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Review

BACTERIOCINS PRODUCED BY LACTIC ACID BACTERIA – A REVIEW

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Lactic acid bacteria (LAB) have an essential role in the production of fermented products. With their metabolic activity, they influence the ripening processes - leading to desired sensory qualities while at the same time inhibiting the growth of undesired microorganisms. Because of their dominant role during fermentation and because of a long tradition of utilization, LAB have been designated as "safe microbiota". Biological protection of LAB, as a naturally present and/or selected and intentionally added microflora, is realized through the production of non-specific (lactic acid, acetic acid and other volatile organic acids, hydrogen peroxide, diacetyl, etc) and specific metabolites, bacteriocins.

Bacteriocins are extracellularly released proteins or peptides which possess certain antibacterial activity towards certain types of microorganisms, usually related to the producing bacteria. Today, bacteriocins represent a very interesting potential for their application in the food industry. Their application can reduce the use of synthetic preservatives and/or the intensity of thermal treatment during food production consumer's need for safe, fresh and minimally-processed food. With the intention of realizing this potential to the fullest, it is necessary to understand the nature of bacteriocins, their production mechanisms, regulations and actions, as well as the influence of external factors on the their antimicrobial activity. The composition of food, i.e. its characteristics (pH, temperature, ingredients and additives, types and quantities of epiphytic microbiota) and the actual technological process used in production, can all influence the stability and activity of the added bacteriocins.

The future research in this field should also aim to clarify this unknown aspect of the application of bacteriocins, to provide the necessary knowledge about the optimization of the external conditions and open up the possibility of discovering their new producers.

KEY WORDS: bacteriocins, lactic acid bacteria, natural preservation, food

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LACTIC ACID BACTERIA AS A "SAFE MICROBIOTA"

Modern methods of food production and processing are based on the application of different forms of protective technologies which aim to assure and protect both the safety of the products and the acceptable and unvaried quality from the moment of production to the moment of consumption. This concept has been the subject of many discussions, not only in developing countries (where the development and use of these technologies is necessary), but also in the industrialized world. The biggest challenge in the food industry right now is the effort to: a) reduce economic losses caused by food spoilage, b) reduce the price of the food production process, c) reduce the possibility of pathogen transfer, and d) satisfy the growing consumer need for ready-to-use food that tastes fresh, has a high nutritional and vitamin value, and has been minimally processed and treated with preservatives (1).

Fermentation is one of the oldest food preservation technologies. The empirical application of microorganisms and/or their natural metabolic products dates back to the early history of human kind, in the Neolithic period, 10,000 years BC (2, 3). The principle of biological preservation, i.e. the reduction of consumer health risks, is based primarily on the action of certain microorganisms and their metabolic products towards the unwanted spoilage bacteria or the food poisoning bacteria, but without changing the quality of the product. Today, the great global market of food and beverages features a wide array of commercial fermented products (over 5,000 of them), in which certain microorganisms play a role in the production and sustainability (4).

It is well known that the wine, beer and alcohol industries would be impossible without certain yeasts. Similarly, lactic acid bacteria (LAB) play an essential role in a great number of products whose production is based on lactic fermentation (milk, meat and vegetable industries) (Table 1).

LAB produce a series of antimicrobial substances (Table 2), such as weak organic acids, diacetyl, acetone, hydrogen peroxide, reuterin, reutericyclin, antifungal peptides and bacteriocins (5-7). The role and the mechanism of action in the majority of final metabolic products of LAB has been described long ago and has its own place in natural food protection (8), while the role of the bacteriocins from LAB is still a subject of intense discussion (9).

LAB BACTERIOCINS AND THEIR APPLICATION IN FOOD PRODUCTION

Bacteriocins are extracellularly released proteins or peptide molecules which show certain antibacterial properties towards certain species of microorganisms, usually congenial with the producing bacteria (12), i.e. they belong to similar ecological niches.

LAB bacteriocins are natural antimicrobial peptides or proteins with very interesting potential applications in the food industry as bioprotectors (15), preserving people's health (16) while enhancing the sustainability of food (17). Hurst (18) described bacteriocins as "biological food preservatives" and that term has been generally accepted.

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Table 1. Fermented foods and beverages and their associated lactic acid bacteria

Type of fermented product	Lactic acid bacteria
Dairy products	
- Hard cheeses without eyes	*Lactococcus lactis* ssp. *lactis, L. lactis* ssp. *cremoris;*
- Cheeses with small eyes	*L. lactis* ssp. *lactis, L. lactis* ssp. *lactis* var. *diacetylactis, L. lactis* ssp. *cremoris, Leuconostoc mesenteroides* ssp. *cremoris;*
- Swiss- and Italian-type cheeses	*Lactobacillus delbrueckii* ssp. *lactis, Lb. helveticus, Lb. casei, Lb. delbrueckii* ssp. *bulgaricus, Streptococcus thermophilus;*
- Butter and buttermilk	*L. lactis* ssp. *lactis, L. lactis* ssp. *lactis* var. *diacetylactis, L. lactis* ssp. *cremoris, Ln. mesenteroides* ssp. *cremoris;*
- Yoghurt	*Lb. delbrueckii* ssp. *bulgaricus, S. thermophilus;*
- Fermented probiotic milk	*Lb. casei, Lb. acidophilus, Lb. rhamnosus, Lb. johnsonii, Bifidobacterium lactis, B. bifidum, B. breve;*
- Kefir	*Lb. kefir, Lb. kefiranofacies, Lb. brevis;*
Fermented meats	
- Fermented sausage (Europe)	*Lb. sakei, Lb. curvatus;*
- Fermented sausage (USA)	*Pediococcus acidilactici, P. pentosaceus;*
Fermented fish products	*Lb. alimentarius, Carnobacterium piscicola;*
Fermented vegetables	
- Sauerkraut	*Ln. mesenteroides, Lb. plantarum, P. acidilactici;*
Pickles	*Ln. mesenteroides, P. cerevisiae, Lb. brevis, Lb.plantarum;*
- Fermented olives	*Ln. mesenteroides, Lb. pentosus, Lb. plantarum;*
- Fermented vegetables	*P. acidilactici, P. pentosaceus, Lb. plantarum, Lb. fermenium;*
Soy sauce	*Tegragenococcus halophilus;*
Fermented cereals	
- Sourdough	*Lb. sanfransiscensis, Lb. farciminis, Lb. fermentum, Lb. brevis, Lb. plantarum, Lb. amylovorus, Lb. reuteri, Lb. pontis, Lb. panis, Lb. alimentarius, Weissella cibaria;*
Alcoholic beverages	
- Wine (malolactic fermentation)	*Oenococcus oeni*
- Rice wine	*Lb. sakei*

L. = *Lactococcus*, Lb. = *Lactobacillus*, Ln. = *Leuconostoc*, S. = *Streptococcus*, B. = *Bifidobacterium*, P. = *Pediococcus*

Table 2. Metabolic products of LAB with antimicrobial effect (10, 11)

LAB product	Main (target) microorganisms
Organic acids	
Lactic acid	Spoilage bacteria and Gram-negative bacteria, certain fungi
Acetic acid	Spoilage bacteria, clostridia, certain yeasts and molds
Hydrogen peroxide (H_2O_2)	Pathogenic and spoilage bacteria
Enzymes	
Lactoperoxidase system with H_2O_2	Pathogenic and spoilage bacteria (milk and dairy)
Lysozymes (tech. recombinations of DNA)	Undesired Gram-positive bacteria
Low molecular weight metabolites	
Reuterin (3-OH-propionaldehyde)	A wide spectrum of bacteria, molds and yeasts
Diacetyl	Gram-negative bacteria
Fatty acids	Various bacteria
Bacteriocins	
Nisin	Some lactic acid- and Gram-positive bacteria, especially the endospore-forming ones
Other bacteriocins	Gram-positive bacteria, inhibitory spectrum

These bacteriocins are ribosome-synthesized polypeptides that have bactericidal activity and are rapidly digested by proteinases from the human digestive tract (19). They are often compared with antibiotics because of their expressed antibacterial properties (18, 20). However, in contrast to therapeutic antibiotics, their application as a rule does not imply the possibility of undesired allergic reactions occurring in humans (15). As the discovery of penicillin by Alexander Fleming in the year 1929 has direct importance for the human race, so the discovery of bacteriocins, in an indirect sense, is important in terms of natural health care and food safety. The comparative view of the synthesis, activity, antimicrobial spectrum, toxicity and resistance of antibiotics and „natural antibiotic substances“– bacteriocins, are presented in Table 3.

Table 3. Bacteriocins and antibiotics – similarities and differences (15)

Characteristic	Bacteriocins	Antibiotics
Application	Food	Clinical
Synthesis	Ribosomal	Secondary metabolite
Activity	Narrow spectrum	Varying spectrum
Host cell immunity	Yes	No
Mechanism of target cell resistance or tolerance	Usually, adaptation affecting cell membrane composition	Usually, a genetically transferable determinant affecting different sites depending the mode of action
Interaction requirements	Sometimes docking molecules	Specific target
Mode of action	Mostly, pore formation, but in a few cases possible cell wall biosynthesis	Cell membrane or intracellular targets
Toxicity/side effects	None known	Yes

The findings regarding the production of bacteriocins represent an important moment in the biological protection of food, considering that their application allows bactericidal and bacteriostatic effect towards certain harmful microorganisms, which belong to the same ecological niches or use the same energy source. This protective role of bacteriocins is supported by the fact that most of them are closely related to the host, i.e. to the producer cell, and that they are much more efficient against the bacteria with which they compete for the same deficitary nutrients.

Although bacteriocins are produced by many Gram-positive and Gram-negative bacteria, those produced by the LAB are the most interesting ones for the food industry. Besides, most of the LAB that produce bacteriocins are natural isolates, which makes them ideal for use in the food industry. Consumers today make very specific demands of the food industry. First of all, there is the need to consume products that have not been through extensive preservation processes and that do not contain too many chemical preservatives. That is why the possibility of using bacteriocins as natural food protectors is considered to be very important.

Bacteriocins are peptide or protein molecules synthesized on ribosomes of the producing bacteria that show antimicrobial properties; especially on Gram-positive microorganisms (21), where the producing cells retain immunity towards its own bacteriocins (12).

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LAB produce different bacteriocins, which can be classified in one of the classes proposed by Klaenhammer in 1993. Today, most of the LAB bacteriocins that find their use in food preservation processes, belong to the classes Ia, II and IV (Table 4).

Table 4. Classification of bacteriocins (22)

Class	**General characteristics**	**Bacteriocins from LAB**
I - Lantibiotics	***Modified, heat stable, <15 kDa***	
Ia - Linear	Pore forming, cationic	Nisin, Lacticin 481, Plantaricin C
Ib - Globular	Enzyme inhibitors, no cationic	None
Ic - Multi-component	Two peptides	Lct3147, Plantaricin W
II – Unmodified peptides	***Heat stable, <15 kDa***	
IIa - Pediocin-like	Anti-listeria, YGNGVconsensus	Pediocin PA1/AcH, Enterocin A, Sakacin A
IIb - Miscellaneous	Non-pediocin-like	Enterocin B, L50, Carnobacteriocin A
Ic - Multi-component	Two peptides	Lactococcin G, Plantaricin S, Lactacin F
III - Large proteins	***Heat labile, >30 kDa***	
IIIa - Bacteriolytic	Cell wall degradation	Enterolysin A, Lcn972
IIIb - Non-lytic	Cytosolic targets	Colicin E2-E9
IV – Circular peptides	***Heat stable, tail-head peptide bond***	AS-48, Gassericin A, Acidocin B

The production of bacteriocins represents the cell's natural advantage, considering that the synthesized peptides/proteins can destroy or inhibit the growth of the other bacteria that compete for the same ecological niches or the same nutrients. This role is supported by the fact that many bacteriocins have a strict relationship with the host cell and that they are much more efficient against the bacteria that compete for the same deficitary nutrients. The statement that „bacteriocins can be considered as a rudimentary form of the food's natural immunity" (21) is quite interesting. The antibacterial spectrum of action often includes spoilage microorganisms and pathogenic bacteria from food, like *Listeria monocytogenes* and *Staphylococcus aureus.* The activity against Gram-negative bacteria, like *Escherichia coli* and *Salmonella* is also noted, usually if the integrity of their outer membrane is breached (osmotic shock, treating the cell with low pH or high pressure, presence of detergents or cleaners, passage of cells through pulsating electric field) (23, 24).

Therefore, the production of bacteriocins by the LAB is an advantage not only for the bacteria, but it can also be used in the food industry as a means to control unwanted microorganisms in food in a natural way, which is in the end, the basic demand of the consumers (25, 2).

Today, the most studied, and at the same time, most used bacteriocin is nisin, isolated from *Lactococcus lactis* spp. *lactis.* It belongs to the class I of bacteriocins (lantibiotics) and is composed of 34 amino acid peptides (26). It was discovered back in 1928 when it was found that a certain matter synthesized from *Streptococus lactis* (today known as *Lc. lactis* spp. *lactis)* inhibits the growth of *Lactobacillus bulgaricus* (27, 28). Not long after, researchers in food-related fields realized that nisin presents great potential in the food safety. It first appeared on the market in England in 1953, and its intensive application started in 1957, in the production of cheese (29). It was found that nisin possesses a strong antibacterial action against Gram-positive bacteria (including *L. monocytogenes, S. aureus,* the bacteria of the *Mycobacterium* genus), but that it also inhibits the creation of spores in some bacteria (*Bacillus* and *Clostridium*) (18). The mechanism of the inhibitory action of nisin on the spore-creating process is not completely explained, but it is believed that it is a result of the action towards the sulfhydride groups of protein molecules (30). The character of the activity shown was more sporostatic than sporocidal.

Today, nisin is used in 48 countries across the globe, as it was registered back in 1948 as an approved food additive (E-234) by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO), Expert Committee on Food Additives (31). It is, at least for now, the only bacteriocin with the GRAS status (*Generally Regarded As Safe*), and the only one that can be freely used as an additive in the food production. It is predominantly used in the production of preserved foods and dairy products. It is particularly efficient in cheese and cheese spread production as a natural antimicrobial factor against thermo-resistant sporogenous bacteria. Nisin is particularly important in the prevention of *Clostridium botulinum* that can lead to drastic health risks due to toxin production.

There are also several other isolated bacteriocins that have been adapted for comerrcial use and described in the scientific literature, such as pediocin PA-1/AcH, lacticin 3147, lacticin 481, enterocin AS-48, variocin, etc. The aforementioned preliminary studies on the activity of bacteriocins *in vitro* or in food systems, have been conducted using partially purified bacteriocin preparations obtained from liquid suspensions of bacterial cultures. During the experiments, the researchers often increase the concentration of additional bacteriocins, which greatly limits the objective interpretation of the results, and therefore also the objectivity of the preliminary tests.

Bacteriocins can be added to food as additives, in which case their synthesis has already been performed *ex situ*, or in the form of "concentrates" of bacteriocin-producing strains cultivated in adequate and favorable substrates (the synthesis of bacteriocins is done *in situ*), or, again, by adding a product that has previously been fermented by a bacteriocin-producing strain (32). The in situ production of bacteriocins has shown several advantages compared to *ex situ* production, seen both from a legal and commercial point of view.

The bacteriocins produced *ex situ* can be applied in the form of immobilized preparations, where a partially purified bacteriocin is bound to a carrier. The carrier acts as a fuel tank and as a diffuser of concentrated bacteriocin molecules, and as such, it provides their continuous supplementation into the food. The carrier also protects the bacteriocin from the negative effects of certain food components and from potential enzyme inactivation. A great number of methods for bacteriocin immobilization have been proposed, including the absorption on the producing cells (33), on the particles of silicon dioxide, on corn starch powder (34), liposomal encapsulation (35), or by building them into gels and films from different materials, such as calcium alginate, gelatin, cellulose, soy protein, corn starch, collagen, cellophane, nylon and other plastic polymer films (36).

In most cases, the immobilized bacteriocins have an important function in terms of the reduction of post-process food contamination, especially in superficial multiplication of undesired bacteria. Antimicrobial packages, such as polyethylene film sheets, which contain bacteriocin isolated from *Lb. curvatus* (32Y), reduce the number of live cells of

L. monocytogenes during storage of packaged pork chops, raw beef and hotdogs (37). Similarly, the cellophane wrap with incorporated nisin reduces the total number of live aerobic cells in beef during storage at 8°C (38), and the number of *Micrococcus luteus* ATCC 10240 cells in raw and pasteurized milk during storage (39).

Reducing the costs of bio-preservation is particularly important for developing countries where, very often, food safety is extremely compromised (40). Therefore, a lot of research today is focused on the selection and development of bacteriocin-producing cultures for application in the food industry (2, 41). The appropriate application of LAB must be based on the careful selection of well-adapted strains that can grow uninhibited in certain kinds of food, their application in those foods must be approved, i.e. it must not compromise or change its nature, they must be able to grow during processing and during food storage, and they must possess the ability to produce enough bacteriocins to completely inhibit pathogenic or spoilage microorganisms. The qualities of the strain and the quantities of the bacteriocins produced can be improved through the heterologous expression of bacteriocin genes (42) and the exact moment of production of bacteriocins can also be altered by using certain inducible factors during the production (43).

In the Serbian meat industry, research was performed in order to eventually introduce the application of semi-purified bacteriocins isolated from *Leuconostoc mesenteroides* E 131 (44, 45), *Ln. mesenteroides* IMAU:10231 (46-48). Since technological application of the bacteria of the *Leuconostoc* strain in the meat industry is limited due to their physiological properties (they produce mucus, i.e. exopolysaccharides, acetoin, diacetate, ethanol, etc), which are not acceptable in this industry from the point of view of quality, the direct application of their isolated and purified bacteriocins was a good solution. Unlike them, the bacteriocin-producing strains of *Lb. sakei* can be added to the meat filling in the form of liquid culture suspensions (49). In addition to protective cultures, the authors have inoculated into the filling for national fermented sausages (Croatian, Bosnian, Serbian, Greek, Hungarian) the pathogenic *L. monocytogenes* as well (50). During the ripening process, which lasted 28 days, the reduction of this pathogen was observed. The investigation showed a slight antilisterial advantage of *Lb. sakei* I 151, compared to other two strains that were applied.

ENVIRONMENTAL FACTORS AND BACTERIOCIN EFFICIENCY IN FOOD SYSTEMS

Food represents a complex ecosystem whose microbiological profile is affected by the type of food, the type of thermal treatment applied during the processing, the type of processing, storage conditions, and others. Because of this, the differences in the microbiological population can be quite large, depending on whether the food is commercially sterile, raw or fermented (51).

On the other hand, the efficiency of bacteriocins added to the food, in addition to the characteristics that come from their own structure and nature, also depends on various external environment factors. In most cases, the action of these factors is limiting and inhibitory in terms of the intensity of the antimicrobial effect and it is based on the quality of the food itself, on the existence of interactions between bacteriocins and the food components, potential processes of precipitation, inactivation or unbalanced diffusion of bacteriocins in the food matrix (Table 5). In other words, the properties of the food system, for example, its structure, its buffer capacity, composition (nutrients, additives, antibiotics), processing conditions (freezing, cooling, exposure to high pressure and temperature, homogenization, etc), pH and others, indirectly damage the producing cell or lead to reduced bacteriocin synthesis (1). The quantity and the type of microorganisms or potential contaminants present in the food have a direct effect on the activity of the added bacteriocins. At the same time, it takes more bacteriocins to inactivate more microorganism cells, and vice versa. In addition, microbiological interactions can have a great impact on the microbiological balance and/or multiplication of useful or harmful bacterial strains.

Table 5. Bacteriocin efficacy in foods: limiting factors (1)

Food-related factor:
– *Food processing conditions;* – *Food storage temperature;* – *Food pH, and bacteriocin unstability to pH changes;* – *Inactivation by food enzymes;* – *Interaction with food additives/ingredients;* – *Bacteriocin adsorption to food components;* – *Low solubility and uneven distribution in the food matrix;* – *Limited stability of bacteriocin during the food shelf life.*
The food microbiota:
– *Microbial load;* – *Microbial diversity;* – *Bacteriocin sensitivity;* – *Microbial interactions in the food system.*
The target bacteria:
– *Microbial load;* – *Bacteriocin sensitivity (Gram-type, genus, species, strains);* – *Physiological stage (growing, resting, starving or viable but non-culturable cells,* – *stressed or sub-lethally injured cells, endospores...);* – *Protection by physico-chemical barriers (microcolonies, biofilms, slime);* – *Development of resistance/adaptation.*

CONCLUSIONS AND FUTURE CONSIDERATIONS

Civilization has been using the benefits of bacteriocins for thousands of years now. However, the only bacteriocin that has found its use today in the food industry as a food preservative and that has an official, legal approval in countries around the world is nisin. After realizing that there are other efficient bacteriocins besides nisin that can be used successfully in different types of foods, one can only wonder why their application in the modern food industry, as of today, is lacking. The primary reasons for this may include difficulties related to different national legislatures and the presence of financial problems in the field of development, formulation and the placement of the commercial preparations of new bacteriocins on the global food market. In other words, those difficulties refer primarily to the implementation of bacteriocins on the market, while the scientific proofs of justifiability and benefits of their application are well procured. The efficiency of bacteriocins that has been shown, as well as the economic and not overly demanding way of incorporating them into food products, represents a great alternative in combination with other natural preservatives. If the aforementioned problems are disregarded, we are left with a wide spectrum of significant reasons for their application in the food industry. On the other hand, it would be too naive to believe that bacteriocins can represent the only and final solution for the current problems in the field of food safety.

Summarizing the previous experience in this field, it can be concluded that in the future it is necessary to attract more consumer interest and to intensify the promotion of the application of these natural substances as a part of the preventive measures against diseases caused by food contamination. The application of bacteriocins and bacteriocin-producing microorganisms can be interesting and very desirable, considering that the trust of the consumers in chemical preservatives is very shaken and even called into question. The composition of food, i.e. its properties (pH, temperature, ingredients and additives, types and quantities of epiphytic microbiota) and the actual technological process used in production, can all influence the stability and activity of the added bacteriocins. Future research in this field should aim to clarify this unknown aspect of their application as well. That way, the necessary knowledge in the field of the optimization of external conditions will be provided, which will, in turn, lead to the maximum antimicrobial effect of the bacteriocins being added into food systems, but it will also open up the possibility of discovering their new producers.

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БАКТЕРИОЦИНИ БАКТЕРИЈА МЛЕЧНЕ КИСЕЛИНЕ – ПРЕГЛЕД

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Бактерије млечне киселине (БМК) имају есенцијалну улогу у току производње ферментисаних производа. Својом метаболичком активношћу утичу на процесе зрења, доводећи до стварања жељених сензорних својстава уз истовремено спречавање раста нежељених микроорганизама. Због своје доминантности током ферментације и традиције дуге употребе, БМК су означене као "здравствено безбедна микробиота". Биолошку заштиту БМК, као природно присутна и/или селекционисана и накнадно додата микробиота, остварују кроз продукцију неспецифичних (млечна, сирћетна и друге испарљиве органске киселине, водоник-пероксид, диацетил итд.) и специфичних метаболита, бактериоцина.

Бактериоцини представљају ванћелијске ослобођене протеине или пептидне молекуле, који поседују извесна бактерицидна својства према одређеној врсти микроорганизама, обично сродним бактеријама произвођача. Данас бактериоцини представљају веома значајну могућност примене у индустрији хране. Њиховом применом може се смањити употреба синтетичких конзерванаса и/или интензитет топлотног третмана током производње хране. Примена бактериоцина може бити алтернатива у настојању да се задовоље потребе потрошача за безбедном, свежом и минимално прерађеном храном.

У намери да се потпуно реализује потенцијал бактериоцина, неопходно је да се схвати њихова природа, механизам продукције, регулације и деловања, као и утицај спољашњих фактора на њихову антимикробну активност. Састав хране, односно њена својстава (pH, температура, састојци, адитиви, врста и број присутних епифитних микробиота) и тип технолошког процеса, који се користи у производњи, могу да утичу на стабилност и активност додатих бактериоцина.

Будућа истраживања у овој области треба да имају за циљ да добро разјасне ове непознате аспекте њихове примене. На тај начин, биће обезбеђена неопходна знања у области оптимизације спољашњих услова, али ће, такође, отворити и могућност откривања њихових нових произвођача.

Кључне речи: бактериоцини, бактерије млечне киселине, природно конзервисање, храна

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