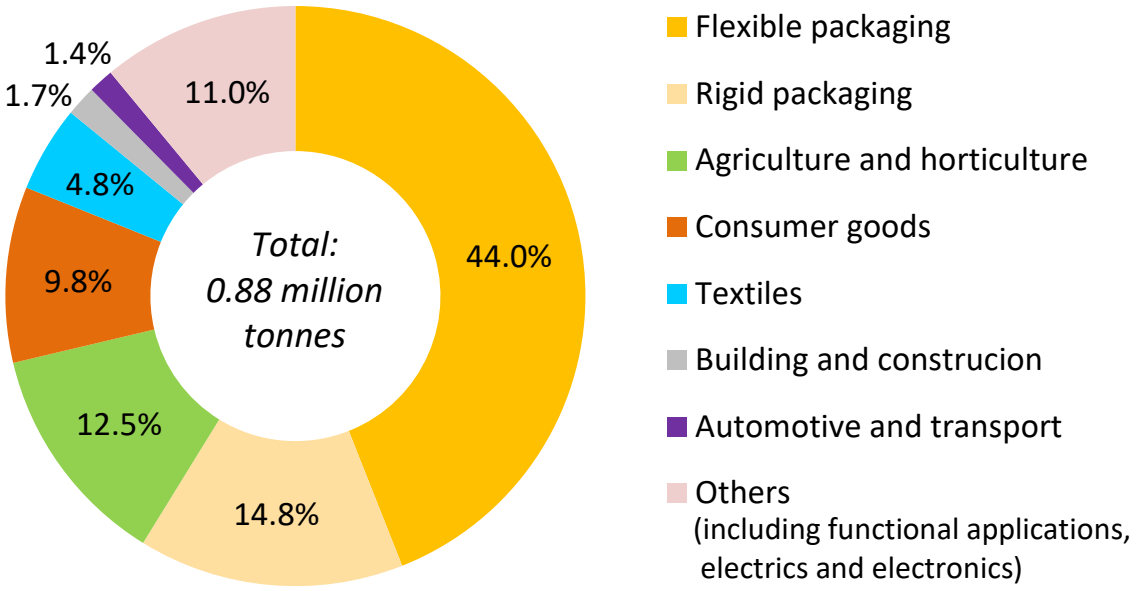

Figure 3. Levels of production of biodegradable plastics in 2017 (by market segment) (European Bioplastics, nova-Institute, 2017) [1].

Global production capacities of biodegradable plastics in 2017 and prediction for the year 2022 are given in Figure 4. As can be seen, according to the latest market data compiled by European Bioplastics in collaboration with the nova-Institute, the largest share in global production took starch blends, PLA, PBAT, PBS and PHA. Moreover, the global production capacities of biodegradable plastics are predicted to grow from around 900 thousand tonnes in 2017 to approximately 1.1 million tonnes by 2022, where PLA and PHA will be the major growth driver in this field [1].

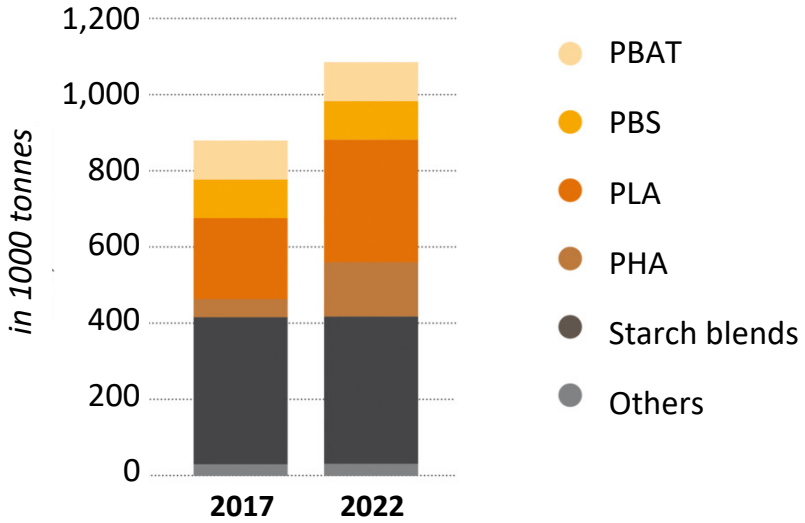

Figure 4. Global levels of production of biodegradable plastics in 2017 and 2022 (European Bioplastics, nova-Institute, 2017) [1].

Natural biodegradable polymers

Natural biodegradable polymers are formed in nature during the growth cycles of all organisms, therefore they are also referred to as biopolymers or agro-polymers [6,17]. The synthesis of natural polymers generally involves enzyme-catalysed, chain growth polymerisation reactions of activated monomers, which are generally formed within cells by complex metabolic processes [6,19].

The most widespread natural polymers directly extracted from biomass are polysaccharides such as cellulose and starch, but also lignin, chitin and chitosans, proteins and others find several applications [18,20].

The second class of natural polymers consists of polymers produced directly by microorganisms. The main representatives of this group are bacterial cellulose and polyhydroxy-alkanoates.

Synthetic biodegradable polymers

Synthetic biodegradable polymers can be distinguished according to the origin of the monomers from which are they synthesised, i.e. bio-derived monomers, monomers of the petrochemical industry (fossil resources) or their combination.

A typical trait for synthetic biodegradable polymers is the presence of hydrolysable bonds (heterochains containing oxygen or nitrogen) within the backbone, such as ester, orthoester, anhydride, urethane or amide [4,21]. However, the most widespread group is represented by polyesters and their copolymers, such as polylactic acid (PLA), polyglycolic acid (PGA), polycaprolactones (PCL), polybutylene succinate (PBS), polybutylene adipate terephthalate (PBAT), etc. In the case of polyurethanes and polyamides, which have a higher resistance to degradation compared to polyesters, the degradability can be supported, for example by introducing the hydrolytically sensitive bond to form polyester-urethanes and polyester-amides, respectively [4].

Another, less numerous group of synthetic biodegradable polymers includes polymers with a C–C backbone chain. Typical examples of this group are polyvinyl acetate (PVAc) and polyvinyl alcohol (PVA) [6].

However, one of the most promising and most widely produced biodegradable synthetic polymers is polylactide acid (see Fig. 4), which has the additional advantage of being based on renewable sources.

Polylactic acid (PLA)

Polylactic acid, or polylactide, is a biodegradable aliphatic polyester synthesised from a lactic acid. Chemically, lactic acid occurs as two optical isomers, L(+) and D(-) stereoisomers. The majority of lactic acid is obtained by fermentation of simple sugars; however, it can be also obtained from petrochemicals [22,23].

This means that not only is PLA biodegradable and biocompatible, but also primarily produced from renewable resources of non-fossil origin, through the fermentation of polysaccharides sourced from common raw, plant materials (e.g. potato, corn, sugar beet and sugar cane) [23–25].

Polylactide production proceeds through direct condensation polymerisation, azeotropic dehydrative condensation polymerisation or ring-opening polymerisation through a lactide intermediate (Fig. 5) [22,25].

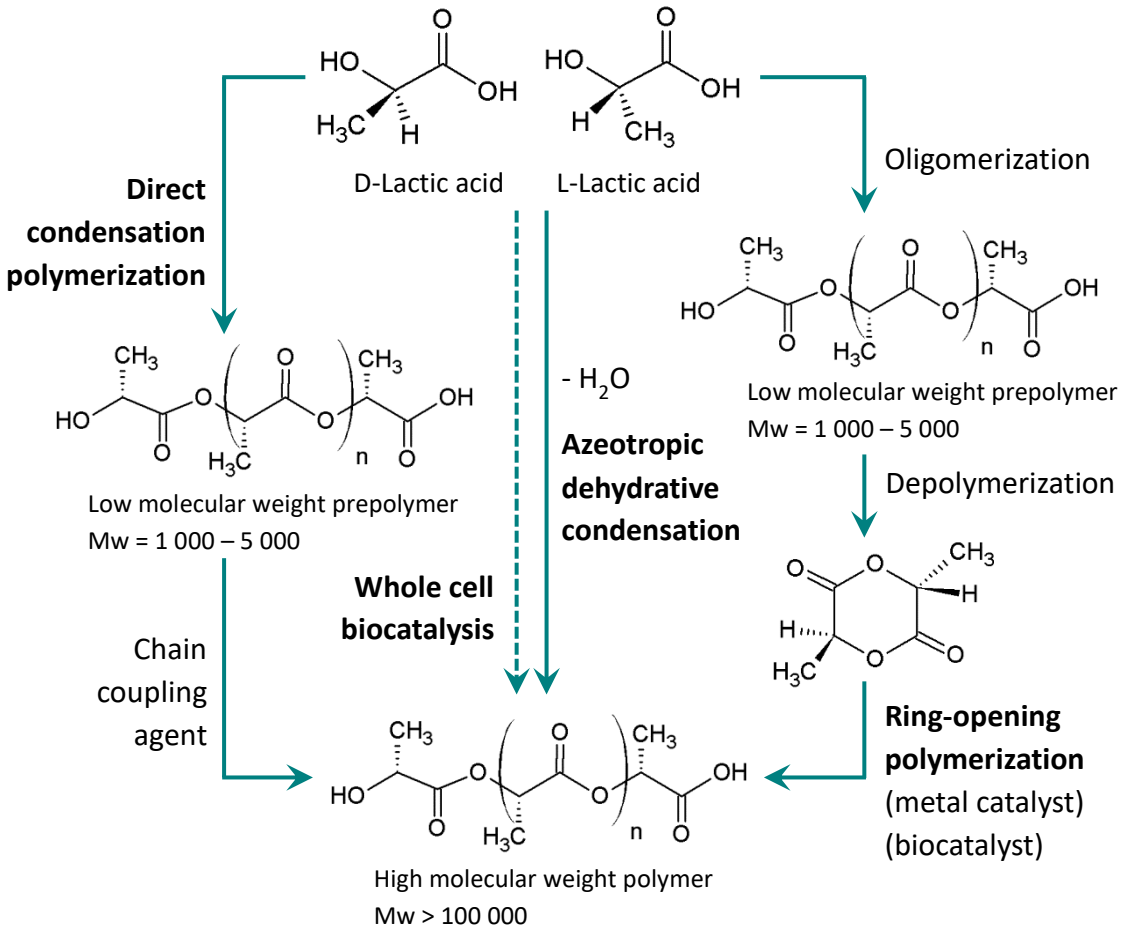

Figure 5. Primary methods for the synthesis of PLA [23].

Polylactide properties are strongly dependent on the molecular weight and stereochemistry. Depending on the enantiomer's composition and thermal history, PLA can be amorphous or semi-crystalline in its solid state. Generally, when the D-isomer is present at less than about 6%, the PLA is semi-crystalline. On the other hand, if the D-isomer content is >6%, then the polymer can be considered as amorphous [22,26]. The typical glass transition temperature (T_g) ranges from 50°C to 80°C, whereas its melting temperature (T_m) ranges from 130°C to 180°C. For example, enantiomerically pure PLA is a semi-crystalline with a T_g of 55°C and T_m of 180°C [27].

The crystallinity of PLA has benefits that include improved chemical and heat resistance, stiffness and permeability. In this context, with decreasing crystallinity (higher D-isomer content) T_m and mechanical properties are also decreasing and it has been reported that the degradation is faster. Therefore, for biomedical devices, amorphous PLA is mostly preferred, whereas semi-crystalline PLA is predominantly used in applications where higher mechanical properties are desired [22].

In the terms of mechanical properties, PLA is generally characterised as a hard polymer exhibiting a high Young´s modulus (~2–3 GPa) and the tensile strength of 50–70 MPa. These characteristics are comparable with or even exceed those of non-biodegradable conventional plastics (i.e. PET, PS, PP and PE). Nevertheless, PLA is relatively brittle, with less than 10% elongation at break which constitutes a factor limiting its use in applications necessitating plastic deformation under high stress [22–25].

As a consequence, considerable efforts have been made to improve the properties of PLA, with the aim of obtaining a more flexible material at a lower cost. This can be achieved either by modifying PLA with biocompatible plasticisers or by blending it with other biodegradable or non-biodegradable polymers [25,28].

1.2 Whey and potential of its use

Whey (milk whey or cheese whey) is a yellowish liquid remaining after the production of cheese. It is obtained after casein precipitation by the action of acids (acid whey) or enzymes (sweet whey). Whey composition is variable and depends on several factors, including the origin and composition of milk, the type of processed cheese, the method of casein precipitation, thermal treatment, storage of product, etc. [29–31]. The main constituents of both sweet and acid whey are water (~93% of total volume), lactose, whey proteins, and minerals (>50% is represented by NaCl, KCl and calcium salts). Whey also contains small quantities of other components, such as lactic and citric acids, non-protein nitrogen compounds and B-group vitamins [32,33].

World production of whey is estimated at around 190 000 000 ton/year, with a yearly increase in volume rate of approximately 2%, but only around 50% is processed [29,32]. Such a high volume of whey as a biological waste is considered harmful to the environment, which is the reason why the disposal of whey by dumping in water bodies is now prohibited in most dairy producing nations by strict environmental legislation [30,32].

On the other hand, this dairy stream also represents an excellent source of bioactive substances, especially proteins and peptides, which have helped transform whey from a waste material to a valuable product containing a multitude of components available for exploitation in the agri-food, biotechnology, medical, pharmaceutical and related markets [30,33–36].

Although there has been an obvious increase in the use of whey produced in recent decades, a significant portion of the whey is still discarded or clearly underutilised. Moreover, the processing has been envisaged mostly for protein recovery, despite the fact that the lactose amount is seven times higher in the whey [31]. Therefore, utilisation of whey as a source of valuable substances seems to be still an interesting subject of research. Examples of technological alternatives for whey utilisation are shown in Figure 6.

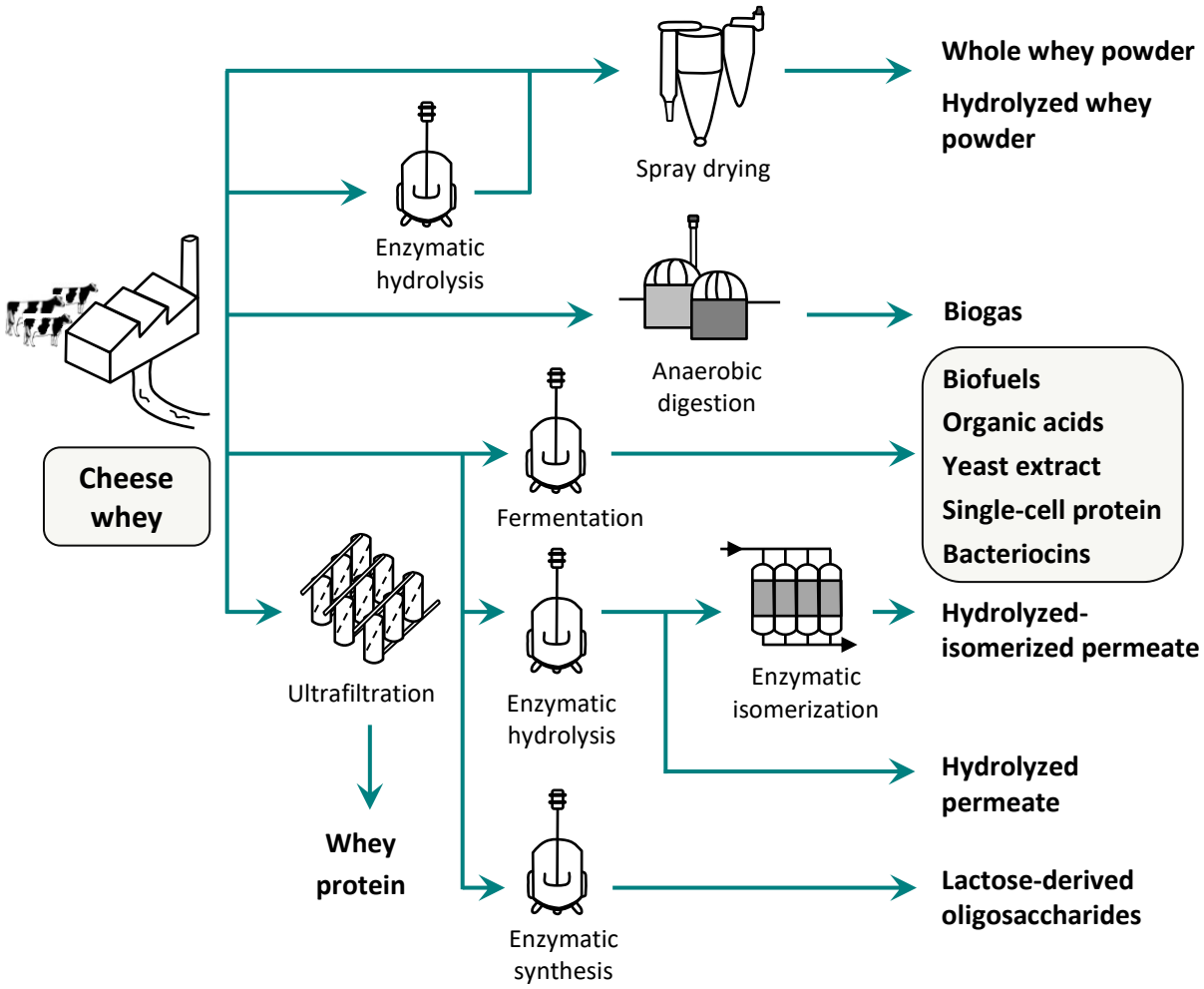

Figure 6. Technical scheme for whey utilisation and upgrading [31].

Whey fermentation products

Since whey contains significant quantities of lactose (approximately 70–72% of the total solids), an interesting way to upgrade this 'waste' liquid could be as a substrate for fermentation. For example, the production of bioethanol, vinegar, yeasts, organic acids (e.g. citric acid, lactic acid), glycerol, xanthan gum, bacteriocins, biosurfactants (sophorolipids), single-cell protein and biodegradable polymers (polyhydroxy-alkanoates) are all due to the bioconversion of lactose/whey [31,32,37].

In the field of antimicrobial modification of polymers, the utilisation of lactic acid bacteria (LAB) and especially their metabolites seems to be beneficial.

LAB are a heterogeneous group of strains of different genera, which synthesise lactic acid as the major product of sugar fermentations. The genera that comprise LAB are at the core *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and others. LAB are classified into two groups according to their hexose metabolic pathways. Homofermentative LAB produce lactic acid as their only fermentation product. On the other hand, heterofermentative LAB may form acetic acid, ethanol, aroma compounds and CO_2 besides lactic acid. The advantage of using LAB is undoubtedly the fact that they have a 'generally recognized as safe (GRAS) status' as defined by the US Food and Drug Administration Agency [37–39].

As mentioned, besides lactic acid, many LAB strains produce a significant amount of non-specific compounds, such as organic acids, diacetyl, hydrogen peroxide, reuterin, bacteriocins, etc., which define the spectrum of their antimicrobial activity [39,40]. The major microbial metabolites that restrict the growth of pathogenic contaminants are given in Table 1.

Table 1. Antimicrobial fermentation metabolites [39]

Compound	**Sensitive microbiota**	**MIC or pH levels**
Lactic acid	All microorganisms	pH dependent; pK_a 5.2
Acetic acid	All microorganisms	pH dependent; pK_a 4.75
Ethanol	All microorganisms	At concentrations approximately ≥ 10%
CO_2	Most microorganisms	Aqueous: at pH ≥ 6 Gas: at 20–50%
Diacetyl	Yeasts, Gram-negative bacteria Non-LAB, Gram-positive bacteria	At 200 ppm At 300 ppm
Hydrogen peroxide	All microorganisms	500 ppm
Reuterin	Many bacteria and fungi	
Bacteriocins (i.e. nisin)	Gram-positive bacteria	50–100 ppm

**MIC = Minimum inhibitory concentration*

Since whey is a renewable agro-industrial by-product, it represents a valuable substrate for producing of antimicrobial metabolites with the potential for subsequent widespread application [41]. However, out of all the fermentation metabolites outlined in Tab. 1, lactic acid and bacteriocins comprise the most promising compounds with antimicrobial properties in the preparation and/or modification of polymers.

Lactic acid (LA)

Lactic acid (2-hydroxypropanoic acid) is a colourless organic acid. It belongs to α-hydroxy acids with an asymmetric carbon atom and exists as L(+) or D(-) stereoisomer (Figure 6). The chemical behaviour of LA is determined by its physico-chemical properties, among which are i) acidic character in an aqueous medium, ii) bifunctional reactivity associated with the presence of a carboxyl and hydroxyl group (great reaction versatility), and iii) asymmetric optical activity [23,42,43].

The majority of LA produced today is obtained by fermentation of simple sugars, using homofermentative bacterial strains of the genus *Lactobacilli*, however, it can be also obtained chemically from renewable resources such as ethanol, acetaldehyde or from petrochemicals [22,23].

When LA is produced by fermentation, it is composed mainly of L-lactic acid, generally 99.5% L-isomer and 0.5% D-isomer. On the other hand, when LA is generated from petrochemicals, it exists in the meso-form (50/50 mixture of the L- and D-isomer). It means that the production of LA by fermentation has advantages over chemical synthesis, as desirable optically pure lactic acid could be produced [22,37].

Bacteriocins

Bacteriocins are ribosomally synthesised antimicrobial peptides produced by bacteria that inhibit the growth of similar or closely related bacterial strains. They are natural substances well-known for exhibiting antibacterial properties even at low concentrations. A number of bacteriocins from a wide variety of bacteria have been discovered, and their diverse structures have been reported [44–46]. To date (October 2018), BACTIBASE, which is an open-access database designed for the characterisation of bacteriocins, contains 230 bacteriocin sequences, most of which are the products of Gram-positive bacteria [47].

In the past few years, interest in bacteriocin research, particularly from the lactic acid bacteria (LAB), has gained great momentum. LAB bacteriocins have a number of positive attributes that have made them attractive for various applications, especially in food science, pharmaceutics and clinical medicine. The main advantage is that they are frequently produced by commercially applicable bacterial strains generally recognised as safe (GRAS) for human consumption [41,44,45,48,49].

Bacteriocins are occasionally confused in the literature with antibiotics, although the two differ fundamentally from each other in several characteristic respects; e.g. synthesis, the spectrum of bioactivity and intensity, the mode of action, stability, degradability, toxicity and resistance mechanisms (Tab. 2) [50].

Table 2. Characteristics of LAB bacteriocins compared to antibiotics [44,49]

Characteristic	Bacteriocins	Antibiotics
Bioactivity spectra	Mostly narrow	Mostly broad
Intensity of bioactivity	nM – μM	μM – mM
Synthesis	Ribosomal	Secondary metabolite
Proteolytic enzyme degradability	High	Moderate-to-none
Thermal stability	High	Low
Active pH range	Wide	Narrow
Mode of action	Pore formation, inhibition of cell wall biosynthesis	Cell membrane or intercellular targets, inhibition of cell wall biosynthesis
Toxicity towards eukaryotic cells	None known	Yes

Over the years, different kind of schemes have been proposed to classify bacteriocins from Gram-positive bacteria including LAB [40,44,45,51,52]. According to Cotter *et al*., bacteriocins are grouped into three categories (Tab. 3): lantibiotics (class I), non-lanthionine-containing bacteriocins (class II) and non-bacteriocin lytic proteins (bacteriolysins) [51].

The best known and most widely characterised bacteriocin is nisin, which is described in more detail in the following chapter.

Table 3. Classifications of LAB bacteriocins [51]

Classification	Remarks	Examples
Class I (Lantibiotics)		
Lanthionine-containing bacteriocins	Subdivided into 11 subgroups based on the sequences of the unmodified pro-peptides	nisin, epidermin, streptin, pep5, mersacidin, lacticin 481, lacticin 3147, cytolysin, lactocin S
Class II		
Non-lanthionine-containing bacteriocins	IIa) pediocin-like	pediocin PA1, leucocin A, lacticin F
	IIb) two-peptide	
	IIc) cyclic	enterocin AS48, reuterin 6
	IId) non-pediocin single linears peptides	lactococcin A, divergicin A
Bacteriolysins		
Non-bacteriocin lytic proteins	Large, heat-labile proteins, often murein hydrolases	lysostaphin, enterolysin A

1.3 Bacteriocin nisin

Nisin is the most well-known and extensively studied bacteriocin. This antimicrobial peptide is produced by a group of Gram-positive bacteria, and constitutes the main representative of the class of lantibiotics. As shown in Figure 7, nisin was discovered in 1928 and first commercially marketed in 1953 as an antimicrobial agent. In 1969, nisin was recognised as biologically safe and approved for use as an antimicrobial agent in food stuffs by a joint committee on food additives, formed of the Food and Agriculture Organisation and World Health Organisation (FAO/WHO). Nearly 20 years on, in 1988, nisin was approved in the United States by the Food and Drug Administration (FDA) as GRAS [53–55].

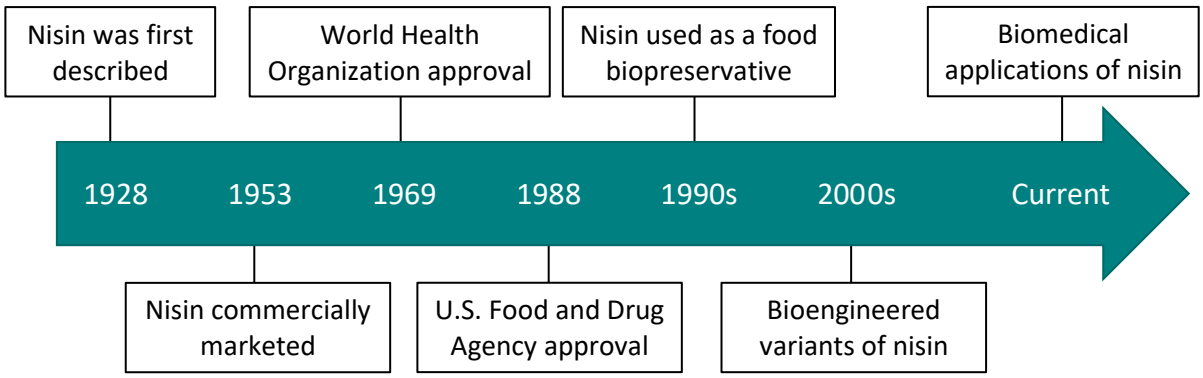

Figure 7. Timeline of nisin development [53].

Currently, nisin is permitted as a food additive (E324) in over 50 countries, including European Union countries, the USA, South America, Australia and Russia, etc. Due to the antibacterial effect it exhibits against *Listeria monocytogenes*, clostridia spores and other bacteria associated with spoiling food, nisin has made a significant impact in the food industry as a natural biopreservative for various types of food [49,54,55].

Over the past two decades, research revealed another potential use for nisin, as a useful therapeutic agent in biomedical applications. It has been reported that nisin can prevent the growth of drug-resistant bacterial strains and other pathogens associated with disease. Moreover, nisin has been shown to possess anti-biofilm properties and the selective cytotoxicity against cancer cells has been explored [44,48,53]. In addition, nisin is employed by the veterinary industry as a preventive drug or a cure for bovine mastitis [56], and its utilisation in cosmetics has also been reported [44,57].

Nisin is effective against a broad spectrum of Gram-positive vegetative bacteria, encompassing numerous foodborne pathogens and spoilage bacteria (e.g. the bacterial genera *Bacillus, Bronchothrix, Clostridium, Enterococcus, Lactobacillus, Desulfotomaculum, Leuconostoc, Listeria, Micrococcus,*

Pediococcus and *Staphylococcus*), and is particularly effective against bacterial spores. Under normal circumstances, nisin does not significantly inhibit Gram-negative bacteria, yeasts or moulds. However, when in combination with a chelating agent, such as ethylenediaminetetraacetic acid (EDTA), and exposed to heat treatment, freezing, hydrostatic pressure, a pulsed electric field or any treatment causing alteration to the cell wall, nisin also proves effective against a variety of Gram-negative bacteria and yeasts [54,55,58].

Studies have shown that the mode of action of nisin against bacteria involves inhibiting of cell wall biosynthesis and pore formation in the cytoplasmic membrane (Fig. 8). Both processes are based on interaction with the lipid II cell wall precursor. Nisin binds with a high affinity to said lipid II, a hydrophobic carrier of peptidoglycan monomers. The occurrence of a nisin-lipid II complex leads to the inhibition of peptidoglycan synthesis and to formation of the highly specific pore exhibited, thereby facilitating the efflux of ions or essential cellular components resulting in inhibition or death of the bacteria [59–61].

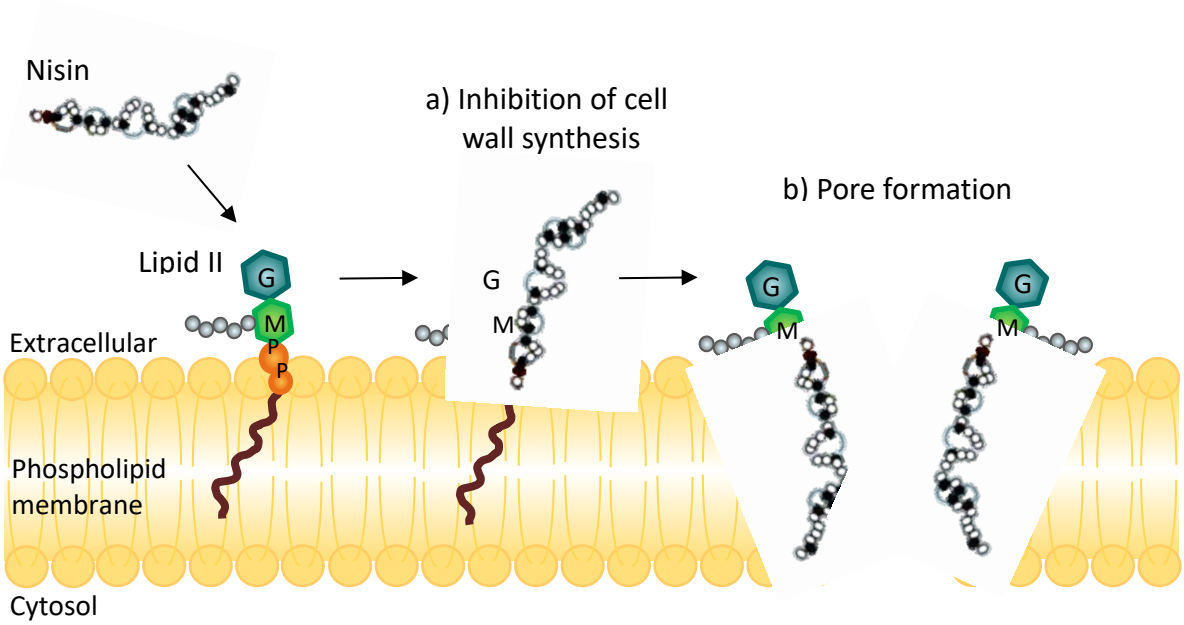

Figure 8. Schematic representation of the mechanisms of nisin action [55].

To date, at least eight natural nisin variants have been discovered, and quite a number of bioengineered forms of nisin have been developed [53]. The natural variants include nisin A, nisin Z, nisin F and nisin Q, which have been isolated from *Lactococcus lactis*, nisins U and U2, from *Streptococcus uberis*, nisin P, which is encoded on nisin operons present in both *Streptococcus gallolyticus* subsp. *pasteurianus* and *Streptococcus suis* and most recently described nisin H, which is produced from *Streptococcus hyointestinalis* [62].

The chemical structure of the variant first discovered, nisin A, together with an indication of sites of amino acid substitutions for natural variants of nisin, are

given in Figure 9. As illustrated, nisin is composed of 34 amino acids, including several unusual modified residues – dehydroalanine (Dha) and dehydrobutyrine (Dhb). These unsaturated amino acid residues can react further with neighbouring nucleophilic groups, and potentially form thioether bridges with cysteine residues [63]. The reaction product of cystein residue and Dha is called lanthionine (Ala–S–Ala), and the reaction product of the same with Dhb is β-methyllanthionine (Ala–S–Abu; where Abu refers to aminobutyric acid). Consequently, nisin represents a polycyclic structure containing thioether bridges in five positions (shown in red).

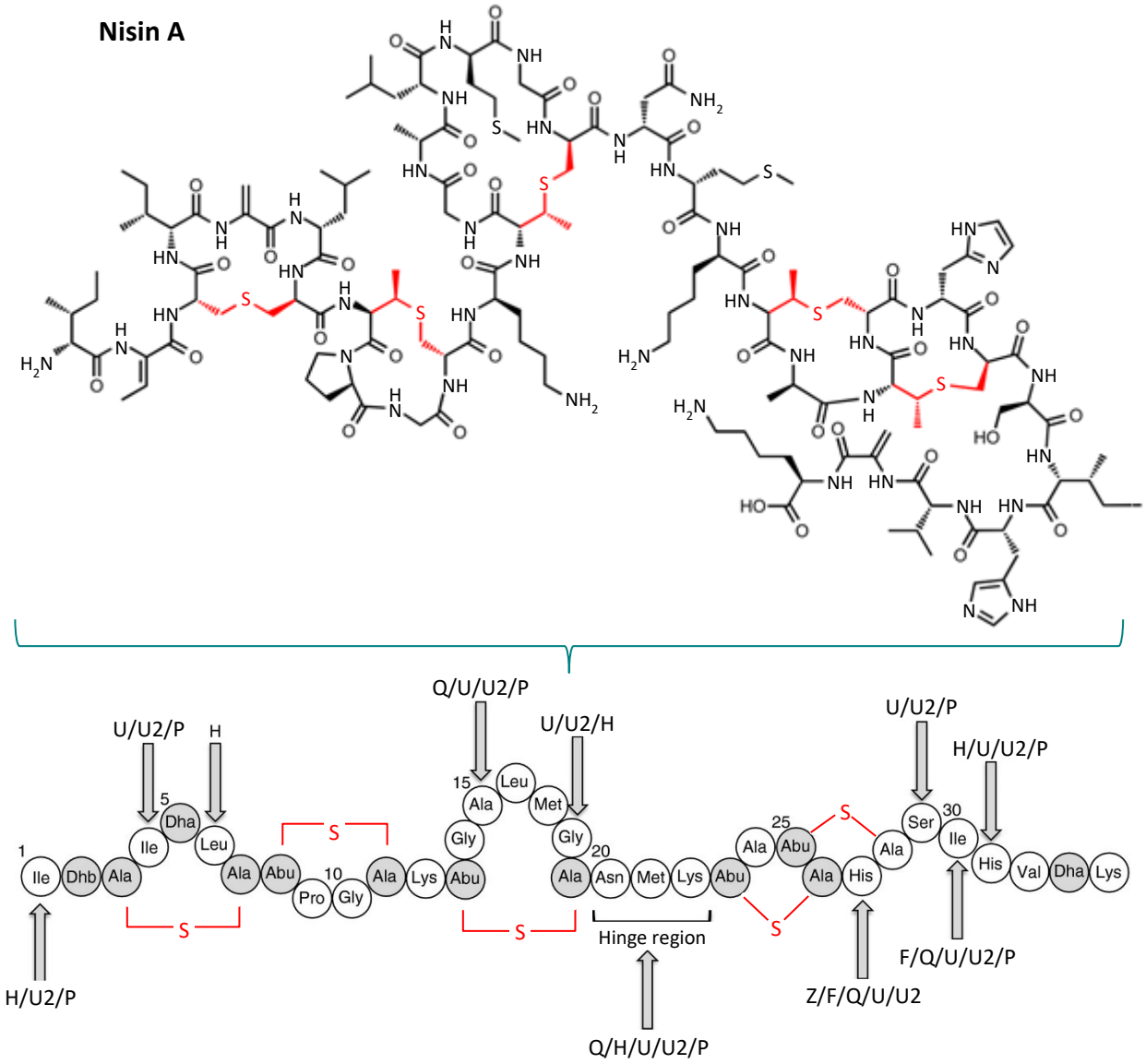

Figure 9. Chemical structure of nisin A; arrows indicate the sites of amino acid substitutions for natural variants of nisin [53].

The activity or potency of nisin can be expressed in terms of International Units (IU), and 1 g of pure nisin is usually equivalent to $40 \cdot 10^6$ IU (meaning 1 IU ≈ 0.025 μg of pure nisin) [54,58].

Commercially available nisin preparations generally do not exceed 2.5 wt. % of pure nisin, and they are standardised to 10^6 IU·g^{-1}, with the remainder made up of added salts (primarily NaCl) and milk solids derived from fermentation of a modified medium by lactic acid bacteria. It can be said that the growth of LAB for industrial production of nisin requires complex nutritional conditions, thereby raising production costs and complicating steps for purification [55].

The method most commonly utilised for nisin quantification is the agar diffusion bioassay [64]. This is based on the measurement of inhibition zones in agar seeded with nisin-sensitive microorganisms. Although the assay is the most widely one applied, agar diffusion techniques have many limitations, such as low sensitivity due to interfering substances in food extracts, long microbial culture times or possible formation of false inhibitory zones [65].

Other more sensitive techniques for quantifying nisin also exist, examples being chemiluminescence [66], electrophoresis [67,68], flow cytometry [69] and the enzyme-linked immunosorbent assay (ELISA) [70,71]. However, these methods also present some limitations.

Currently, high-performance liquid chromatography (HPLC) [68,72], especially in combination with mass spectrometry (LC-MS) [63,65,73], appears to be a very promising and effective method, which permit accurate molecular mass determination of target nisin molecules in crude samples.

Stability of nisin

The activity and stability of nisin depend on various factors. In its dry state, nisin powder is highly stable under the given recommended storage conditions (i.e. at temperatures below 25°C, in a dark, dry area). However, the solubility and stability of nisin solutions – especially concerning thermal stress – are largely reliant on pH. For instance, nisin solutions are at their most stable for autoclaving (121°C for 15 min) within the pH range 3.0–3.5 (<15% activity loss); values of pH below outside of this range induce a significant decrease in activity (>90% loss in activity at pH 1 or 7) [54,55,58,74].

Other factors that may affect nisin activity include proteolytic enzymes, such as pancreatin and α-chymotrypsin, which have the capacity to break the peptidic chain [75]. Similarly, degradation of nisin may occur in the presence of certain food additives, e.g. titanium dioxide (a whitener) or sodium metabisulphite (an antioxidant, bleach and antimicrobial agent) [54,58].

Regarding conditions *in vivo*, bacteriocins are generally liable to degrade in the gastrointestinal tract, tissue, serum and organs such as the liver and kidneys [76]. However, a few studies report that cerain bacteriocins, including nisin, may survive *in vivo* and still inhibit bacterial growth [77–79].

1.4 Antimicrobial modifications of polymers

For some applications, in addition to the passive function of polymer products (e.g. packaging or structural function), the active function (e.g. protective or/and indicative) is also required. In this sense, the polymeric materials with resistance to microbial colonisation and with the potential to control the growth of spoilage and pathogenic microorganisms (antimicrobial polymers) have been one of the examples of the active material functionality [9,80].

Research on incorporating antimicrobial agents into polymers has gained in importance in order to aid development of active and intelligent packaging material. Moreover, the antibacterial activity demonstrated by such material represents a desirable property for the medical sector (medical devices, drug delivery, hospital surfaces/furniture and surgical equipment), as well as in other areas – e.g. textiles, automotive or hygienic purposes [11–13,81–86].

Mechanisms and types of antimicrobial polymers

Several options exist for classifying polymeric materials that exhibit antimicrobial properties. Depending on the mechanism of action, antimicrobial activity of polymers can be categorised as either passive or active (Fig. 10).

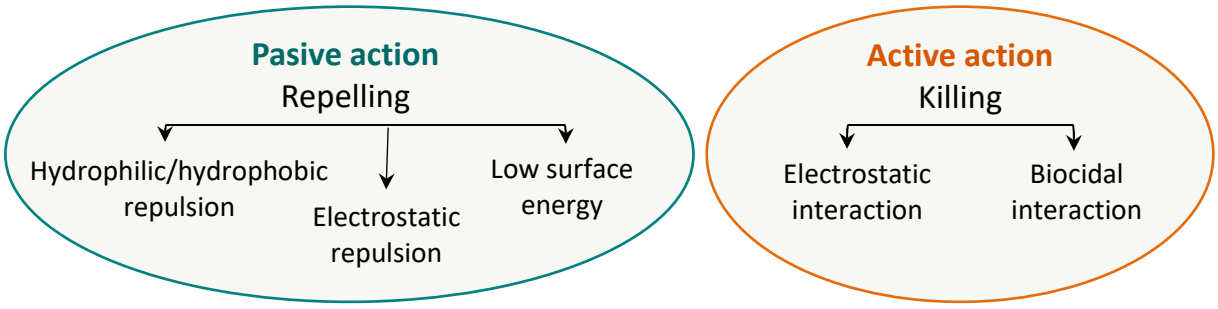

Figure 10. Mechanisms of action of antimicrobial polymers [87].

Passive action means that the material can prevent adhesion of bacteria on its surface through repulsion. Largely as a consequence of the hydrophobic and negatively-charged properties of microbes, passive polymers should be hydrophilic, negatively-charged or possess low surface free energy [87,88].

In contrast, antimicrobial polymers displaying an active mechanism of action function by killing bacteria directly. According to the type of polymeric system, they are generally divided into three categories (Fig. 11) [12,89,90]:

- biocidal polymers (polymers with intrinsic antimicrobial activity),
- polymer biocides (polymers that carry active molecules covalently linked to their backbone),
- biocide-releasing polymers (systems where the polymer acts as a delivery platform for transferring small biocides to the environment).

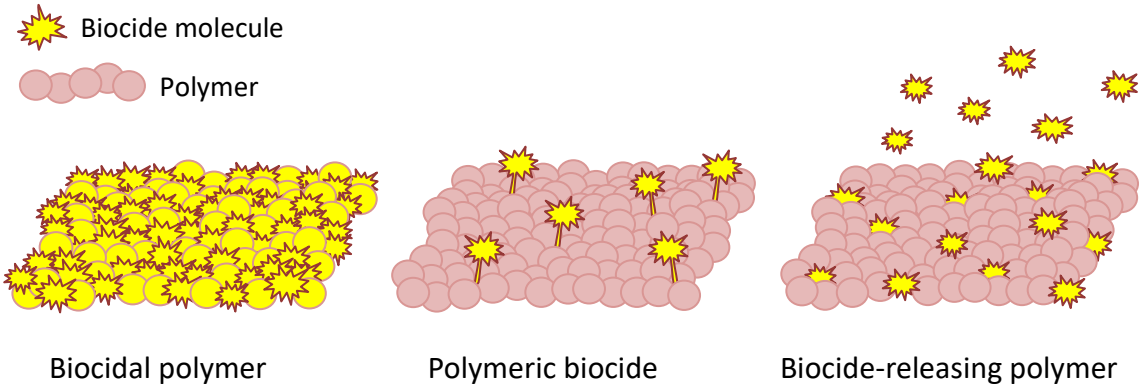

Figure 11. Schematic representation of antimicrobial polymeric systems [12,89].

Notably, surfaces based on biocidal polymers or polymeric biocides kill microorganisms upon contact (thereby constituting contact-killing surfaces), in contrast with the mechanism of action of releasing systems, i.e. releasing surfaces [89].

Biocide-releasing polymers are usually the most active systems, due to the high concentration of biocides realised during release and their proximity to the target bacteria cells. However, the toxicity associated with biocide molecules is disadvantage of this strategy, and their efficiency reduces significantly over time [12,87,90]. Regarding this type of system, a multitude of biocide molecules (antimicrobial agents) of organic or inorganic origin have been successfully incorporated into or coated onto several types of polymer, e.g. those undergoing modifications with some metals (Ag, Cu), metal oxides (ZnO, MgO, TiO_2), quaternary ammonium compounds, antibiotics, chlorine, nitric oxide, phenols, organic acids, essential oils, etc. [9,82,83,89–94].

Antimicrobial modification of biodegradable polymers

The development and use of biodegradable polymers have risen significantly over the past few years. However, other 'added value' functionality is needed in order to increase their competitiveness on the market and to facilitate further specialised applications.

Any material with a capacity for degradation by living organisms while exhibiting antimicrobial properties is a highly promising one, especially in environmental and medical fields.

Generally, the most relevant areas for application of biodegradable antimicrobial materials are those where: a) the biodegradable polymer acts partially as protection of the antimicrobial agent and permits controlled release of the substance into the environment; or b) disposable products or equipment come into contact with microbial contamination. The former of the two mainly relates to medicine (drug-delivery, resorbable sutures and implants) [89,95,96] or agriculture (biodegradable systems for incorporation of pesticides or

herbicides) [97], while the latter pertains to disposable packaging (bags or containers for perishable food stuffs, medical packaging for single-use products) [5,13] or disposable products in medicine (syringes, catheters, tubing, non-woven textiles) [9].

Indeed, the real-world potential of biodegradable materials enhanced with antibacterial activity is considerable. However, the design of these systems can be quite challenging in some respects. Regarding the selection of an appropriate biodegradable matrix, it is necessary to attend to polymer degradation rates, as these affect the release profile of the antimicrobial agent and other properties besides (e.g. mechanical), as well as the shelf life of the material. Generally, low water soluble matrices degrade slowly but offer better protection from an environmental standpoint, although they are not usually effective for long-term prevention. Consequently, partially water-soluble matrices (e.g. PLA/PEG blends among others) are preferred as they offer better control over the release of the antibacterial compound [28,89].

Polymers modified by whey fermentation antimicrobials

Although many different chemicals of organic and inorganic origin have been successfully employed for antimicrobial modification of polymers [12,13,89], legislative restrictions exist regarding the safety of various antibacterial additives, pertaining to their toxicity and potential carcinogenic and teratogenic effects [14]. Therefore, in alignment with new trends, stress is increasingly being put on the need to apply naturally derived antimicrobial compounds, especially in food packaging, cosmetics and biomedical applications [8]. Examples of beneficial compounds for the antimicrobial modification of polymers are whey fermentation products, e.g. lactic acid and bacteriocins.

Polymer systems modified by lactic acid (LA)

The use of LA for modification of polymers has been investigated in several studies. For example, Sedlarik *et al*. [98] prepared and characterised polymeric biodegradable films based on polyvinyl alcohol (PVA) and LA with possible multiple applications, such as medical or packaging materials. The films showed a rapid increase in elasticity and a decrease in glass transition temperatures due to the presence of LA. Moreover, the bacteriostatic effect on *Pseudomonas putida*, *Staphylococcus epidermidis* and *Micrococcu*s sp. was proved. The total biodegradation of samples was not influenced by LA content. This work was followed by a study carried out by Hrabalikova *et al.* [99], where the effect of the degree of hydrolysis of PVA under extended interaction with LA was investigated. Regarding the antibacterial properties of prepared PVA/LA films, the great antibacterial effectiveness of the proposed systems against *Escherichia coli* and *Staphylococcus aureus* was proved and described by the mathematical model.

As another example, LA-loaded gelatin microspheres were prepared by the chemical cross-linking technique by Dinarvand *et al.* [100]. Drug release experiments showed a distinct biphasic release pattern. That behaviour may be desirable for topical drug-delivery since a therapeutic loading dose can be provided initially and the sustained drug release could maintain the therapeutic drug level. Moreover, the desired LA-release rate can be achieved by controlling the amount of the cross-linking agent and the duration of cross-linking time.

Polymer systems modified by bacteriocins

Considerable attention has been paid to applying bacteriocins in the generation of active plastic materials in the past decade. Due to the fact that bacteriocins are nonvolatile substances, they can act upon contact with the target cell of the given microorganism. The advantage of supplementing a polymer matrix with bacteriocins compared to direct incorporation into the food matrix is that the bacteriocin is partially protected from direct interaction with food components, thereby reducing the risk of its inactivation. Additionally, employing encapsulation techniques as well as incorporating bacteriocin directly into the polymer matrix can ensure the controlled release of the antimicrobial substance. Bacteriocins of various type, such as nisins, lacticins, lactocins, enterocins, pediocins and sakacins, have been utilised to modify different polymers, both synthetic and natural [85,101,102].

Considering the scope of the experimental part of the dissertation, the following chapter is given over to describing how bacteriocin nisin may be employed for the modification of polymer systems.

1.5 Polymer systems modified by bacteriocin nisin

Nisin has been used for the modification of many different polymers, both synthetic and natural. For example, in the latter case, polymers such as cellulose [103,104], chitosan [105], and protein-based polymers [106,107] were used. In contrast, synthetic polymers and copolymers such as polyethylene (PE) [108–112], ethylene vinyl acetate (EVA) [113–115], polyvinyl chloride (PVC) [116], polyvinyl alcohol (PVA) [117,118], and poly(butylene adipate-co-terephthalate (PBAT) [119] have been handled to investigate antimicrobial polymer systems using nisin.

However, in relation to biodegradable polymers, the vast majority of works in the literature deal with the modification of natural biodegradable polymers for utilisation in the food industry. In the case of synthetic biodegradable polymers, only a few works have investigated modification with nisin. Examples of these systems and brief descriptions of the same are summarized in Table 4.

Table 4. Synthetic biodegradable polymers modified with nisin

Polymer matrix	Method of preparation	Target microorganism	References
PVA cross-linked with glutaric acid	solvent casting	*Escherichia coli, Staphylococcus aureus*	[117] (2016)
PBAT	extrusion	*Listeria monocytogenes, Staphylococcus aureus, Clostridium perfringens Bacillus cereus*	[119] (2015)
PLA; PLA/GTA	extrusion	*Escherichia coli* O157:H7	[120] (2010)
PLA; PLA/lactic acid	extrusion	*Listeria monocytogenes*	[121] (2009)
PLA/pectin	coating method (post-extrusion)	*Listeria monocytogenes*	[122] (2009)
PLA	solvent casting	*Listeria monocytogenes, Escherichia coli* O157:H7, *Salmonella enteritidis*	[123] (2008)
PLA; PLA/pectin	coating method (post-extrusion)	*Lactobacillus plantarum*	[124] (2007)
PVA cross-linked with glyoxal	solvent casting	Antimicrobial activity was not determined	[118] (2003)

**GTA = glycerol triacetate*

For example, immobilisation of nisin into a PVA matrix cross-linked with nontoxic glutaric acid was carried out by Hrabalikova *et al*. [117]. The samples were prepared by the solvent casting technique, wherein 150 $\mu g.g^{-1}$ of pure nisin was added into the polymer matrix. Results showed the suitability of glutaric acid as an effective cross-linking agent of PVA, which acted synergistically with nisin against both of the tested Gram-positive and Gram-negative strains.

As another example, biodegradable PBAT films were prepared by the melt processing technique by Zehetmeyer *et al*. [119]. It was found that the addition of nisin had no demonstrable effect on thermal properties. However, mechanical characteristics differed significantly between the samples. Prepared films containing 3 wt. % of Nisaplin® displayed antibacterial properties against all the tested Gram-positive bacterial strains, indicating that the antibacterial activity of nisin was retained after film preparation by melt intercalation at 140°C.

Regarding the modification of PLA, samples have primarily been prepared by the solvent casting method [123] or the post-extrusion coating method [122,124]. Only two studies have involved the preparation of PLA with nisin by an extrusion process, wherein Liu *et al*. utilised inclusion of a plasticiser to allow extrusion of PLA at a temperature as low as 120°C [120,121]. The earlier of the two studies dealt with the preparation of PLA coextruded with lactic acid,

lactide or GTA. The prepared film membranes modified with 5 wt. % of Nisaplin® were effective against *L. monocytogenes,* and they possessed mechanical properties which matched those of some commercially available petroleum-derived plastics [121]. On the other hand, the second study examined inclusion of EDTA into the Nisaplin® formulation. The prepared blend systems exhibited a synergistic effect of the given components on suppression of growth of the Gram-negative bacteria, *E. coli* O157:H7 [120].

Discovering that only a few works in the literature dealt with nisin modification of PLA (the best known and most promising bio-based synthetic biodegradable polymer) provides an opening for research on nisin incorporation into PLA-based polymer matrices and their subsequent detailed characterisation, which is necessary for the commercial utilisation of the investigated materials.

Moreover, biodegradable polymers (both natural and synthetic) modified with nisin are generally obtained by carrying out the solvent-casting technique [104–107,117,118,123] or via surface modification [103,124–126]. From an industrial point of view, it is preferable to obtain active polymer films by applying standard thermoplastic technological processes, due to the possibility of producing large volumes of material in a single step without the use of harmful organic solvents, which is also associated with lower production costs.

Only a few studies have investigated formulating nisin-incorporated biodegradable films using thermoplastic processing [119–121]. This fact is primarily because bacteriocins as proteins are sensitive to various conditions (temperature, pH, water activity, etc.), in addition to which deterioration in antimicrobial activity can occur during extrusion at high temperature and high shear rates, hence also under high pressure [127].

According to the literature, the maximal temperature at which nisin can retain its bioactivity is 120°C [113]. Nevertheless, Scaffaro *et al.* [114] reported that no reduction in antibacterial activity was observed at even higher processing temperatures (140°C and 160°C) in the EVA-based material prepared therein, probably as a consequence of the short processing time employed.

The processing temperature of polymers appears to be an important factor affecting the chemical stability of nisin, related to loss of efficacy against food-spoilage microorganisms. In connection with this, more detailed investigation of the effect of high temperatures (approaching the thermoplastic processing temperatures of PLA) on antibacterial activity of nisin in polymer systems seems to be a potential issue for further research.

Finally, it is necessary to highlight another important factor limiting the effectiveness of nisin as a food preservative. Loss in nisin activity occurs in some foods through possible interactions with food components like proteins and fat particles. A possible solution for improving the stability and efficiency of nisin may be the use of encapsulation techniques and the preparation of novel systems, e.g. those based on biodegradable particles or other similar systems.

AIMS OF WORK

The doctoral thesis is devoted to utilisation of whey fermentation products for antimicrobial modification of biodegradable polymers. The aims of work defined based on the state of the art study and conclusions made out of that are following:

- Preparation of novel biodegradable PLA-based polymer systems modified with bacteriocin nisin:
  - in the form of films (solvent casting method)
  - in the form of microparticles (solvent evaporation method)

- Characterisation of structural, mechanical, and thermal properties of prepared polymer systems

- Evaluation of release kinetics of nisin from polymer matrix by chromatographic separation techniques (HPLC)

- Evaluation of antibacterial properties by different testing methods
  - agar diffusion testing
  - dilution and spread-plate technique
  - measurement of antibacterial activity on plastic surfaces (international standard test method)

- Evaluation of long-term and high-temperature stability of antibacterial activity of nisin-containing polymer systems

The results obtained herein shall be presented in scientific journals and at international conferences.

2. EXPERIMENTAL PART

The doctoral thesis is aimed at the utilisation of whey fermentation products (bacteriocins) for antimicrobial modifications of biodegradable polymers.

First of all, the optimisation of bacteriocins production from whey-based fermentation media by using a nisin-producing strain *Lactococcus lactis* subsp. *lactis* was carried out. On this basis, novel alternative extraction of bacteriocin nisin from a whey fermentation media and its stabilisation by using polyethylen glycol (PEG) as the matrix with high practical applicability was performed [68]. Based on these results, the utility model was accepted by Industrial Property Office of the Czech Republic.

In the next step, as the main part of the dissertation, novel biodegradable PLA-based polymer systems with nisin were prepared in the form of blend films and microparticles.

The work regarding the prepared films describes the synergetic effect of polyethylene glycol in polylactide blends, wherein the polyether acts as both the plasticiser and functional additive. Nisin at concentrations ranging between 0.02–0.15 wt.% was incorporated into the samples by the solvent cast technique. The effect of various PEG on the structural, mechanical, and thermal properties of the PLA-based blends was investigated by scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR-ATR), stress-strain analysis (according to the ASTM D882-12 standard test method), differential scanning calorimetry (DSC) and dynamic mechanical analysis (DMA), respectively. Antibacterial activity of the samples was detected by the agar diffusion technique against *Micrococcus luteus*. Furthermore, the antibacterial properties of the samples were tested according to the ISO 22196 standard against Gram-negative (*Escherichia coli*) and Gram-positive (S*taphylococcus aureus, Listeria monocytogenes*) bacterial strains. The nisin detection and release study were performed by using high-performance liquid chromatography (HPLC).

The next part of the work is closely linked to the previous work. Research on the thermal stability of nisin in PLA and PLA/PEG blends was carried out by exposing the given films to various temperatures (90°C, 120°C, 160°C, and 180°C) for a duration of up to 48 hours. Assessment of the antibacterial activity of the samples was carried out by the agar diffusion method against *Micrococcus luteus*, while structural analysis involved the use of high-performance liquid chromatography with mass detection (HPLC-QTOFMS). Structural changes in the polymer matrix were evaluated by gel permeation chromatography (GPC) and scanning electron microscopy (SEM).

The last part of work is focused on the preparation of novel porous type of core-shell-structured microparticles base on polylactide (shell) and polyvinyl alcohol cross-linked with glutaric acid (core) prepared by water-in-oil-in-water solvent evaporation technique. The effect of cross-linking and the initial amount of nisin on their morphology was investigated using scanning electron microscopy (SEM), BET surface area analysis, zeta potential measurements, and Fourier transform infrared spectroscopy (FTIR-ATR). Encapsulation efficiency and release profile of nisin from the microparticles were studied by high-performance liquid chromatography (HPLC). Antibacterial activity of prepared systems was tested by dilution and spread plate technique.

Selected results and conclusions of the experimental work are summarised in the next chapter in the following three sections:

- Effect of polyethylene glycol plasticiser on long-term antibacterial activity and the release profile of bacteriocin nisin from polylactide blends (published in *Polymers Advanced Technologies*, 2018, 1–11)
- Thermal stability of bacteriocin nisin in polylactide-based films (submitted to *Polymer Degradation and Stability*, PDST-D-18-00041)
- Core-shell PLA-PVA porous microparticles as carriers for bacteriocin nisin (published in *Journal of Microencapsulation*, 2017, 34, 243–249)

3. SUMMARY OF RESULTS

3.1 Effect of polyethylene glycol plasticiser on long-term antibacterial activity and the release profile of bacteriocin nisin from polylactide blends

Biodegradable polymer blends based on polylactic acid modified by polyethylene glycol (20 wt.%) with bacteriocin nisin (0.02–0.15 wt.%) were prepared by the solvent casting technique.

The results show that the incompatibility of the polymer matrices causes the formation of a porous structure (Figure 12 c,d), which aids the incorporation of nisin and positively affects its diffusion from the prepared blends.

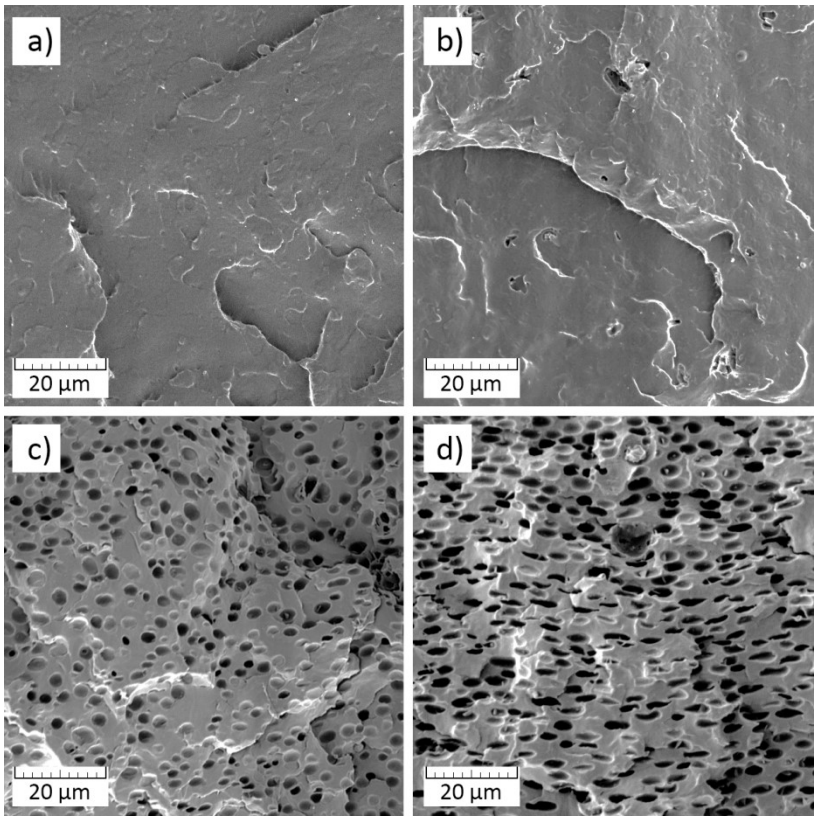

Figure 12. SEM micrographs of the cross-sections of the prepared blend systems containing a) PLA, b) PLA/nisin, c) PLA/PEG1000/nisin, and d) PLA/PEG6000/nisin after leaching in demineralized water.

The presence of PEG as a plasticiser positively influences tensile properties by improving the ductility of the blends, while also reducing their tensile strength and Young´s modulus. The plasticising effect was seen to heighten alongside a parallel decrease in the molecular weight of PEG as proved by DMA analysis.

All samples containing nisin exhibited antibacterial activity against both of the Gram-positive bacterial strains tested. The minimal effective concentration

of nisin in the PLA/nisin and PLA/PEG/nisin samples was between 0.02–0.05 wt. % for *Staphylococcus aureus* and below 0.02 wt. % for *Listeria monocytogenes*. In the case of Gram-negative *Escherichia coli*, no significant antibacterial activity was observed.

The presence of PEG also affected the release kinetics of nisin (Fig. 13). Increase in the molecular weight of PEG led to a concurrent rise in the total amount of nisin released from the investigated systems.

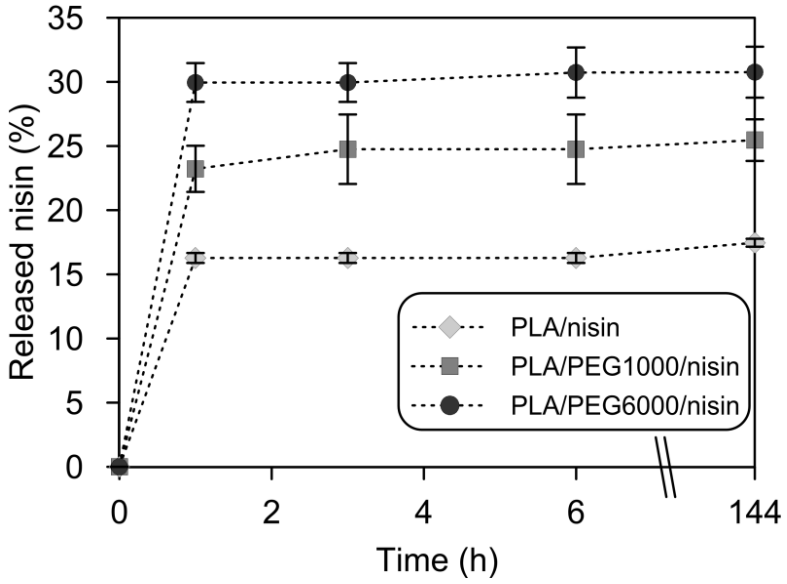

Figure 13. The release profile of nisin from the prepared blend samples.

Regarding the stability testing, all the prepared biodegradable blends showed antibacterial activity – even after 9 months of storage – against both of the Gram-positive bacterial strains tested (Tab. 5).

Table 5. Antibacterial activity (*R*) and efficiency (*AE*) of samples containing nisin against Gram-positive bacterial strains after 9 months of storage

Sample (concentration of nisin)	***Staphylococcus aureus***			***Listeria monocytogenes***		
	CFU·cm^{-2}	R	AE (%)	CFU·cm^{-2}	R	AE (%)
0.15 wt. % nisin (i.e. 60 000 IU·g^{-1})						
PLA	6.3E+05	-	-	1.5E+06	-	-
PLA/nisin	<4	5.3	100	<1	6.2	100
PLA/PEG1000/nisin	0	5.8	100	0	6.2	100
PLA/PEG6000/nisin	0	5.8	100	0	6.2	100

Thus, the prepared PLA/PEG/nisin blends can be conveniently stored at room temperature for a long period of time without any adverse effect impacting on nisin activity. Consequently, they show potential for antimicrobial application in food packaging or biomedicine.

3.2 Thermal stability of bacteriocin nisin in polylactide-based films

The primary aim of this work was to evaluate the thermal stability of bacteriocin nisin in PLA and PLA/PEG blend films. The nisin-incorporated PLA and PLA/PEG films that were cast underwent treatment at various temperatures (90–180°C) for up to 48 hours.

The results show that the PLA-based films displayed antimicrobial properties even when they were thermally treated above the melting temperature of PLA. However, antibacterial activity was reduced by about 25% under treatment at 160°C for 5 min and by more than 60% under treatment at 180°C for 5 min (Fig. 14).

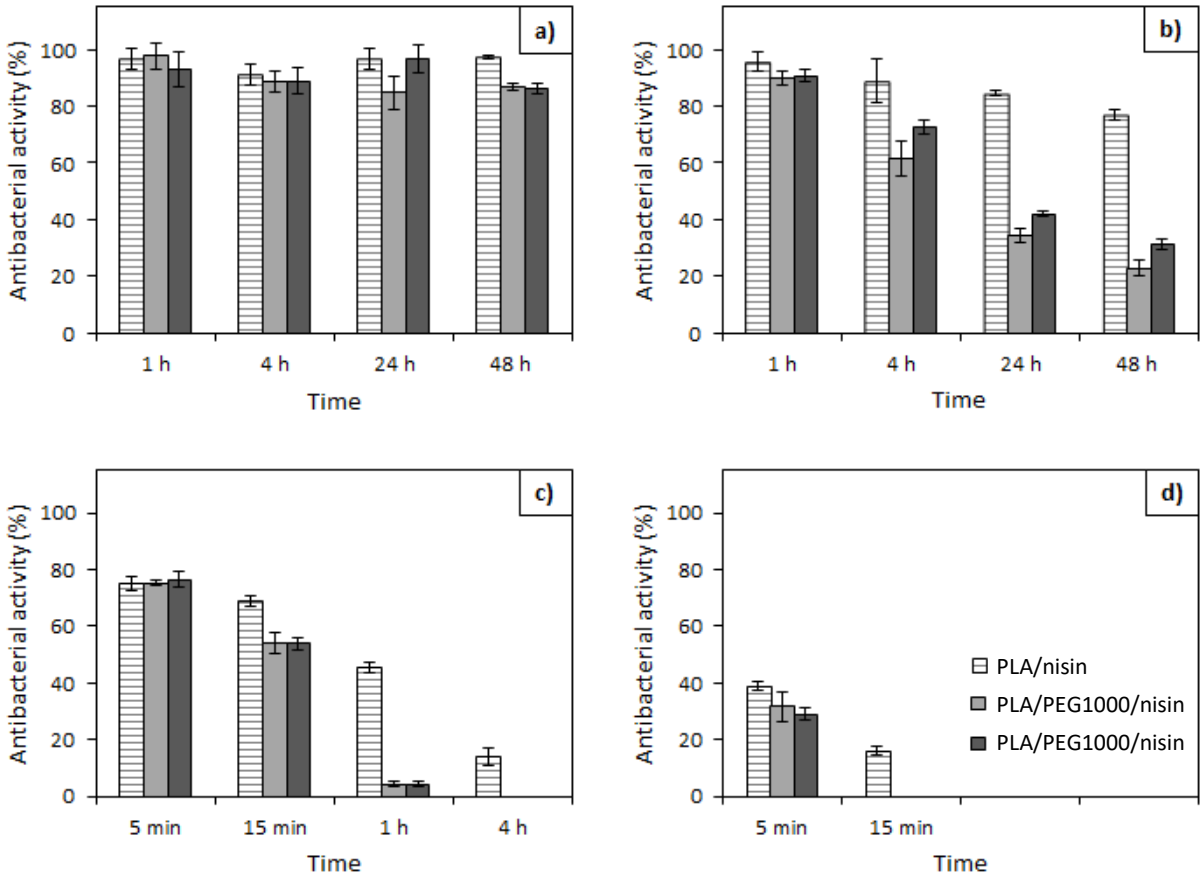

Figure 14. Antibacterial activity of PLA/nisin and PLA/PEG/nisin blends treated at a) 90°C, b) 120°C, c) 160°C, and d) 180°C for different durations; antibacterial activity of thermally untreated samples is taken as 100%.

These results reveal that nisin incorporated in polymer matrix can maintain its antibacterial activity at temperatures relevant to processing window of plastics used for packaging and/or medical devices production.

One of the objectives of the present study was to confirm the presence of the nisin molecule in the thermally treated samples. For this purpose, the HPLC-QTOFMS method was optimised and validated. In order to detect the target

analyte, the $[M+5H]^{5+}$ (m/z 671.3163) molecule ion was monitored. The limit of detection (LOD) was 50 $ng \cdot mL^{-1}$. Employing the HPLC-QTOFMS method, nisin was determined in extracts of the PLA/nisin blend films. The levels of free nisin in the extracts of PEG-incorporated samples were under the LOD for the method used (the vast proportion of the nisin probably occurred in the form of nisin-PEG conjugates).

The effect of high temperature on structural changes in the polymer matrix was investigated using GPC and SEM. It was found that no significant effect exerted by the content of nisin was noticeable on the PLA matrix (Fig. 15).

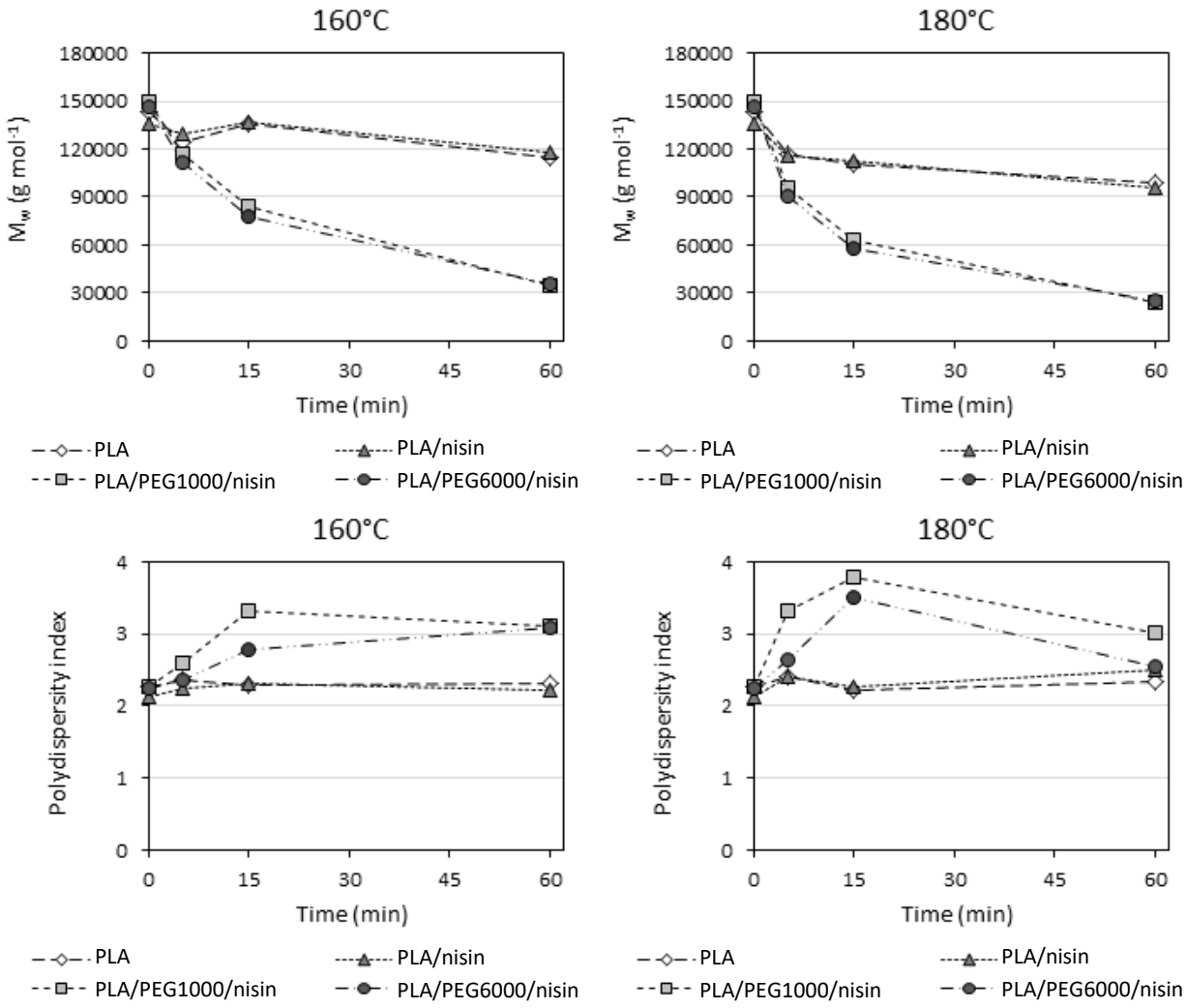

Figure 15. GPC data on PLA-based blend samples during thermal treatment exceeding the T_m of PLA.

On the other hand, despite the fact that PEG improves the stability of nisin in long-term storage, and can effectively modify mechanical properties of PLA, the adverse effect of PEG on the stability of nisin as well as the polymeric matrix treated above 120°C was evident.

3.3 Core-shell PLA-PVA porous microparticles as carriers for bacteriocin nisin

In this study, microparticles made from PLA shell and cross-linked PVA core were prepared for encapsulation and controlled release of the model peptide – nisin. The microparticles were synthesised via solvent evaporation technique with some modifications. PVA was modified by chemical cross-linking reaction using GA as the cross-linking agent. FTIR-ATR analysis confirmed the formation of an ester bond between the two functional groups.

It was found that the particle morphology, zeta potential and encapsulation efficiency were influenced by GA concentration and the nisin content. With the increased nisin concentration, the particle size raised (9–16 μm). On the other hand, the decreasing encapsulation efficiency value with the increasing nisin content arose probably due to the saturation of nisin into microparticles. SEM analysis showed the spherical shape and micro dimensions of the particles (Fig. 16).

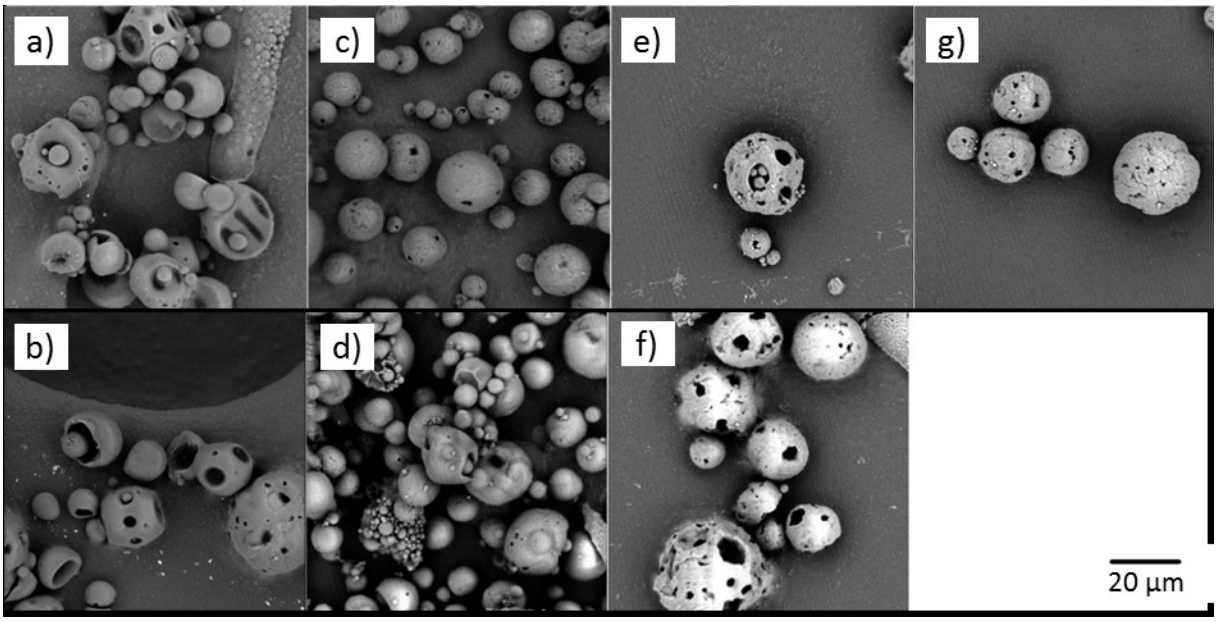

Figure 16. SEM analysis of a) PLA/PVA/1.5 NIS, b) PLA/PVA/GA/1.5 NIS, c) PLA/PVA/3.0 NIS, d) PLA/PVA/GA/3.0 NIS, e) PLA/PVA/5.0 NIS, f) PLA/PVA/GA/5.0 NIS, and g) PLA/PVA microparticles.

Futhermore, BET surface analysis reveal that the cross-linked systems exhibit lower values of pore diameter, total pore volume and surface area in comparison with the non-cross-linked ones. This behaviour could be connected with the limited water molecules absorption into the cross-linked PVA during the microparticles formation.

In vitro release study of nisin from PLA/PVA microparticles exhibited gradual release depending on the initial nisin concentration (Fig. 17). Due to the

presence of larger pores in the non-cross-linked formulations, a larger amount of aqueous medium is likely to come in contact with the particles and facilitate the release of the nisin from the microparticles.

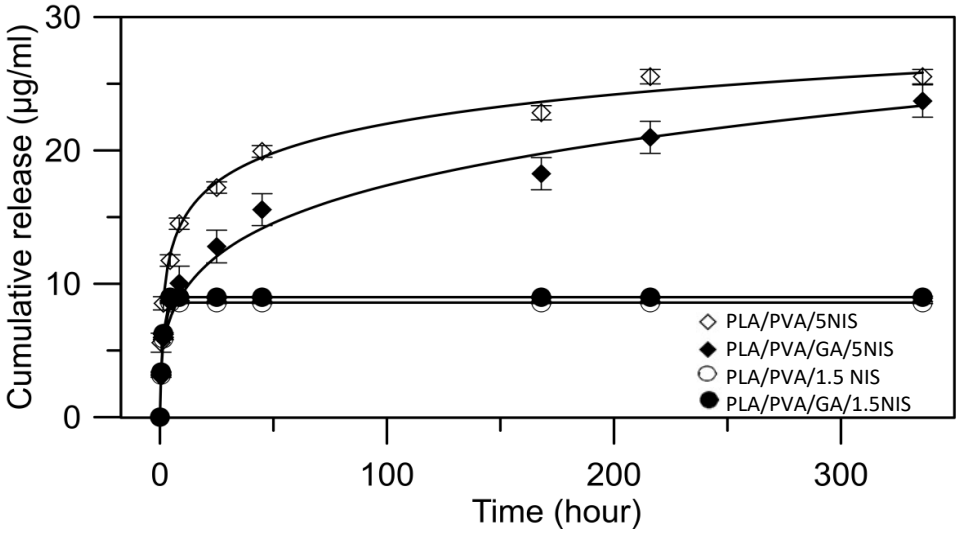

Figure 17. Cumulative release of nisin from microparticles.

Antibacterial activity (*AA*) of prepared systems was tested by dilution and spread plate technique. With the increasing nisin concentration, also *AA* increased in the microparticles (Tab. 6). Exception occurred in the case of PLA/PVA/GA/5.0 NIS, where *AA* decreased likely because of the gradual nisin release during the first 24 h from cross-linked microparticles with low mean pore diameter and total pore volume. In addition, antibacterial testing revealed synergistic effect of nisin and GA against Gram-positive *Listeria monocytogenes* growth.

Table 6. Antibacterial activity (*AA*) of microparticles containing various amounts of nisin and glutaric acid

Sample	**AA (%)**
PLA/PVA	0.0
PLA/PVA/1.5 NIS	42.0
PLA/PVA/GA/1.5 NIS	76.1
PLA/PVA/3.0 NIS	59.4
PLA/PVA/GA/3.0 NIS	79.1
PLA/PVA/5.0 NIS	64.8
PLA/PVA/GA/5.0 NIS	42.6

CONCLUSIONS

The development and use of biodegradable polymers have been significantly growing in the last years, especially in the environmental and medical fields. Current demand for bio-based and biodegradable plastics makes polylactide (PLA) one of the most promising materials for providing interesting alternatives to conventional petrol-based plastics. Besides, enhancement of PLA with antibacterial activity can further increase its applicability in the market.

In alignment with new trends, the use of naturally derived antimicrobial compounds obtained by fermentation of whey (e.g. bacteriocins) is very advantageous, particularly in food packaging, cosmetics and biomedical applications.

According to the current state of knowledge, the overview of this issue was drawn in the theoretical part, and research aims of work were defined. On the basis of this, the experimental part of work was devoted to the preparation of novel biodegradable PLA-based polymer systems modified with bacteriocin nisin in the form of blend films as well as microparticles.

In the first part, PLA and PLA/PEG blend films with nisin were prepared and characterised. The results showed that the incompatibility of the polymer matrices caused the formation of a porous structure, which aids the incorporation of nisin and positively affects its diffusion from the prepared blends. The presence of PEG, besides its plasticising effect, significantly enhanced the release profile and sustained the long-term antibacterial activity of nisin in a PLA matrix. Thus, the prepared blends can be conveniently stored for a long period without any adverse effect impacting on nisin activity. Consequently, the prepared PLA/PEG/nisin blend films show potential for antimicrobial application in food packaging or biomedicine.

The second part was focused on the evaluation of thermal stability on bacteriocin nisin in PLA and PLA/PEG blends at temperatures of 90°C to 180°C. The results showed that nisin retained almost 70% of its antibacterial activity in the PLA matrix, even after treatment at 160°C for 15 minutes. Moreover, it was found that the presence of PEG significantly enhanced the degradation of nisin above 120°C.

The last part of work was dealing with preparation of microparticles based on PLA and PVA cross-linked with glutaric acid. Results showed the microparticles in size range of 9–16 μm in diameter with spherical multi-hollow core-shell structure. The presence of cross-linking agent influenced the release profile of nisin and showed the synergistic effect on *L. monocytogenes* growth reduction. The prepared systems provide the effective encapsulation of nisin allowing its the controlled release into the environment and showing potential for application in biomedicine or agriculture.

CONTRIBUTIONS TO SCIENCE AND PRACTICE

The presented doctoral thesis brings novel approaches to modification of biopolymers in order to achieve a biodegradable material with antibacterial properties, which is the current topic in both academic and industrial research.

Moreover, this work is dealing with the use of antimicrobial substances which can be produced from whey (a dairy waste product). This fact is very promising, particularly regarding the application in practice.

The main contribution to the science of the reported work can be found in the preparation and characterisation of novel biodegradable PLA-based systems modified with bacteriocin nisin in the form of:

- blend films with potential for antimicrobial applications in food packaging or biomedicine,
- core-shell porous microparticles providing controlled release with potential in biomedicine or agriculture.

Furthermore, new highly sensitive methods for nisin detection regarding chromatographic techniques (e.g. high performance liquid chromatography with mass detection – LC-QTOFMS) have been optimised and can be further successfully used within the Centre of Polymer Systems infrastructure.

The results reported in this thesis were presented at several national and international conferences and were (or will be) published in international journals with impact factor.

Besides, other outputs of applied research have been achieved, such as adoption of a utility model (concerning nisin-containing stabilised polymeric composition based on PEG as the matrix with high practical applicability) and preparation of functional samples for use in cosmetics (in the form of hydrogel and an ointment).

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LIST OF FIGURES

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LIST OF SYMBOLS AND ABBREVIATIONS

AA	Antibacterial activity
Abu	Aminobutyric acid
AE	Antibacterial efficiency
ASTM	American society for testing and materials
ATR	Attenuated total reflection
CFU	Colony-forming unit
Dha	2,3-didehydroalanine
Dhb	2,3-didehydrobutyrine
DMA	Dynamic mechanical analysis
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EVA	Ethylene vinyl acetate
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
GA	Glutaric acid
GPC	Gel permeation chromatography
GRAS	Generally recognized as safe
GTA	Glycerol triacetate
HPLC	High-performance liquid chromatography
HPLC-QTOFMS	High-performance liquid chromatography quadrupole time of flight mass spectrometry

ISO	International organization for standardization
IU	International unit
KCl	Potassium chloride
LA	Lactic acid
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography-mass spectrometry
LMW	Low-molecular-weight
MIC	Minimum inhibitory concentration
NaCl	Sodium chloride
NIS	Nisin
PBAT	Polybutylene adipate-co-terephthalate
PBS	Polybutylene succinate
PBSA	Polybutylene succinate-co-adipate
PCL	Polycaprolactone
PE	Polyethylene
PEG	Polyethelene glycol
PET	Polyethylene terephthalate
PGA	Polyglycolic acid
pH	Potential of hydrogen
PHA	Polyhydroxy-alkanoates
PHB	Polyhydroxy butyrate
PHBV	Polyhydroxybutyrate-co-hydroxyvalerate
PHV	Polyhydroxyvalerate
pK_a	Acid dissociation constant
PLA	Polylactic acid, Polylactide
PLGA	Polylactic-co-glycolic acid
PP	Polypropylene glycol
ppm	Parts per million
PS	Polystyrene
PVA	Polyvinyl alcohol
PVAc	Polyvinyl acetate
PVC	Polyvinyl chloride
R	Antimicrobial activity (according to ISO 22196)
SEM	Scanning electron microscopy
T_g	Glass transition temperature
T_m	Melting temperature
WHO	World Health Organisation

CURRICULUM VITAE

Personal information

Ing. Pavlína Holčapková

Zabrání 250, 763 02, Zlín 4, Czech Republic

holcapkova@utb.cz

Date of birth 17/12/1989 ‖ *Nationality* Czech

Education

2014–to date Doctoral degree studies

Tomas Bata University in Zlín, Faculty of Technology, Zlín
Technology of Macromolecular Compounds

2012–2014 Master´s degree

Tomas Bata University in Zlín, Faculty of Technology, Zlín
Technology and Economics of Fat, Detergent and Cosmetics Production

2009–2012 Bachelor´s degree

Tomas Bata University in Zlín, Faculty of Technology, Zlín
Technology of Fat, Detergent and Cosmetics Production

2005–2009 High school with graduation

Secondary Health School and Higher Medical School, Zlín
Medical Lyceum

Training abroad

16/04/2018–16/07/2018 Erasmus traineeship

AIMPLAS, Plastics technology centre
Valencia (Spain)

Characterization of novel additives for tailoring of polylactide properties

01/10/2016–01/11/2016 Erasmus traineeship

WOOD K PLUS, Kompetenzzentrum Holz GmbH
Linz (Austria)

Preparation and characterization of biocomposites based on biodegradable polylactic acid (PLA) and natural fibres

Work experience on projects within Centre of Polymer Systems (CPS)

10/2014–to date	Member of research team Project TE02000006 – Centre for alternative environment friendly high effective polymer antimicrobial agents for industrial applications (2014-2019, TA0/TE)
05/2015–10/2017	Member of research team Project QJ1310254 – Research into the use of whey as dairy industry waste product, the production of antimicrobial compounds for the modification of hydrophilic polymer systems with the use in cosmetic and medical applications (2013-2017, MZE/QJ)

Work on projects within Internal Grant Agency (IGA)

01/2018–08/2018	Member of research team Project IGA/CPS/2018/003 – Functionalized polymer composites Tomas Bata University in Zlín, Centre of Polymer Systems
01/2017–12/2017	Member of research team/Principal investigator Project IGA/CPS/2017/005 – Bioactive systems for medical and industrial applications Tomas Bata University in Zlín, Centre of Polymer Systems
01/2016–12/2016	Principal investigator Project IGA/CPS/2016/004 – Preparation and characterization of bioactive polymer systems Tomas Bata University in Zlín, Centre of Polymer Systems
03/2015–12/2015	Principal investigator Project IGA/CPS/2015/003 – Development of advanced biodegradable and antimicrobial polymer systems Tomas Bata University in Zlín, Centre of Polymer Systems
09/2014–12/2014	Member of research team Project IGA/FT/2014/012 – Study on biodegradable and antimicrobial polymer systems Tomas Bata University in Zlín, Faculty of Technology

LIST OF PUBLICATIONS

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1. HOLCAPKOVA, Pavlina; HURAJOVA, Anna; BAZANT, Pavel; SEDLARIK, Vladimir. Thermal Stability of bacteriocin nisin in polylactide-based films. Submitted to *Polymer Degradation and Stability*, PDST-D-18-00041, 8th January, 2018.

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2. HOLCAPKOVA, Pavlina; VESELA, Daniela; SEDLARIK, Vladimir; PANTUCEK, Jiri. Hydrogel stabilized by bacteriocin for cosmetic applications, functional sample, ID 43876766, 2017.

3. HOLCAPKOVA, Pavlina; VESELA, Daniela; SEDLARIK, Vladimir; PANTUCEK, Jiri. Ointment containing nisin-PEG conjugate, functional sample, ID 43876765, 2017.

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2. HOLČAPKOVÁ, Pavlína. Interaction of polymers with surfactants (bachelor thesis), Tomas Bata University in Zlín, Faculty of Technology, Zlín, 2012.

Pavlína Holčapková

Utilisation of whey fermentation products for antimicrobial modification of biodegradable polymers

Využití fermentačních produktů syrovátky pro antimikrobiální modifikace biorozložitelných polymerů

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