

Inhibition Effect of Lactic Acid Bacteria against Food Born Pathogen, *Listeria monocytogenes*

Rouha Kasra-Kermanshahi^{1*}, Elahe Mobarak-Qamsari¹

¹Department of Microbiology, Faculty of Biological Sciences, University of Alzahra, Tehran, Iran

Abstract

In recent years due to changes in lifestyle and eating behavior of the human populations, disease caused by contaminated food has increased significantly. *Listeria monocytogenes*, *Escherichia coli* O157:H7 and *Salmonella enteric* are three of the most important food borne bacterial pathogens and can lead to food borne diseases. Also today wide spread of resistance to antibiotics among bacteria occurs due to increased consumption of antibiotics. Therefore, there is a dire need for development of new types of safe antimicrobial compounds. In this field, the most extensive research and commercial practices are based on probiotic bacteria. Probiotics, specifically lactic acid bacteria, are broadly used in the food industry for fermentation. Furthermore, probiotics produce valuable antimicrobial products that results to health effects. Now, the use of probiotic for treatment of disease is thought to be an effective way to improve the gut health and an alternative for treatment by antibiotics. Probiotics contribute to food safety by inhibition of the growth of other bacteria. Lactic acid bacteria can be used as protective cultures to compete with several pathogens and undesired organisms. Since food safety has become a significant international concern, here we investigated application of lactic acid bacteria for controlling the growth of *Listeria monocytogenes*.

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Correspondence to:

Rouha Kasra-Kermanshahi

Department of Microbiology, Faculty of Biological Sciences, University of Alzahra, Tehran, Iran. P.O. Box: 1993893973

Tel: +98-21-88058912

Fax: +98-21-88058912

E-mail: rouha.kasra@gmail.com

1. Introduction

Researchers in the field of food safety and regulatory agencies have been concerned with the growing number of food-borne illness outbreaks caused by several pathogens and/or their enterotoxins. Increased use of antibiotics has led to expansion of bacterial resistance to antibiotics. Therefore, there is increasing interest in the development of new types of efficient and safe antimicrobial compounds [1]. Moreover, in food industry a microbiological problem has very important economic effect. In recent years, there is aversion towards the use of chemical preservatives in food while application of lactic acid bacteria (LAB) as natural preservatives has increased due to the potential production of metabolites with antimicrobial activity. Antagonistic effect of LAB is primarily related to resource competition, production of different low molecular weight substances (e.g. diac-

etyl, acetaldehyde, hydrogen peroxide, etc.); production of different organic acids; pH lowering effect, production of bacteriocin; and releasing bacteriocin-like substances [2].

Application of LAB is effective in food safety, preserving food quality, developing characteristic new flavors, and to advance the nutritional qualities of food. LAB show drastic antagonistic activity against many microorganisms, including spoilage organisms in food and pathogenic bacteria such as *Listeria*, *Staphylococcus*, *Clostridium* and *Bacillus* spp. The antagonistic effect of LAB is mainly due to decrease the pH of the food, competition for nutrients, and production of inhibitory metabolites [3,4].

As mentioned, in recent years several studies have been carried out on the application of pure culture or metabolites of LAB to control the growth of food

born bacteria [5,6]. One of the most famous food born bacteria is *Listeria monocytogenes*. Listeriosis is a food borne disease that presents severe hazards to susceptible groups, in particular, to the elderly, new born infants, pregnant women and those who are immune compromised. The causative agent, *L. monocytogenes*, has been isolated from a wide range of food sources [7].

L. monocytogenes is able to survive and grow from under 0 to 45°C. This bacterium is one of several important hazards in this kind of product due to growing at storage temperatures. The real hazard is the growth of *Listeria* in the product when the storage temperature is above 5°C. Similar to *Listeria* spp, LAB can grow under the same storage conditions. Thus, different studies have performed on the isolation of this Gram positive species and its metabolites (bacteriocins) to assess them either as competitive flora against the pathogenic organisms or as antimicrobials, respectively [3]. Due to increased consumer demands for foods that contain lower concentrations of chemical preservatives, there has been great focus on the identification of natural antimicrobials, such as bacteriocins that can be applied to control the growth of pathogens in food.

Today, food safety has great importance in international community, so application of antimicrobial proteins produced by LAB is favorable. Because these kinds of proteins have been effective on different pathogens in food without side effects on the features of food; here, the application of LAB and their metabolites are investigated to inhibit the growth of *L. monocytogenes*.

2. Lactic acid bacteria

LAB are important organisms in food industry for their fermentative ability, as well as health and nutritional benefits. Food fermentation is done using species from different genera such as *Lactococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Lactobacillus*, and the newly recognized *Carnobacterium*. Naturally, raw material such as milk and meat contain LAB. They can be applied to produce many fermented foods [8]. One important attribute of LAB is their ability to produce antimicrobial compounds like organic acids, ethanol, diacetyl, hydrogen peroxide, bacteriocins or bacteri-

cidal proteins [9,10]. Table 1 shows a summary of these compounds.

The homo-fermentative LAB produce lactic acid as the major metabolic end product of carbohydrate fermentation that leads to pH reduction of food and also directly growth inhibition of many microorganisms. LAB are characterized by their tolerance to low pH and growth under this circumstance in which other bacteria are unable to grow, and thereby ensuring safety of food. Furthermore, penetration of lactic acid to the membranes leads to lowering the intracellular pH. Other feature of lactic acid is interference with metabolic processes such as oxidative phosphorylation. The hetero-fermentative LAB produce lactic acid and additional products such as acetic acid, ethanol and carbon dioxide. Ethanol is a part of intermediary products that are converted into produce CO₂ and H₂. Carbon dioxide interacts with cell membranes by reducing internal and external pH levels. Diacetyl is a product of citrate metabolism, and is responsible for the aroma and flavor of certain fermented dairy products. Known action for diacetyl is interference with arginine binding proteins. Peroxide oxidizes membrane lipids and cell proteins [11].

About bacteriocins, these molecules are proteins or protein complexes with bactericidal activity against a number of other species closely and not closely related to the producer bacterium. Bacteriocins affect membranes, DNA synthesis, and protein synthesis. Bacteriocins are produced by some LAB strains and contribute to the biological control of pathogenic and spoilage microorganisms. It is to be noted that these metabolic compounds probably act synergistically [11].

Recently, interest in bacteriocins has grown significantly due to their potential usefulness as natural food preservatives in addition to promoting good health. Many LAB, including members of the genera *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Leuconostoc*, *Pediococcus*, and *Carnobacterium* are known to secrete bacteriocins [11].

Starter cultures are commonly used for fermentation of foods. Recently, starter cultures such as LAB have received great attention due to their significant effect on food safety.

Table 1. Inhibitory compounds produced by Lactic acid bacteria

Inhibitory compound	Mechanism of action	References
Lactic acid and other volatile acids	Disruption of cellular metabolism	[3,10]
Hydrogen peroxide	Inactivation of essential biomolecules by superoxide anion chain reaction Activation of lactoperoxidase system	[10,11,13]
Carbon dioxide	Anaerobic environment and/or inhibition of enzyme decarboxylation and/or disruption of the cell membrane	[9,10,13]
Diacetyl	Interference with arginine utilization	[10,13]
Bacteriocin	Disruption of cytoplasmic membrane	[17, 25,26]

Furthermore, this new kind of starter culture can be effective on nutritional, sensory and health features of food [13].

3. Food borne pathogens

More than 250 toxins and pathogens are known to be transmitted by food, and this list continues to grow steadily [11,12]. Several reports indicates that food-borne pathogens are one of the main causes of death in the world. For example, in the United States, 1300 deaths are caused by 31 foodborne pathogens each year, in addition to 56000 hospitalizations and 9.4 million illnesses [14]. *Campylobacter*, *Clostridium perfringens*, *E. coli*, *L. monocytogenes*, Norovirus and *Salmonella spp.* are responsible for more than 90 percent of all symptomatic foodrelated illnesses (Table 2) [15,16]. Control of these foodborne enteric pathogens is a real challenge for food industry and public health agency. Moreover, it is very difficult to protect safety of food chains due to resurgence of multidrug resistant strains of foodborne pathogens [14].

Now probiotic therapy is thought to be an efficient way to improve the gut health and an alternative to antibiotic treatments. Probiotics, specifically LAB, are widely used in the food industry for fermentation but have gained attention from health professionals because of their potential useful effects. They contain many safe bioactive compounds such as bacteriocin to combat with bacterial pathogens [14].

4. *Listeria monocytogenes*

L. monocytogenes is a bacterium that is widely distributed in nature. It is commonly found in soil, surface water, foods and plants, and is carried by a variety of animals. Most infections are acquired by ingestion of spoiled food or feed. Then contaminated

animals can discard the bacterium in feces, milk, and uterine discharges. In humans, listerial infection can result in the fairly rare but hazardous disease Listeriosis, which has a case fatality rate of approximately 20 %. This disease primarily affects the very young or old and pregnant women; however, it can also affect healthy individuals. *Listeria* is well adapted to food-processing and food storage environments. It can grow and multiply at low “refrigeration” temperatures, and establish persistent infections on tools for processing of food. *L. monocytogenes* may grow in biofilms that defend them against environmental stress and can be screened from surfaces after cleaning and disinfection. Pasteurization and cooking can result to kill *Listeria*; however, in some ready-to-eat foods, contamination may take place before packaging. Unpasteurized milk, soft cheeses, deli meats (sliced cooked meat used for making sandwiches or some other meals) and hot dogs are common sources of *Listeria* infections [9,18].

5. Control of *Listeria monocytogenes* contaminant by LAB

L. monocytogenes is a bacterium that causes a rare but severe disease in susceptible individuals such as pregnant women, newborn infants, the elderly and immune compromised patients with a mortality rate between 20 to 30% [19]. The consumption of raw milk and raw milk products has caused several listeriosis outbreaks resulting in several hundred cases [20]. For example, in numerous reports, cheese is known as a source of listeriosis outbreaks [18-21]. Although *L. monocytogenes* is a regular food pathogen in cheese and red meatbased products. It can also be encountered in chicken, where the most important health concerns are associated with salmonella and campylobacter [25].

Table 2. Most common foodborne pathogens

Bacteria	Symptoms	Sources	References
<i>Campylobacter spp.</i>	Diarrhea (sometimes bloody), muscle pain, abdominal pain, headache and nausea.	Raw milk and chicken, shellfish	[11,25]
<i>Clostridium botulinum</i>	Dry mouth, double vision, weakness, muscle paralysis, and breathing problems may develop. Botulism can be fatal. It's important to get immediate medical help.	Homecanned and prepared foods	[11,15]
<i>Clostridium perfringens</i>	Abdominal pain, diarrhea, and sometimes nausea and vomiting.	Meat and meat products	[11,15]
Pathogenic <i>Escherichia coli</i>	Severe stomach cramps, bloody diarrhea, and nausea. <i>E.coli</i> 0157:H7 can cause permanent kidney damage, which can lead to death in young children.	Meat (undercooked or raw hamburger), uncooked produce, raw milk, unpasteurized juice, and contaminated water	[15,16]
<i>Listeria monocytogenes</i>	Nausea, vomiting, diarrhea, influenza-like symptoms, meningitis	Refrigerated, ready-to-eat foods, raw milk, cheeses, raw vegetables	[18-21]
Norovirus (Norwalk-like Virus)	Diarrhea, nausea, vomiting, stomach cramps, headache, and fever.	Raw oysters, shellfish, coleslaw, salads, baked goods, frosting, contaminated water, and ice. It can also spread via person-to-person.	[11,12]
<i>Salmonella spp.</i>	Diarrhea, fever, vomiting, headache, nausea, and stomach cramps	Raw meat, poultry, seafood, raw milk, dairy products, and produce	[12,25]

LAB constitute the most suitable choice for application as protective cultures, since they are present in all fermented foods with long history of safe use, and form part of the gut microflora of humans and animals. Some LAB strains or their metabolic products have been used in food against pathogens, mainly *L. monocytogenes* in dairy products such as soft and hard cheese and meat products such as sausages and ham [6,26].

For fermentation of foods, different kinds of starter cultures are used. In this field, application of some LAB strains as starter culture is very significant. LAB are classified as producers a variety of antimicrobial metabolites such as bacteriocins. They are secondary metabolites of LAB that can affect on other bacteria (except for their producers) especially gram positive bacteria such as *L. monocytogenes* [27]. In the past years, there has been significant application of bacteriocin or bacteriocin producer strains in controlling the growth of pathogenic bacteria in foods. Although bacteriocins may be found in many bacteria, those produced by LAB are GRAS (Generally Recognized As Safe) that have received particular attention in recent years because of their potential application in the food industry as natural preservatives [28].

6. Classification of bacteriocins

Bacteriocins are peptides produced by some bacteria and archaea that are active against other bacteria. Classification of bacteriocins can be done based on different features such as molecular weight, conformational structure, mechanism of action etc. [29].

Bacteriocins are usually classified into three or four groups [30,31]. Nisin was discovered in 1928 [32] and subtilin, analogue of nisin differing by 12 amino acid residues, was discovered in 1948 [33]. Both belong to Class I, termed lantibiotics. Class Ia bacteriocins, which include nisin, consist of cationic and hydrophobic

peptides that form pores in bacterial membranes [34]. In terms of structure, Class Ia and Ib are different. Bacteriocins belonging to Class Ia are flexible while Class Ib are rigid. Also class Ib bacteriocins are globular peptides, and have negative net charge or no net charge [35,36].

Class II bacteriocins contain small heat-stable, non-modified peptides, and can be more subdivided, Class IIa or pediocin-like bacteriocin, Class IIb and IIc [37,38]. Class IIa bacteriocins display anti-*Listeria* activity. Based on primary structures, Class IIa bacteriocins contain a conserved N-terminal region and two cysteine residues joined by a disulfide bridge. Class IIb bacteriocins have two different peptides. In this case, activation of both peptides is necessary. The primary amino acid sequences of the peptides are different. Although, separate genes encode each of the two amino acid sequences, but for immunity activation, only one gene is sufficient. It has been suggested that bacteriocins of Class IIc are secreted by universal sec-system [31]. Since this suggestion, it has been shown that Class IIa bacteriocins can apply this secretory system and thus the sub-class IIc should be eradicated [39]. The large and heat resistance bacteriocins compose the Class III bacteriocins for which there is much less information available [40]. The fourth class of bacteriocins also contains mixture of bacteriocins with other macromolecules (Table 3) [30,41,42]. It is worth noting that among the different kinds of bacteriocins, Class I and II have been further investigated and more applicable in food industry due to specificity of target and robustness [43].

7. Effectiveness of LAB bacteriocins against *Listeria monocytogenes* in food systems

LAB and, specifically lactococci, are a common option as host by virtue of their generally regarded as safe status [7]. LAB are the principal microorganisms involved in the fermentative conversion of food.

Table 3. Classification and characteristics of bacteriocins

Bacteriocin classes	Bacteriocin subclasses	Molecular mass	Characterization of class/subclass	Bacteriocin	References
Class I	a	<5 kDa	Lantibiotics/ peptides containing lanthionine and -methyl lanthionine	Nisin	[32,34]
	b		Globular peptides with no net charge or net negative charge	Mersacidin	[34,36]
Class II	a	<10 kDa	Small heat-stable peptides, synthesized in a form of precursor	Pediocin PA-1, Sakacins A and P	[27,35,37]
	b		Two component systems: two different peptides required to form an active poration complex	Lactacin F, Plantaricin EF and JK	[31,37,38]
	c		Sec-dependent bacteriocin	Carno-bactericin A, Helveticins J and V, Lactacins A and B	[31,37]
Class III		>30 kDa	Large molecules sensitive to heat	Acidophilucin A, Lactacins A and B	[30,40]
Class IV		Large protein	Mixture of protein(s), lipid(s) and carbohydrate(s) in bacteriocin molecule	Leucocin S, Mesenterocin 52	[41,42]

The production of antimicrobial peptides, called bacteriocins, is one reason that is responsible for the antagonistic effects of LAB against other organisms [44, 45].

Since LAB are usually used as starter cultures in fermentation of food, investigators have explored using producers of bacteriocin as starter cultures. In some cases, usual bacteriocin producers, such as *Lactobacillus plantarum*, *Pediococcus acidilactici* and *Enterococcus faecalis* have been used in such studies [46, 47]. Nunez et al. found that inoculation of Manchego cheese with a bacteriocin-producing *E. Faecaliss* strain decreased the number of *L. monocytogenes* by 6 logs in 7 days, whereas application of commercial starter culture had no effect on the survival of the organism in cheese. In the same way, in a naturally contaminated salami sausage, the surviving number of *L. monocytogenes* decreased when the product was inoculated with *L. plantarum* MCS1 that produces bacteriocin [47]. However, reports showed that many starter cultures do not produce bacteriocin, but today a few number of bacteriocins starter cultures are sold for meat industry [17].

Mahdavi et al. investigated the effect of nisin produced by *Lactococcus lactis* against *L. monocytogenes* and *Bacillus cereus* in order to compare the isolated strain. According to their results, the bacteriocin could be added to liquid foods directly or produced as a byproduct of starter cultures in fermented foods. This would inhibit the growth of *L. monocytogenes* [48].

In another study, application of two strains, *Enterococcus faecium* PCD71 and *Lactobacillus fermentum* ACA-DC179 in chicken meat resulted in significant reduction of two pathogenic bacteria, *L. monocytogenes* and *S. enteritidis*, respectively. The anti-listerial activity of these two LAB, *E. faecium* PCD71 and *L. fermentum* ACA-DC179 is closely related with the production of Class II bacteriocins, such as enterocins and pediocins [25]. Also in this study, the potential spoilage effect of protective cultures in meat was investigated. Since LAB may contribute in spoilage of food, so it is important to assess whether a candidate protective culture has harmful effects on food quality. In this study, total antioxidant capacity, crude protein content and pH of the meat samples were determined as markers of meat spoilage [25]. The results showed that two strains as used in this study, *E. faecium* PCD71 and *L. fermentum* ACA-DC179 did not show any detrimental effect on biochemical parameters related to chicken meat spoilage [25].

Bacteriocins have been directly inoculated to foods such as cheese to prevent against Clostridium and Listeria. To date, among the LAB bacteriocins commercially marketed nisin groups produced by *L. lactis* subsp. *lactis*, and pediocins produced by *Pediococcus* sp. are the most known by their antilisterial property [5,49].

In long-life cottage cheese spiked with 10^4 viable *Listeria* g^{-1} , the addition of 2000 IU/g nisin resulted in a 1000-fold decrease in *L. monocytogenes* after 7 days storage at 20°C, compared to a 10-fold decrease in the control [50]. Since LAB are also found in meat, bacteriocins produced by these bacteria have been explored and isolated. Though some researchers concluded that nisin is not efficient in meat applications due to high pH [51], inability to uniformly distribute nisin, and interference by meat components (for instance phospholipids) [52].

As mentioned above, application of nisin in raw meat material faced with some problems, so using other bacteriocins has been investigated. Increasing the shelf life of fresh meat is obtained by using sakacins, enterocins, leucocin A and the carno bacteriocins A and B. Also decreasing the number of investigated organisms in meat by pediocin PA-1 was very remarkable [53] but is not yet an approved food additive in the United States [17].

Studies have shown that changes in microflora in human or in environment due to application of bacteriocins in food are unlikely. Because of few amount of bacteriocins needed for elimination or reduction of spoilage or pathogenic organisms in food, no significant effect on non-target microorganisms is possible. In any event, bacteriocins are unlikely to endure gastric condition, as they are sensitive to proteolytic degradation [26].

8. Bacteriocin resistance

Protection of foodstuffs by using LAB that produce bacteriocin or purified bacteriocins has been investigated [28,36,54]. Nisin from Class I and pediocin like bacteriocin belonging to class IIa are favorable bacteriocins that can act against *L. monocytogenes* [27]. Most bacteriocins are able to kill target cells by changing the permeability of cell membrane. It is well known that the antimicrobial activity of bacteriocins is very particular, since they use specific receptors such as lipid II or mannose phosphotransferase system on the sensitive target cell surfaces [55-57]. The sensitivity to nisin and pediocin-like bacteriocin has been shown among different strains of *L. monocytogenes* and this sensitivity is depending to strain [58]. The use of bacteriocins in food requires awareness of the sensitivity of food microorganisms. The intrinsic characteristics of the food could be affected by bacteriocins [59].

The big concern in this part is the hazard of increasing strains that are resistant to bacteriocins. Laboratory studies have shown that among listeria spp., resistance to some bacteriocins such as nisin and pediocin-like bacteriocins is expanding [60]. In addition, the resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes and thus form multi-resistant *Listeria* strains [61].

L. monocytogenes shows resistance to nisin through change in the structure of membrane fatty

acids and with alterations in the cell wall [6,62]. In *L. monocytogenes*, reduction ratio C15:C17 that leads to thickening of the membrane increases resistance to nisin [63]. Ming and Daeschel also found that nisin resistant *L. monocytogenes* have decreased the amounts of phosphatidyl glycerol, diphosphatidyl glycerol and bisphosphatidyl glycerol phosphate [64]. In addition of change in the composition of the cell membrane, also production of nisinase, an enzyme that degrades nisin, could be a factor for nisin resistance in some mutants [65]. Gravesen et al. reported that in *L. monocytogenes*, mutation in parts of gene that code for β -glucoside-specific phosphoenolpyruvate-dependent phosphotransferase systems results to high expression, and therefore, resistance to pediocin PA-1 [66]. According to this report, understanding the exact role of phosphotransferase systems in resistance to pediocin requires more investigation [67]. Although as mentioned earlier, resistance is a result of structural or genetical mutation, there are reports that show using bacteriocin from different classes results to cross-resistance [67-70].

9. Control and removal biofilm of *Listeria monocytogenes* by LAB

Microbial biofilms may be unfavorable and undesirable in food processing premises. *L. monocytogenes* are well suited for proliferate in cold wet environments that are best for biofilm formation. Biofilm formation by *Listeria* occurs in pure culture, and can endure and grow in multispecies biofilms. *L. monocytogenes* forms biofilms on stainless steel, polycarbonate surfaces, plastic and many other materials in contact with food. Therefore, *Listeria* spp. could be survived and grow in many sectors of food processing companies [71]. In biofilms, microorganism is drastically more resistant to disinfection than its free-living counterparts, and complex biofilms are more difficult to remove than adherent single cells of microorganism [18].

LAB adherence to surfaces may have a potential for preventing the adherence and biofilm growth of *L. monocytogenes*. Such strategies have been investigated in a number of studies. One study reports effect of a surface-adhering LAB against *L. monocytogenes*. It reported the inhibition of growth and biofilm formation of *L. monocytogenes* by a surface-adhering nisin-producing *L. lactis* [72].

Microbial molecules, usually used as biopreservatives such as nisin, lauricidin, reuterin and pediocin, have been well recognized for their ability to control the biofilm of different microorganisms including *L. monocytogenes* that can grow in dairy products [73].

Food packaging can be done by using materials that contain nisin. This kind of application would prevent foodborne pathogens. Carballo and Araujo evaluated the effect of the adsorption of nisin containing materials on the adhesion of *L. monocytogenes* to them. They concluded that high severity of rubber results to the more adsorption of nisin onto this material, and this would be the reason for the

high reduction of bacterial adherence [74]. Mahdavi et al. evaluated the effect of diverse nisin concentrations on biofilm forming pathogenic bacteria *Staphylococcus enteritidis*, *S. aureus* and *L. monocytogenes* by microtiter plate method. In this study, the effective level of nisin against listerial biofilm was 4×10^3 IUml⁻¹ [75].

10. Conclusion

It is important to encourage consumers' attention to the existence of natural substances that can protect against food-related illnesses. Due to the health benefits of probiotic bacteria, these microorganisms are popular among consumers as natural cultures. Likewise, using natural cultures that produce bacteriocins or addition of purified bacteriocin to foods is also attractive, chiefly as a result of consumers' concern for chemical preservatives. The use of probiotic strains can be considered as an alternative approach for reducing the formation of biofilm. So research in this field is increasing, especially in the field of food and medical trials. Probiotic strains known as nonpathogenic, safe and health beneficial are defined as live supplements for improving the intestinal microbial balance.

Microorganisms associated in biofilm formation have great growth and durability, and this is a problematic issue in the food industry due to protection against disinfectants. For example, growth of *L. monocytogenes* in biofilms leads to protection against the environment.

There are new procedures to prevent adhesion of *L. monocytogenes*, but some problems such as high costs and resistant strains caused practical limitation. Despite considerable research on the adhesive properties and resistance of *L. monocytogenes* for survival in the food production environment, no applicable solution for avoiding establishment of the bacterium has yet been found.

Probiotic lactobacilli with various applications are now the best choice to treat many infectious diseases in the food industry. These bacteria are characterized by many helpful properties to control pathogens. These properties contain competence with pathogens in cell attachment, high adherence ability to human intestinal epithelium, production of antibacterial substances such as organic acids, hydrogen peroxides, bacteriocins and etc.

11. Conflicts of Interest

The authors declare no conflict of interest.

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