10.14751/SZIE.2016.040

SZENT ISTVÁN UNIVERSITY
FACULTY OF FOOD SCIENCE

INTERACTIONS BETWEEN *LISTERIA* SPP. AND LACTIC ACID BACTERIA PRODUCING ANTILISTERIAL BACTERIOCINS

PhD Dissertation by

Tekla Engelhardt

Under the supervision of

Professor Csilla Mohácsi-Farkas and

Dr. Gabriella Kiskó

Szent István University

Faculty of Food Science

Department of Microbiology and Biotechnology

Budapest, 2016

10.14751/SZIE.2016.040

PhD School

Name: PhD School of Food Science

Field: Food Science

Head: Prof. Gyula Vatai
Professor, DSc
Szent István University, Faculty of Food Science
Department of Food Engineering

Supervisors: Prof. Csilla Mohácsi-Farkas
Professor, PhD
Szent István University, Faculty of Food Science
Department of Microbiology and Biotechnology

Dr. Gabriella Kiskó
Associate Professor, PhD
Szent István University, Faculty of Food Science
Department of Microbiology and Biotechnology

The applicant met the requirement of the PhD regulations of the Szent István University and the thesis is accepted for the defence process.

.....
Signature of Head of School

.....
Signature of Supervisors

TABLE OF CONTENTS

10.14751/SZIE.2016.040

10.14751/SZIE.2016.040

LIST OF ABBREVIATIONS

ALOA	Agar *Listeria* Ottaviani and Agosti
ATCC	American Type Culture Collection
AU	Arbitrary Unit
B.	*Bifidobacterium*
BH	Brain-Heart
CCM	Czech Collection of Microorganisms
CFU	Colony Forming Unit
E.	*Escherichia*
EC	European Commission
EFSA	European Food Safety Authority
ESB	Escola Superior de Biotecnologia
EU	European Union
FB	Fraser Broth
FDA	U.S. Food and Drug Administration
GT	Generation Time
hFB	Half Fraser Broth
ISO	International Organization for Standardization
L.	*Listeria*
LAB	lactic acid bacteria
Lb.	*Lactobacillus*
Lc.	*Lactococcu*s
L. i.	*Listeria innocua*
LL	lettuce leaves
L. m.	*Listeria monocytogenes*
Ln.	*Leuconostoc*
LRCESB	*Listeria* Research Center of Escola Superior de Biotecnologia
MID	Minimal Infectious Dose
mMRS-BPB	modified MRS supplemented with bromophenol blue
MRS	de Man, Rogosa Sharpe
NCTC	National Collection of Type Cultures

10.14751/SZIE.2016.040

OEK	Országos Epidemiológiai Központ (National Center for Epidemiology)
P.	*Pediococcus*
Ps.	*Pseudomonas*
RTE	Ready-To-Eat
S.	*Staphylococcus*
Str.	*Streptococcus*
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SS	Stainless Steel
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TTD	Time to Detection
UCP	Universidade Católica Portuguesa
US	United States
YE	Yeast Extract
YOPI	Young, Old, Pregnant, Immunosuppressed

10.14751/SZIE.2016.040

CHAPTER 1 – INTRODUCTION

It is estimated that millions of people suffer from food-borne diseases and 2 million deaths occur each year due to the consumption of contaminated food and water. The responsible organisms causing most of these diseases are: *Salmonella* spp., *Campylobacter spp*., Shiga-toxin producing *Escherichia coli*, norovirus and *Listeria monocytogenes*. According to the General Food Law in the European Union the food business operators have to ensure food safety at each point of the food chain, 'from farm to fork'. Pathogens are able to enter into the food system e.g. via humans, animals, plants, soil, waste water systems, air, contaminated equipment. All of the stakeholders are interested in to produce safe foods besides satisfying the consumer's demands. In the past decade new trends appeared in the food industry. From the side of consumers, foods with high nutritional value, mildly treated, natural, fresh products are requested. To fulfil these requirements, food business operators are needed to develop new methodologies, new hurdles to ensure safe products, by eliminating food-borne pathogens. One of the most traditional food preservation techniques is heat treatment, which has several benefits and disadvantages, too. The most remarkable disadvantage of heat treatment is the loss in the nutritional value of the product, however if it is applied carefully, the heat can eliminate pathogenic bacteria. The food industry turns toward novel preservation techniques such as high-hydrostatic pressure, modified atmosphere packaging, food irradiation, pulsed electric field technique, addition of antimicrobial compounds e.g.: nisin, bacteriocin, carvacrol etc. With these novel techniques safe products with high quality can be produced. In order to apply them properly, the food industry needs reliable information about the response of the pathogens to the treatments. If the preservation techniques are not applied in a right way, the pathogens might survive and/or adapt to the new environmental conditions, which may lead to presence of pathogens. The beneficial bacteria – e.g. lactic acid bacteria – are commonly used to prolong the shelf life and they also contribute to the organoleptic properties of the food products. The most common LAB genera used in the food industry are *Lactobacillus*, *Lactococcus, Leuconostoc*, *Pediococcus.* Lactic acid bacteria are able to control the closely related Gram-positive species, like *L. monocytogenes*. *L. monocytogenes* as mentioned above is an emerging food-borne pathogen which can cause serious disease, and its occurrence in ready-to-eat, fermented, fresh food products is high.

Microorganisms have a complex ecosystem in food, which also influences the behaviour of the microbes. It is essential to understand the interrelationships of microbes. For example, LAB strains are able to inhibit the growth of *L. monocytogenes* by their different metabolites.

10.14751/SZIE.2016.040

Moreover, *L. monocytogenes* is a well-known bad competitor, thus some other microbes are able to overgrow it.

The overall focus of my dissertation was to better understand the interactions between the beneficial LAB strains and the pathogen, *L. monocytogenes*. *L. innocua* is commonly used as a surrogate of *L. monocytogenes*, therefore *L. innocua* is also involved in these studies. The influence of *L. innocua* on the growth of *L. monocytogenes* was also investigated.

The specific objectives of my research were:

1. to evaluate the antilisterial activity of *Lactobacillus sakei* and *Lactobacillus plantarum;*
2. to understand the behaviour of lactic acid bacteria against *L. monocytogenes* under different environmental conditions;
3. to study the antilisterial activity of *Lb. plantarum* at low temperature and at different NaCl concentrations;
4. to characterise the biofilm formation of *P. acidilactici, L. monocytogenes* and *L. innocua* in different media on different surfaces;
5. to understand the behaviour of *L. monocytogenes* and *L. innocua* during the traditional detection method.

A variety of conventional and rapid microbiological techniques, molecular microbiological approaches were applied to reveal the underlying questions.

10.14751/SZIE.2016.040

CHAPTER 2 - LITERATURE REVIEW

2.1. Background of *Listeria genus*

Listeria spp. are Gram-positive, non spore-forming, facultative anaerobic, catalase positive, oxidase negative bacteria. *Listeria* spp. have four well supported monophyletic clades, as follows: (i): *Listeria sensu stricto*: *L. monocytogenes, L. marthii, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii;* (ii): *L. fleischmannii, L. aquatica* sp. nov.*, L. floridensis* sp. nov.; (iii): *L. rocourtiae, L. weihenstephanensis, L. cornellensis* sp. nov.*, L. grandensis* sp. nov., *L. riparia* sp. nov.; (iv): *L. grayi* (den Bakker et al., 2014). *L. monocytogenes* is a human pathogen bacterium, but it was recently found that *L. ivanovii* can also cause human infections (Guillet et al., 2010). *L. innocua* is an apathogenic species sharing similar environments to *L. monocytogenes* (Rodríguez-Lázaro and Hernández, 2014); thus several studies work with *L. innocua* as a surrogate instead of *L. monocytogenes*. *L. monocytogenes* is an intracellular bacterial pathogen able to cause serious infection, namely listeriosis in humans. Occurrence of listeriosis is rare, but the mortality rate is quite high, usually up to 20-30%. The most sensitive group is the YOPI group and the symptoms are different within the groups and healthy adult humans. Listeriosis in pregnant women occurs usually in the third trimester. The infection of the mother characterized by a flu-like illness with fever, myalgia or headache. Listeriosis has serious consequences for the infant, including spontaneous abortion, fetal death, stillbirth, severe neonatal septicemia and meningitis (Rocourt et al., 2000). In case of adult humans *L. monocytogenes* has a particular tropism for the central nervous system and meningeal and/or brain parenchymal infections are thus frequent in cases of listeriosis. Gastro-enteritis symptoms (vomiting and diarrhea) are usually observed before the diagnosis (Rocourt et al., 2000). The infectious dose depends on several factors, such as the food matrices, virulence factors. According to the FDA (2014) the minimal infectious dose (MID) is 1000 cells but for the YOPI group is probably lower. The Public Health Agency of Canada (2011) reported that the estimated MID is 10 to 10^6 million CFU in healthy hosts, but for YOPI individuals it is 0.1 to 10^6 CFU.

L. monocytogenes is able to survive non-favorable conditions and can occur not just in the environment, soils, but in the food production line and in the foods, as well. *L. monocytogenes* typically causes listeriosis by consumption of RTE foods, such as soft cheeses, fishery products (Buchanan et al., 2009). In the past few years the fresh produce was increasingly associated with *L. monocytogenes*; a multistate outbreak was associated with cantaloupe in the US at 2011. The mortality rate was more than 20 % (Upadhyay et al.,

10.14751/SZIE.2016.040

2014). Meal with beef from meals-on-wheels delivery catering company caused listeriosis in Denmark, 2009 with 25 % mortality rate. The patients were elderly people and half of them linked to the immunocompromised group too (Smith et al., 2011).

In the European Union the number of listeriosis increased and the confirmed cases were 2161 in 2014 (EFSA, 2016) (Fig. 2.1).

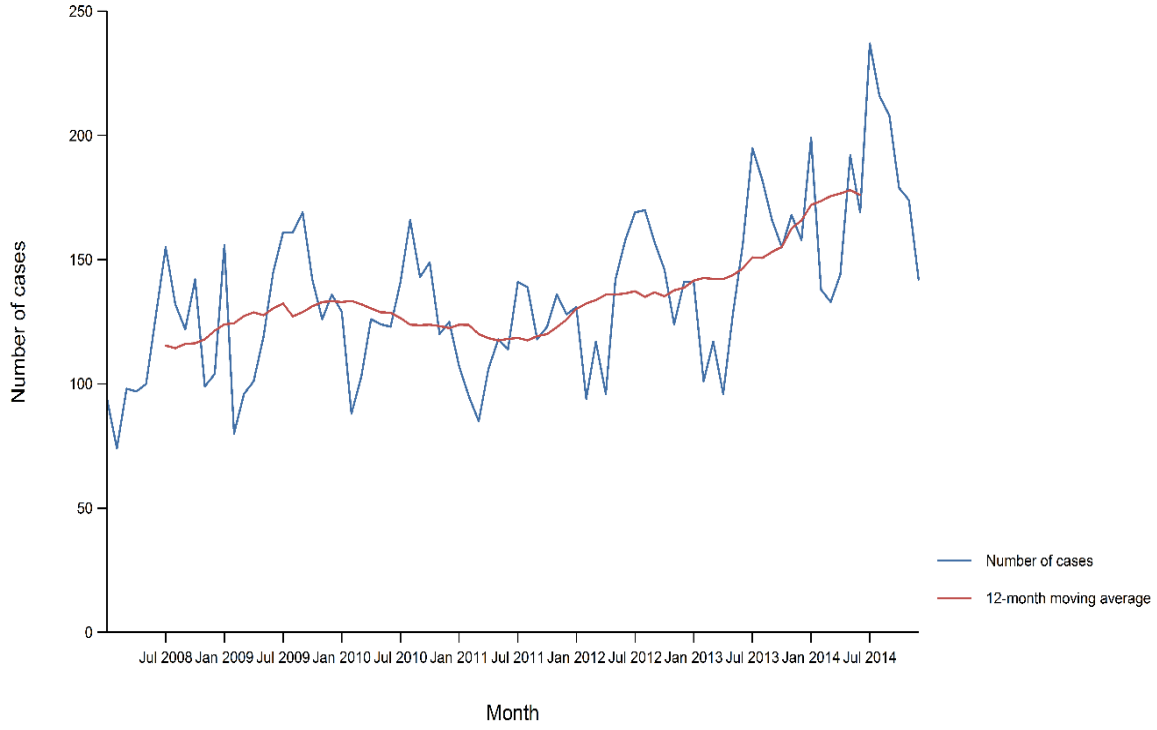


Fig. 2.1. Trend in reported confirmed cases of human listeriosis in the EU/EEA, 2008-2014 (EFSA, 2016)

The number of listeriosis significantly increased in Hungary in the past two years. Until 2013 approx. 12-13 cases per year were reported, at the end of 2013 the cases started to increase and lead to 37-39 cases per year in 2014 and 2015 (OEK, 2015). Unfortunately, no information can be found about the reason.

According to the EU law, the European Commission Regulation No. 2073/2005 establish microbiological criteria for foods. In case of *L. monocytogenes* three categories were proposed, (i): absence in 25 g for RTE products for infants and special medical use; (ii): absence in 25 g before the product leaves the food business operator and 100 CFU/g during the shelf-life for RTE products which are able to support the growth of *L. monocytogenes*; (iii): 100 CFU/g in RTE products which are unable to support the growth of *L. monocytogenes*.

2.1.1. Behaviour of *L. monocytogenes* in food

Table 2.1. shows the growth and inactivation characteristics of *L. monocytogenes*. It is proposed that *L. monocytogenes* is widespread in the food environment, thus it is able to enter into the food production chain almost anywhere.

Table 2.1. Some growth and inactivation characteristics of *L. monocytogenes* in lab media (SANCO, 2008)

Factors	**Growth**		
	Minimum	Optimum	Maximum
Temperature (°C)	-1.5	30-37	45
pH	4.2-4.3	7.0	9.4-9.5
a_w	0.93 (0.90 with glycerol)	0.99	> 0.99
Salt concentration (%)	< 0.5	0.7	12-16
Atmosphere	Facultative anaerobe (it can grow in the presence or absence of oxygen, e.g. in a vacuum or modified atmosphere package)		
	Thermal inactivation		
$D_{65\,°C}$	0.2 to 2 min		
z	7.5°C (4 to 11°C)		
	High pressure inactivation		
400 MPa for 10 min at 20°C → 2 log10 reductions in phosphate buffer (pH 7) 400 MPa for 10 min at 20°C → 8 log10 reductions in citrate buffer (pH 5.6) 400 to 500 MPa for 5 to 10 min at 20°C → 3 to 5 log10 reductions in meat products. 350 MPa for 5 to 10 min at 20°C → 3 to 5 log10 reductions in acidic products (e.g. fruit juices, jams).			

In the literature numerous study can be found how to control *L. monocytogenes* (Bigwood et al., 2012; Luo and Oh, 2016; Strydom et al., 2016) and to understand its behavior in the food system (Melo et al., 2015; Smet et al., 2015). *L. monocytogenes* can be controlled with hurdle technology. Luo and Oh (2016) used the acidic electrolyzed water, ultrasound and mild heat treatment (60 °C, 1 min) against *L. monocytogenes* in bell pepper samples and the

10.14751/SZIE.2016.040

combined treatment caused 3 log cycles reduction. Bover-Cid and co-workers (2015) modeled the inactivation of *L. monocytogenes* in dry-cured ham as a function of pressure (347–852 MPa, 5 min/15 °C), water activity (a_w, 0.86–0.96) and fat content (10–50%). At lower pressure, higher inactivation of *L. monocytogenes* was observed by increasing the fat content above 30%. The results indicate the relevant influence of intrinsic factors, which have to be known to inactivate *L. monocytogenes*.

To control *L. monocytogenes* different antimicrobial substances such as nisin, different bacteriocins, carvacrol, chitosan etc. were also used (Esteban and Palop, 2011; Samelis et al., 2003).

Samelis et al. (2003) used nisin successfully against *L. monocytogenes* in Greek whey cheese. The authors inoculated the cheese samples with 10^4 CFU/g of *L. monocytogenes* and stored at 4 °C. After 10 days *L. monocytogenes* exceeded 7 log CFU/g in cheese samples without nisin in contrary to the samples with nisin where the number of *L. monocytogenes* was below the inoculation level for 45 days.

2.2. Lactic acid bacteria

Lactic acid bacteria are Gram-positive, non-spore forming rods or cocci; mostly aerotolerant anaerobes, therefore they are catalase- and oxidase–negative. LAB have a complicated taxonomy, because they do not have strict taxonomy and possibly they are most numerous group of bacteria linked to humans (Makarova and Koonin, 2007). Most of the LAB belongs to the order *Lactobacillales*, but a few LAB species belong to the *Actinobacteria.* In Fig. 2.2. the phylogenetic tree of *Lactobacillales* can be seen according to Makarova and Koonin (2007).

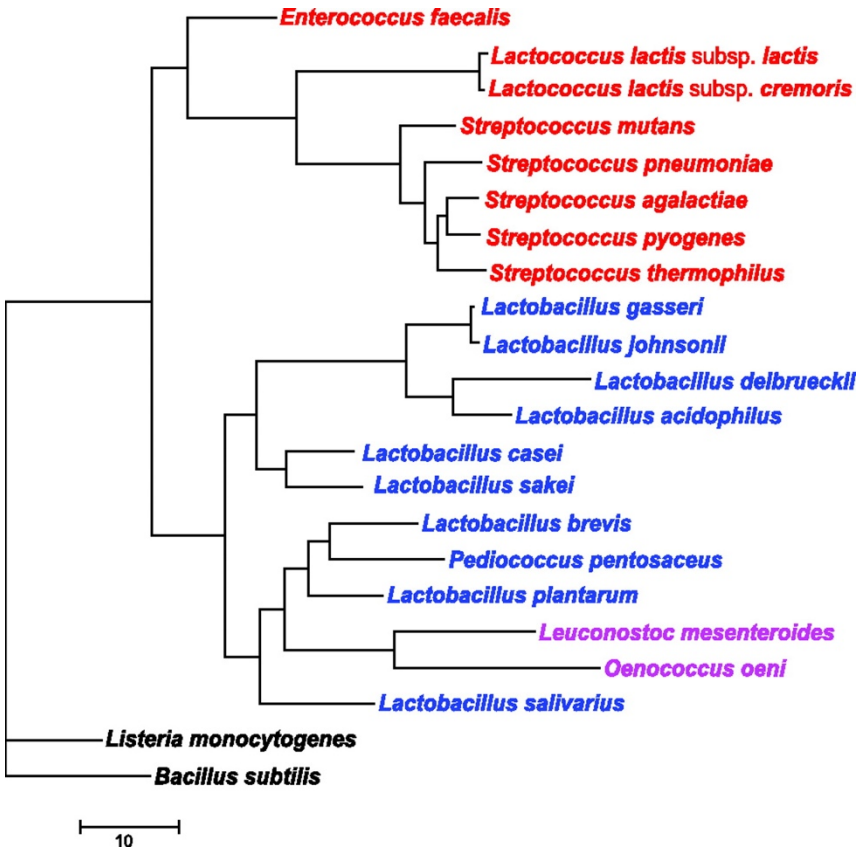


Figure 2.2. The phylogenetic tree of *Lactobacillales*. The species are colored according to the current taxonomy: *Lactobacillaceae* blue; *Leuconostocaceae* magenta; *Streptococcaceae* red. (Makarova and Koonin, 2007)

The *Lactobacillales* clade has closely related species such as *Listeria spp*., *Bacillus spp*., *Staphylococcus spp*. LAB strains have two groups according to the fermentation of carbohydrates. The homofermentative LAB (*Pediococcus spp*., *Lactoccus spp*.) produce lactate and the heterofermentative LAB (*Leuconostoc spp.,* certain *Lactobacillus spp.*) produce lactate, acetate and ethanol. During growth LAB strains are able to produce several antimicrobial substances like bacteriocin, lactate (which leads to drop of pH), H_2O_2, ethanol,

10.14751/SZIE.2016.040

diacetyl, organic acid (Adams and Moss, 2008). In the past decade numerous research (Albano et al., 2007; Callewaert et al., 2000; Martinez et al., 2013) focused on bacteriocins which are biologically active proteins/polipeptides with antibacterial behavior and are usually active against the closely related strains. Bacteriocins can be grouped into three different classes (Table 2.2.).

Table 2.2. Classification of bacteriocins according to Yang et al., 2014

Classification	Features		Bacteriocins	Molecular weight (KDa)	Producing strain
Class I	The bacteriocins are post-translationally modified, linear or globular peptides containing lanthionine, β-methyl lanthionine and dehydrated amino acids		Nisin A, Z	< 5	*Lactococcus lactis* subsp. *lactic*
			Nisin U		*Streptococcus uberis*
Class II	Heat stable, unmodified, non-lanthione-containing bacteriocins, heterogeneous class of small peptides	Class IIa – pediocin PA-1 like bacteriocins	Pediocin PA-1	< 10	*Pediococcus acidilactici*
		Class IIb – composed of two peptides	Lactacin F		*Lactobacillus spp.*
			Plantaricin A, S, EF, JK		*Lactobacillus plantarum*
		Class IIc – circular peptide	Carnocyclin A		*Carnobacterium maltaromaticum*
		Class IId – linear, non-pediocin like, single-peptide	Enterocin AS		*Enterococcus faecalis*
Class III	Large, non heat stable protein		Helveticin J	> 30	*Lactobacillus helveticus*

Because of the above mentioned advantages, it has a long tradition to use *Lactobacillales* in the food industry. LAB strains are used for preservation of fermented foods. They are either present in the raw materials as contaminant and/or added as starters to control the fermentation. The metabolites and enzymatic reactions of LAB contribute to the organoleptic, rheological and nutritional properties of fermented foods (Mayo et al., 2010).

2.2.1. Application of lactic acid bacteria in the food industry

The LAB strains are commonly used as starter cultures in the fermented food industry – e.g. dairy, meat, vegetable products – (Table 2.3.) because of the above mentioned beneficial

properties. The commonly used LAB strains are the following: in dairy industry *Lc. lactis, Lb. casei, Streptococcus thermophilus, Lb. acidophilus*; in meat industry *Lb. sakei, Lb. curvatus, P. acidilactici, P. pentosaceus*; in fermented vegetables *Leuconostoc mesenteroides, Lb. plantarum, P. acidilactici, Lb. brevis*. In recent years the researchers are focusing the LAB strains as protective cultures. In the following part the current status of using LAB strains against pathogens and spoilage microorganism will be presented.

Table 2.3. Fermented food products and beverages and their associated lactic acid bacteria (Vesković Moračanin et al., 2014)

Type of fermented product	Lactic acid bacteria
Dairy products	
Hard cheeses without eyes	*Lactococcus lactis* ssp. *lactis, Lc. lactis* ssp. *cremoris*
Cheeses with small eyes	*Lc. lactis* ssp. *lactis, Lc. lactis* ssp. *lactis* var. *diacetylactis, Lc. 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	*Lc. lactis* ssp. *lactis, Lc. lactis* ssp. *lactis* var. *diacetylactis, Lc. lactis* ssp. *cremoris, Ln. mesenteroides* ssp. *cremoris*
Yoghurt	*Lb. delbrueckii* ssp. *bulgaricus, Str. 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*
Other 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*

10.14751/SZIE.2016.040

In cheese (Argentinian Port Salut cheese) the nisin and natamycin improved the microbiological stability during refrigeration. Edible film was prepared with nisin and natamycin and covered the surface of the cheese samples. The film inhibited the growth of yeasts and moulds and controlled the growth of psychrotrophic bacteria originally present in the cheese and also inhibited the artificially inoculated *Saccharomyces cerevisiae* and *L. innocua* throughout the entire storage (Ollé Resa et al., 2016). Geremew et al. (2015) examined the effect of lactic acid bacteria with spices in cottage cheese to extend the shelf life. Lactobacillus isolates showed antimicrobial activity against *E. coli, S. aureus, Shigella flexneri* and *Streptococcus pneumoniae*.

The synergistic effect of spices together with LAB might contribute a lot to preserve and extend shelf life of cottage cheese, thus, the preservation technique can reduce the risk of spoilage and pathogenesis.

In their study Jones et al., (2008) identified candidate strains suitable to screen meat and meat process-related LAB strains for inhibition of pathogens and spoilage organisms. Meat-derived strains including *Lb. sakei* and *Lactococcus lactis*, were found to have inhibitory effect against one or more of the target strains such as *Listeria monocytogenes*, *Brochothrix thermosphacta*, *Campylobacter jejuni* and *Clostridium estertheticum*. Variations were detected in the antimicrobial activity of LAB strains.

10.14751/SZIE.2016.040

2.3. Biofilms in the food industry

Bacteria generally exist in two forms: planktonic (freely floating in the media) and sessile (attached to a surface or within a biofilm). Biofilms were observed as early as 1674, when Antonie van Leuwenhoek used his microscope to describe aggregates of "animalcules" that he scraped from human tooth surfaces (Garrett et al., 2008). Bacterial communities such as biofilm have an important role in food safety. Different pathogenic and non-pathogenic bacteria are able to produce biofilms. The formation of biofilms has 5 different stages as it can be seen on Fig. 2.3. The 5 steps are the followings: (1) initial attachment, (2) irreversible attachment, (3) micro-colony development by proliferation, (4) biofilm formation, and (5) dispersal (Mizan et al., 2015).

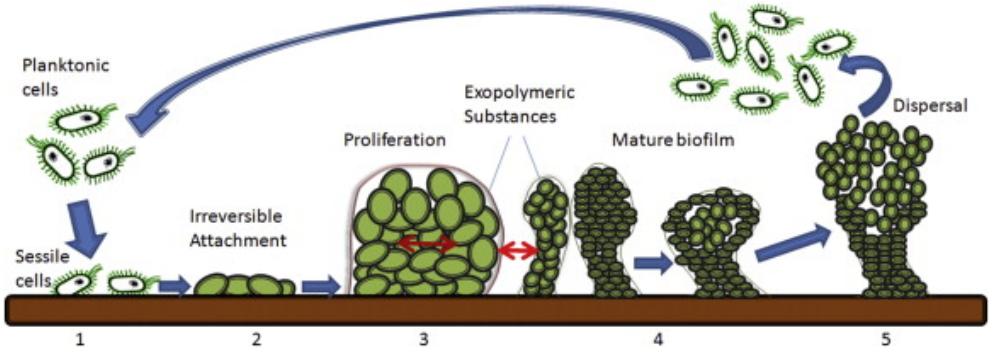


Fig. 2.3. The formation of biofilm (Mizan et al., 2015)

The formation of biofilms is the attachment of microorganisms to the conditioned surface and the two main steps are the reversible and irreversible adhesions. In irreversible adhesion, various short-range forces (hydrogen, ionic and covalent bonding) and exopolysaccharide (EPS) production are involved (Kumar and Anand, 1998). Living within the biofilm has several benefits for microorganisms. These advantages are protection from: antibiotics (i), disinfectants (ii) and different environment conditions (lack of nutrients, extreme conditions etc.) (iii). According to Garrett and co-workers (2008) about 99% of the world's population of bacteria are found in the form of a biofilm at various stages of growth and the films are as diverse as numerous the bacteria are.

Linked to the food industry the most studied pathogenic biofilm producers are *Pseudomonas aeruginosa, E. coli, L. monocytogenes, Campylobacter jejuni* and *Vibrio cholerae.*

The resistance of *L. monocytogenes* was examined in mono-cultured biofilms against hydrogen peroxide-based agent, a mixture of quaternary ammonium compounds and

10.14751/SZIE.2016.040

chlorine. The response of *L. monocytogenes* has changed in the biofilm, which led to resistant *L. monocytogenes* cultures (Pan et al., 2006).

Giaouris et al. (2013) studied the resistance to benzalkonium chloride of co-cultured *L. monocytegenes* with *Ps. putida*. In mixed conditions, the *L. monocytogenes* strongly increased the resistance of *P. putida* in biofilm to benzalkonium chloride. After disinfection with benzalkonium chloride, the majority of killed cells belonged to *L. monocytogenes*, while the remaining viable community was more than 90% composed by *P. putida* cells. When *L. monocytogenes* was co-cultured with *Ps. aeruginosa*, the latter species was found to be dominant during the formation of biofilm. The response of the mixed culture to the commercial dairy sanitizers was examined at two different temperatures (12 and 37 °C) by Lourenço and co-workers (2011). The authors found that the resistance of *L. monocytogenes* increased, when it was co-cultured with *Ps. aeruginosa*. The results also showed the importance of the temperature, the biofilm formed at 12 °C was more resistant than biofilm formed at 37 °C. Based on these facts the behavior of mixed-culture biofilms could provide the information necessary to control them, thus to reduce the contamination in the food.

Winkelströter et al. (2015) examined the effect of *Lb. paraplantarum* on biofilm formation of *L. monocytogenes*. The *Lb. paraplantarum* isolated from cheese and its bacteriocin production was confirmed; it belonged to the class II bacteriocins. The *Lb. paraplantarum* has an ability to perform biofilm itself and in mixed culture with *L. monocytogenes*, too. The *L. monocytogenes* cells were significantly reduced in the mixed biofilm, however there was no information about bacteriocin activity in this biofilm formation. In other paper Winkelströter and De Martinis (2013) examined the biofilm formation of *L. monocytogenes* in the supernatant of lactic acid bacteria, which probably contained bacteriocins. The inhibition of biofilm formation was observed by the authors. It was also demonstrated by Winkelströter and De Martinis (2013) that the supernatants of LAB strains inhibited the invasion of Caco-2 cells by *L. monocytogenes*.

Speranza and co-workers (2009) considered the non-starter lactic acid bacteria biofilms to control the growth of *Listeria monocytogenes* in soft cheeses. In the study they investigated the following strains: *Lb. plantarum* DSM1055, *Lb. casei* DSM20011, *Lb. curvatus* DSM20019 and *Lb. paracasei* DSM20207. All tested strains were able to form biofilm in mono- and co-culture on stainless steel. Experimental cheeses were made in presence of chips containing 7-days LAB biofilms and inoculated with *L. monocytogenes*. Results showed that LAB biofilms can delay the growth of *L. monocytogenes* in soft cheeses: the maximum cell load attained at the stationary phase was about 6 Log CFU/g in experimental cheeses against about 7 Log CFU/g observed in control samples.

10.14751/SZIE.2016.040

Guerrieri et al. (2009) examined the biofilm formation of two LAB bacteriocin producers (*Lactobacillus plantarum* 35d, Enterococcus casseliflavus IM 416K1) and by two non-producers (*Lb. plantarum* 396/1, *Enterococcus faecalis*) against *Listeria monocytogenes* NCTC 10888 in lab media (TSB broth). It was found that the LAB biofilms were able to influence the survival and the multiplication of *L. monocytogenes*. The bacteriocin producer *Lb. plantarum* 35d showed the highest efficacy in reducing *L. monocytogenes* by the end of the experiment (10 days). The non bacteriocin producer *Lb. plantarum* 396/1 was also able to reduce the biofilm formation of *L. monocytogenes* which can be explained by the pH reduction. Slight capability to influence the *L. monocytogenes* survival by the non-bacteriocinogenic *Enterococcus faecalis* was observed.

10.14751/SZIE.2016.040

CHAPTER 3 - CHARACTERIZATION OF TWO BACTERIOCINS ACTIVE AGAINST *LISTERIA* SPECIES PRODUCED BY *LACTOBACILLUS PLANTARUM* AND *LACTOBACILLUS SAKEI*

3.1. Introduction

Fermented meat products are part of daily diet in Europe and fermented sausages have become more popular in urban centers in Hungary and Portugal, too. Meat products are excellent substrates for growth of food-borne pathogen microorganisms. To prevent the growth of pathogenic microbes the industry uses several preservatives (nitrite, nitrate) (Leroy et al., 2006). There is an increasing consumer demand for naturally treated food products, bio-preservatives and innovative foods with potential health benefits (Sidira et al., 2014). In the recent years, there has been an interest in the use of protective lactic acid cultures as starter cultures.

Numerous Gram-positive bacteria produce ribosomally-synthesized peptides that have antimicrobial activity against microorganisms closely related to the producer bacteria. The most well-known antimicrobial substances producing Gram-positive bacteria are lactic acid bacteria (LAB) (Zacharof and Lovitt, 2012), which produce bacteriocins. Application of LAB (*Pediococcus* spp., *Lactococcus* spp., *Lactobacillus* spp. and *Leuconostoc* spp.) could inhibit the foodborne pathogens by acid production and pH reduction, as well as bacteriocin production (Dortu et al., 2008). In the recent years usage of bacteriocinogenic *Lactobacillus* strains as biopreservative cultures is extended to inhibit pathogenic microbiota for meat industry (Todorov et al., 2013). *Lb. sakei* is one of the most important species of the starter cultures used for fermented sausages production (Dortu et al., 2008).

Listeria monocytogenes is a foodborne pathogen, which frequently occures in meat products. Unfortunately, *Listeria* species are able to grow at refrigerated temperature. Numerous scientific articles discuss the possibility to inhibit *L. monocytogenes* by LAB (Balciunas et al., 2013; Martinez et al., 2013; Rodríguez et al., 2000). The aim of my study was to characterize the anti-listerial bacteriocins produced by *Lb. plantarum* ST202Ch and *Lb. sakei* ST153Ch.

Lactobacillus plantarum ST202Ch and *Lb. sakei* ST153Ch were previously isolated from Portuguese fermented sausages (Todorov et al., 2010).

3.2. Materials and methods

3.2.1. Origin of lactic acid bacteria isolates

Lactobacillus plantarum ST202Ch and *Lb. sakei* ST153Ch – (kindly supplied by Svetoslav D. Todorov; Todorov et al., 2010), both strains were previously isolated from fermented meat sausages and deposited in the culture collection of Escola Superior de Biotecnologia (ESB), Universidade Católica Portuguesa (UCP) – were selected for this study. Strains were stored in de Man, Rogosa Sharpe broth (MRS, Biokar Diagnostics) containing 30% (v/v) glycerol at -20 °C, and sub-cultured twice before use in assays.

3.2.2. Pathogenic and indicator strains

Listeria monocytogenes ATCC 7946*, L. monocytogenes* 1486/1 (Listeria Research Center ESB, UCP), *L. innocua* NCTC 11288*, Enterococcus faecalis* ATCC 29212, *E. coli* ATCC 25922, *Klebsiella pneumoniae* (ESB, UCP)*, Pseudomonas aeruginosa* (ESB, UCP)*, Staphylococcus aureus* ATCC 29213*, Salmonella* Typhimurium ATCC 14028 and *Candida albicans* UZ247460 were used as target bacteria for the inhibitory effects of LAB. Bacteria were grown in Tryptone Soy Yeast Extract Broth (TSB-YE; Tryptone Soy Broth (Biokar Diagnostics) + 6 g/l yeast extract (Lab M), at 30 °C or 37 °C for 24 h.
All strains were stored at - 20 °C in TSB containing 30% (v/v) glycerol, and sub-cultured twice before use in assays.

3.2.3. Testing the antibacterial activity

MRS broth (10 ml, Biokar Diagnostics) was inoculated with 1% (v/v) of an overnight culture of each LAB and incubated at 30 °C. After incubation the MRS broth was centrifuged for 10 min at 7000 rpm and the supernatant was kept. The pH of the supernatant was adjusted to pH 5-6 with sterile 1M NaOH and heat treated at 80 °C for 10 min.
TSB broth (10 ml, Biokar Diagnostics) was inoculated with 1% (v/v) of an overnight culture of each indicator organisms (see Chapter 3.2.2.) and incubated at appropriate temperature. After the incubation dilution was performed. The soft TSA agar (TSB broth supplemented with 1% agar, Biokar Diagnostics) was used for pour plating, the final concentration of target strains were 10^6 CFU/ml. After solidification, 10 µl of LAB supernatants were dropped onto

10.14751/SZIE.2016.040

the agar surface, which were incubated at adequate temperatures for 24 hours (van Reenen et al., 2008). After the incubation time an inhibition zone clearly indicated the inhibitory effect.

3.2.4. Bacteriocin production during growth

MRS broth (100 ml, Biokar Diagnostics) was inoculated with 1% (v/v) of an overnight culture of each LAB and incubated at 30 °C. Changes in pH and optical density (OD) (600 nm) were recorded in every hour, for 24 h. Bacteriocin activity (AU/ml) in the cell-free supernatant was recorded in every 3 h for 24 h, as described by Van Reenen et al. (1998). *Listeria monocytogenes* 1486/1 (serogroup IIb; isolated from cheese) and *L. innocua* NCTC 11288 were used as target strains in all measurements.

3.2.5. Effect of enzymes, temperature, pH and detergents on bacteriocin activity

The agar-spot test method (Van Reenen et al., 1998) was used in all tests, as described before by Albano and co-workers (2007). The effect of pH on the activity of bacteriocins was tested by adjusting cell-free supernatants from pH 2.0 to 12.0, with sterile 1 M NaOH or 1 M HCl. After 1 h of incubation at room temperature (25 °C), the samples were re-adjusted to pH 6.0 with sterile 1 M NaOH or 1 M HCl and tested for antimicrobial activity. The effect of temperature on bacteriocin activity was tested by incubating the cell-free supernatants adjusted to pH 6.0 and kept at 4, 25, 30, 37, 60, 80, and 100 °C, respectively, for 1 and 2 h. Bacteriocin activity was also tested after 20 min at 121 °C.

Strains were grown in MRS broth for 15-18 hours at 37 °C. The cells were harvested by centrifugation (7000 rpm, 10 min, 4 °C) and the pH of the cell-free supernatant was adjusted to pH 5-6 with 1 M NaOH. One milliliter cell-free supernatant was incubated at 37 °C for 2 h in the presence of 1 mg/ml and 0.1 mg/ml of each of proteinase K, pepsin and tyrosinase (from mushroom), peroxidase, trypsin, trypsin (from hog), catalase, respectively. To test the effect of detergents, surfactants and protease inhibitors, 1% (w/v) sodium dodecyl sulphate (SDS), Tween 20, Tween 80, urea, Triton X-100, bile salt, urea and NaCl were added to bacteriocin-containing cell-free supernatants. EDTA was added to cell-free supernatants to final concentrations of 0.1, 2.0 and 5.0 mM. Samples were incubated at 37 °C for 5 h and tested for antimicrobial activity.

Listeria monocytogenes (serogroup IIb; isolated from cheese) and *L. innocua* NCTC 11288 were used as target strains for all of the measurements.

3.2.6. Mode of action

Twenty millilitres of a bacteriocin-containing cell-free supernatant (25600 AU/ml, pH 6.0) was filter-sterilized (Frilabo, Cellulose Acetate 0,22 μm Syringe Filter) and added to 100 ml of early exponential phase (3.5-h-old) cultures of *L. monocytogenes* and *L. innocua*, respectively. Growth of *Listeria* strains was monitored before and after the addition of the bacteriocin-containing cell-free supernatant using a spectrophotometer. Optical densities were measured at 600 nm in every hour, for 11 h.

3.2.7. Partial purification and molecular size of bacteriocins

Lb. plantarum ST202Ch and *Lb. sakei* ST153Ch were grown in 300 ml MRS broth for 18 h at 30 °C. Cells were harvested by centrifugation (7000 rpm for 15 min, 4 °C) and the bacteriocin was precipitated from the cell-free supernatant with 70% saturated ammonium sulphate (Yang et al., 1992). After 4 h of slow stirring at 4 °C, proteins were harvested by centrifugation (7000 rpm, 15 min, 4 °C). Precipitated proteins in the pellet and floating on the surface were collected and dissolved in 25 mM ammonium acetate buffer (pH 6.5). All samples were stored at -20 °C.

Molecular size of bacteriocins was determined by tricine - sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) with a Bio-Rad Mini Protean 3 Cell apparatus (Bio - Rad), as described by Schägger and Von Jagow (1987). A molecular weight marker with sizes ranging from 14.4 to 97.4 kDa (SDS-PAGE Standards Low Range, Bio-Rad) was used. The gels were fixed and one half was stained with Coomassie Brilliant Blue G250 (Bio-Rad). The positions of the active bacteriocins were determined by overlaying the other half of the gel (not stained and pre-washed with sterile distilled water) with cells of the test organism (*L. monocytogenes* 1486/1) and TSB+YE agar (1% w/v agar).

3.3. Results and discussion

Cell free supernatant of *Lactobacillus plantarum* ST202Ch and *Lb. sakei* ST153Ch inhibited the growth of *Listeria monocytogenes* ATCC 7946, *L. monocytogenes 1486/1*, *L. innocua* NCTC 11288 and *Enterococcus faecalis* ATCC 29212. No inhibition was observed against *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* ATCC 29213, *Salmonella* Typhimurium ATCC 14028 and *Candida*

10.14751/SZIE.2016.040

albicans UZ247460 (data are not shown). Wide range of class IIa bacteriocins produced by *Lb. plantarum* and *Lb. sakei* are well-knonw, that are active against *Listeria* spp. and *Enterococci* (Kjos et al., 2011). Class IIa bacteriocins have a narrow spectrum of activity but they are especially potent against *Listeria* spp. (Collins et al., 2010), however recent studies reported inhibition activity of class IIa bacteriocins against Gram negative bacteria, eg. *Pseudomonas* spp. and *E. coli* (Gong et al., 2010; Gupta & Tiwari, 2014).

Lactobacillus plantarum ST202Ch produced bacteriocin at the highest level (25600 AU/ml) against *L. monocytogenes* after 15 hours and against *L. innocua* after 18 hours (Fig. 3.1. A-B). For all further investigation the bacteriocins were harvested after 15-18 hours incubation in MRS broth at 30 °C.

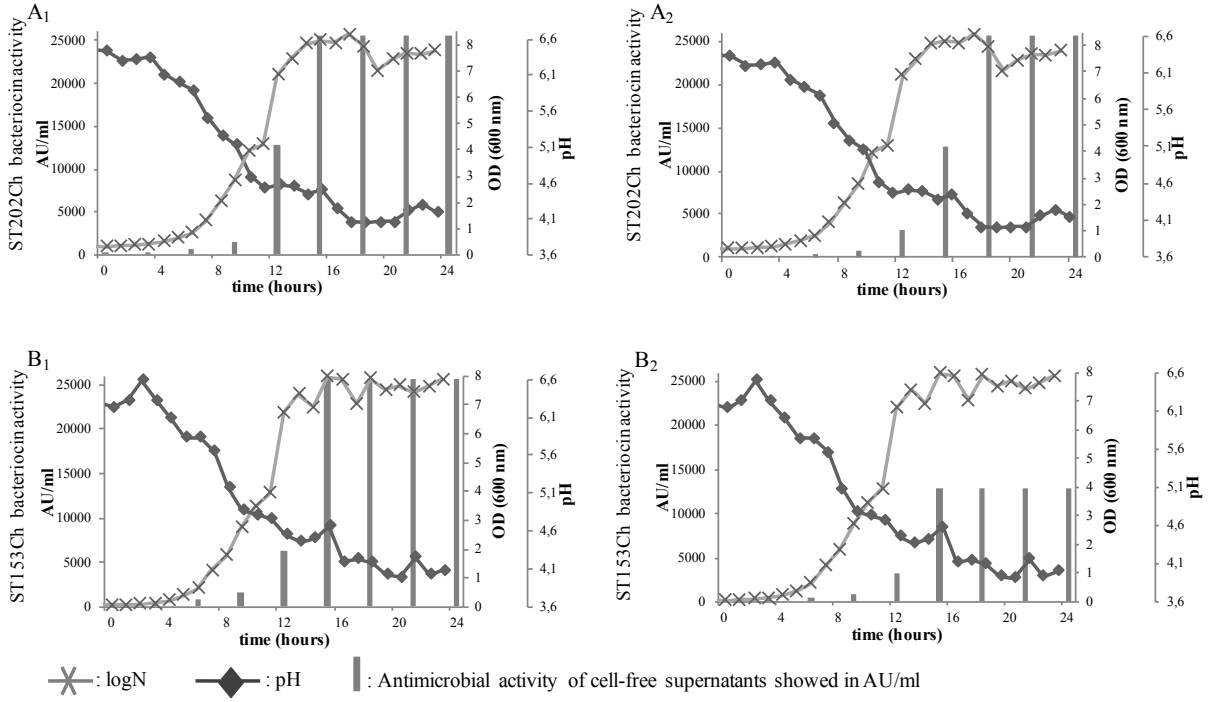


Figure 3.1. Antilisterial activity of neutralized cell-free supernatant produced by *Lb. plantarum* ST202Ch (graph A) and *Lb. sakei* ST153Ch (graph B) in MRS broth at 37 °C. 1: Sensitive strain *L. monocytogenes* 1486/1; 2: Sensitive strain *L. innocua* NCTC 11288.

The maximum bacteriocin activity level was recorded when the pH was around 4.3. The pH decreased from 6.3 to 4 by *Lb. plantarum* ST202Ch and from 6.3 to 3.9 by *Lb. sakei* ST153Ch. In both cases, the cell density increased in the same way, *Lb. plantarum* ST202Ch increased from 0,33 to 7,79 logN and *Lb. sakei* ST153Ch increased from 0,06 to 7,86 logN count. Similar results were recorded by Dortu et al. (2008) and Martinez et al. (2013).

10.14751/SZIE.2016.040

The effect of enzymes, temperature, pH and detergents on bacteriocin activity are shown in Table 3.1.

Table 3.1. Reduction of antimicrobial activity of bacteriocins produced by *Lb. plantarum* ST202Ch and *Lb. sakei* ST153Ch (expressed in percentage values) after incubation under different conditions

		L. plantarum ST202Ch				*L. sakei* ST153Ch			
		L. monocytogenes 1486/1		*L. innocua* NCTC 11288		*L. monocytogenes* 1486/1		*L. innocua* NCTC 11288	
		after 1hour	after 2 hours	after 1hour	after 2 hours	after 1hour	after 2 hours	after 1hour	after 2 hours
Temperature	4 °C	0%	0%	50%	50%	0%	0%	25%	25%
	25 °C	0%	0%	75%	75%	0%	0%	75%	75%
	30 °C	0%	0%	75%	88%	0%	0%	50%	50%
	37 °C	0%	0%	75%	75%	0%	0%	50%	50%
	60 °C	0%	50%	75%	88%	0%	0%	50%	75%
	80 °C	0%	50%	88%	94%	0%	0%	50%	75%
	100 °C	50%	88%	88%	97%	50%	94%	75%	94%
	121 °C after 15 min	97%		99%		97%		98%	
	4 °C after 1 month	0%		75%		0%		50%	
pH (after 2h, at 25 °C)	2	50%		75%		75%		75%	
	4	50%		88%		50%		50%	
	6	75%		94%		75%		75%	
	8	50%		88%		75%		75%	
	10	0%		75%		0%		50%	
	12	94%		98%		88%		94%	
Detergents 1mg/ml (after 5h, at 30 °C)	Tween 20	75%		94%		0%		75%	
	Tween 80	75%		94%		0%		75%	
	Triton X-100	75%		88%		50%		75%	
	SDS	0%		75%		0%		75%	
	Bile salt	75%		94%		50%		88%	
	Urea	75%		94%		0%		75%	
	NaCl	0%		88%		0%		75%	
	0.1 mM EDTA	0%		88%		0%		88%	
	2 mM EDTA	50%		94%		0%		88%	
	5 mM EDTA	75%		94%		0%		88%	
Enzymes (after 2h, at 30 °C)		1 mg/ml	0.1 mg/ml	1 mg/ml	0.1 mg/ml	1 mg/ml	0.1 mg/ml	1 mg/ml	0.1 mg/ml
	protease K	100%	100%	100%	100%	100%	100%	100%	100%
	tyrosinase from mushrooms	100%	94%	100%	99%	100%	88%	100%	97%
	peroxidase	97%	88%	98%	94%	99%	50%	100%	88%
	pepsin	99%	75%	100%	94%	99%	75%	100%	88%
	trypsin	100%	98%	100%	99%	100%	98%	100%	98%
	catalase	88%	50%	94%	94%	75%	0%	88%	50%

10.14751/SZIE.2016.040

Complete inactivation or significant reduction in antimicrobial activity was observed after treatment of bacteriocin *Lb. plantarum* ST202Ch and *Lb. sakei* ST153Ch with protease K, trypsin, tyrosinase at 1mg/ml, pepsin at 1mg/ml and peroxidase at 1mg/ml. No complete inactivation was recorded when treated with catalase and peroxidase.

Treatment of the bacteriocin produced by *Lb. sakei* ST153Ch with 1% (w/v%) Tween 20, Tween 80, SDS, urea, NaCl, 0.1 mM EDTA, 0.2 mM EDTA, 5 mM EDTA and bacteriocin produced by *Lb. plantarum* ST202Ch with SDS, NaCl, 0.1 mM EDTA did not reduced the activity against *L. monocytogenes*. Significant reduction was observed against *L. innocua*. Reduction in activity also observed when bacteriocin ST202Ch was treated with Tween 20, Tween 80, Triton X-100, bile salt, urea, 0.2 mM EDTA, 5 mM EDTA. Similar results were reported by Martinez et al. (2013) and Milioni et al. (2015).

Antimicrobial activity against *L. innocua* was reduced after incubation at 25, 30, 37, 60, 80, 100 °C for 2 hours. Bacteriocin effect against *L. monocytogenes* remained stable after incubation at 4, 25, 30, 37, 60, 80, 100 °C for 2 hours. No complete inactivation was observed after treatment at 121 °C for 15 min. Both bacteriocins against *L. monocytogenes* remained stable after storage at 4 °C for 1 month. It is one of the main advantages, when *Lb. plantarum* and *Lb. sakei* used in the meat industry.

Antimicrobial activity was reduced at pH values (2, 4, 6, 8, 12), suggesting the peptides are sensitive to alkaline and acidic conditions.

The effects of detergents were different to the bacteriocin produced by *Lb. plantarum* ST202Ch and *Lb. sakei* ST153Ch, no change in activity was observed against *L. monocytogenes* in case of bacteriocin produced by *Lb. sakei* ST153Ch (except Triton X-100 and bile salt). Similar results reported by Jiang et al. (2012), Martinez et al. (2013) and Zhu et al. (2014).

It should be highlighted, that *L. innocua* showed to be more resistant in every case.

The addition of bacteriocin produced by *Lb. plantarum* ST202Ch and *Lb. sakei* ST153Ch (25600 AU/ml) (Fig. 3.2.) to mid-log phase (3.5 hours old) *L. monocytogenes* ($OD_{600nm} \approx 0.091$) and *L. innocua* ($OD_{600nm} \approx 0.068$) cultures showed a clear growth inhibition. Bacteriocins had bacteriostatic effect on *L. monocytogenes* and *L. innocua* (Figure 3.2.). Similar results were reported by Hernández et al. (2005) and Katla et al. (2001).

10.14751/SZIE.2016.040

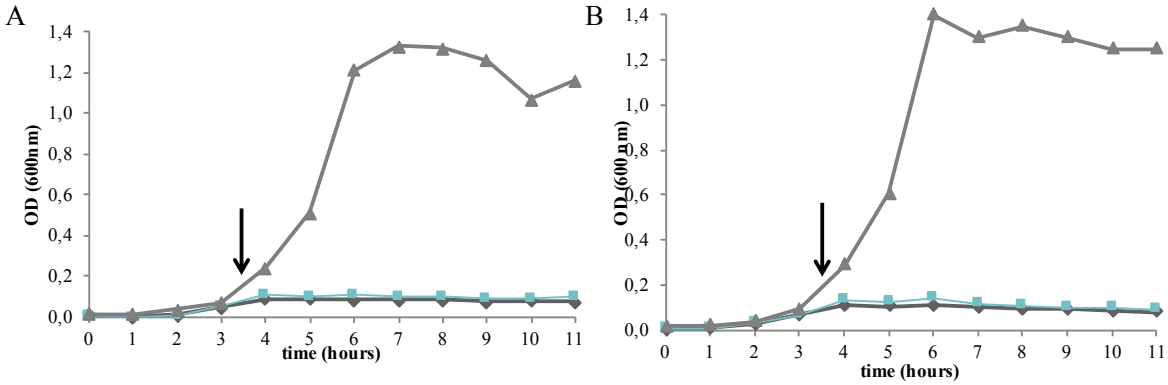


Figure 3.2. Effect of bacteriocins ST202Ch (♦) and ST153Ch (▪) on *L. monocytogenes* 1486/1 (A) and *L. innocua* NCTC 11288 (B) cultured at 37 °C. The symbol (▲) represents the growth of *L. monocytogenes* 1486/1 (A) and *L. innocua* NCTC 11288 (B) without added bacteriocins (controls). The arrow indicates the point at which the bacteriocins were added (3.5 h).

No effect was recorded in untreated (controls) samples in case of the tested *Listeria* species. Bacteriocin activity was detected after the treatment of *Lb. plantarum* and *Lb. sakei* strains with 100 mM NaCl at pH 2.0, suggesting that the bacteriocins did adhere to the surface of *Lb. plantarum* and *Lb. sakei* (data are not shown). Different results were reported for *Lb. plantarum* (Todorov and Dicks, 2006; Xie et al., 2011) and *Lb. sakei* (Todorov and Dicks, 2004).

10.14751/SZIE.2016.040

Bacteriocins produced by *Lb. plantarum* ST202Ch (Fig 3.3.) and *Lb. sakei* ST153Ch are lower than 14.4 kDa (data are not shown), determined by Tricine-SDS-PAGE.

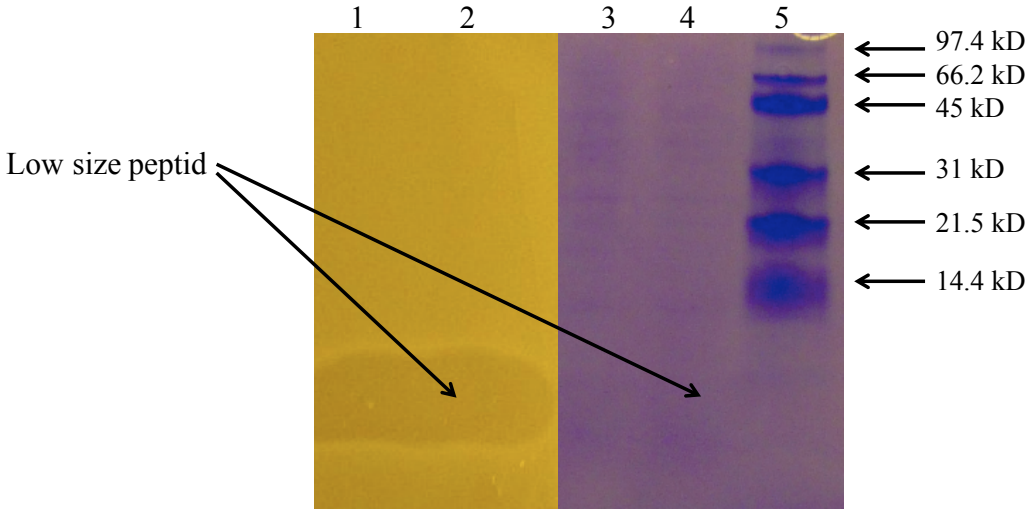


Figure 3.3. Tricine-SDS-PAGE of bacteriocins ST202Ch. Lane 1: zone of growth inhibition, corresponding to the position of the peptide bands in lane 3; lane 2: zone of growth inhibition, corresponding to the position of the peptide bands in lane 4; lane 3: peptide bands stained with Coomassie Blue R250 (40% ammonium sulphate saturated); lane 4: peptide bands stained with Coomassie Blue R250 (60% ammonium sulphate saturated); lane 5: molecular mass marker; The gel in lanes 1 and 2 was covered with viable cells of *L. monocytogenes* 1486/1 (approx. 10^6 CFU/ml), embedded in TSA agar. Incubation was at 37 °C for 24 h.

3.4. Conclusions

Most bacteriocins produced by different LAB strains showed antilisterial activity as well as lactobacilli strains (Martinez et al., 2013; Dortu et al., 2008; Rodríguez et al., 2000). Both bacteriocins - which were tested in this study – may be used in the food industry as bio-preservative agents since they seem to be stable to environmental circumstances e.g.: wide range of pH, low temperature. Further measurements are needed to understand which environmental factors may influence the bacteriocin production, which could increase the application capability of both strains.

10.14751/SZIE.2016.040

CHAPTER 4 - ANTILISTERIAL ACTIVITY OF BACTERIOCINOGENIC *PEDIOCOCCUS ACIDILACTICI* HA6111-2 AND *LACTOBACILLUS PLANTARUM* ST202Ch GROWN UNDER PH, THERMAL AND OSMOTIC STRESS CONDITIONS*

4.1. Introduction

Potential applications of bacteriocins produced by lactic acid bacteria (LAB) - *Lactobacillus spp., Leuconostoc spp., Lactococcus spp., Pediococcus spp*. - by the food industry have been extensively investigated (Balciunas et al., 2013; Callewaert et al., 2000; Devi and Halami, 2011; Kouakou et al., 2010; Mills et al., 2011; Työppönen et al., 2002). Several antilisterial bacteriocins from *Lactobacillus plantarum* (Martinez et al., 2013; Powell et al., 2007; Xie et al., 2011; Zacharof and Lovitt, 2012) and *Pediococcus acidilactici* (Albano et al., 2007; Altuntas et al., 2010) have been described and characterized in the last years. Pediocin PA-1/AcH (Devi and Halami, 2011), pediocin SA-1 (Anastasiadou et al., 2008), plantaricin ASM1 (Hata et al., 2010), plantaricin S (Martinez et al., 2013) and plantaricin MG (Gong et al., 2010) are heat stable and show stability at wide range of pH values. This stability potentiates their application in the food industry as bio-preservatives. Työppönen et al. (2002) successfully used *Lb. plantarum* - as starter culture - to inhibit *Listeria monocytogenes* in dry sausages. *L. monocytogenes* was not detected during the ripening process. Kouakou et al. (2010) studied the effect of *P. acidilactici* in *L. monocytogenes*-seeded raw pork meat. Populations of *L. monocytogenes* were initially reduced, but repair of *L. monocytogenes* was observed after one week.

Increasing consumer demand for less processed and traditional food products has led to the use of biopreservatives to inhibit spoilage and pathogenic microorganisms (Balciunas et al., 2013). Bacteriocin producing LAB can be used as biopreservatives in starter or adjuvant cultures in food fermentations (Cui et al., 2012; Ghanbari et al., 2013; Jiang et al., 2012). Consequently, the food industry is interested in stable cultures that retain the ability to produce bacteriocins under a range of stresses that may be encountered in food products.

Several reviews have addressed the responses of LAB to environmental stresses (Champomier-Vergés et al., 2010; Van de Guchte et al., 2002). The majority of previous publications deal with gene expression in LAB under different environmental conditions (De Angelis et al., 2001; Fadda et al., 2010; Lemos et al., 2001). To my knowledge there

*: Engelhardt T. et al. (2015), *Food Microbiology* 48, 109-115.

10.14751/SZIE.2016.040

have been few attempts to examine the production of bacteriocins by *Lb. plantarum* and *P. acidilactici* during growth under stressful conditions. Production of the bacteriocin amylovorin by *Lactobacillus amylovorus* in MRS broth was stimulated by stressful conditions, resulting in lower growth rates and increased activity against *Lactobacillus delbrueckii* subsp. *bulgaricus* (De Vuyst et al., 1996). Neysens et al. (2003) - based on observations by De Vuyst et al. (1996) - studied the growth limits and bacteriocin production of *Lb. amylovorus* under different stresses (temperature, pH, NaCl) during sourdough fermentation. Stress was found to induce biphasic fermentation kinetics and moderate production of amylovorin.

Bacteriocin production by LAB strains and their application as natural antimicrobials are well known (Balciunas et al., 2013). For industrial applications, it is important to know whether bacteriocins are produced under unfavorable environmental circumstances. Detailed examination of bacteriocin production under defined conditions in laboratory media is prerequisite to their consideration for use in food products. The latter should serve to clarify reasons for low bacteriocin production that are often reported when bacteriocin-producing strains are added to food matrices (Kouakou et al., 2010). To understand this phenomenon, we studied the capacity of *P. acidilactici* HA-6111-2 and *Lb. plantarum* ST202Ch to inhibit *L. monocytogenes* and non-pathogenic *Listeria innocua* when grown in a laboratory culture medium under controlled stress conditions. These strains have been shown to produce antilisterial bacteriocins (Albano et al., 2007; Todorov et al., 2010).

4.2. Materials and methods

4.2.1. Origin of bacterial isolates

Bacteriocin producer *P. acidilactici* HA-6111-2 (Albano et al., 2007) and *Lb. plantarum* ST202Ch (kindly supplied by Svetoslav D. Todorov; Todorov et al., 2010), both previously isolated from fermented meat sausages and deposited in the culture collection of Escola Superior de Biotecnologia (ESB), were selected for this study.

Three isolates of *L. monocytogenes* from the culture collection of the Listeria Research Center of ESB (LRCESB) (1486/1, serogroup IIb, isolated from cheese; 1604/2, serogroup IVb, isolated from cheese; 971, serogroup IIb, isolated from ground beef) and *L. innocua* NCTC 11288 were selected as target strains.

10.14751/SZIE.2016.040

4.2.2. Growth and storage conditions

Lactic acid bacteria were cultured in de Man, Rogosa and Sharpe (MRS) broth (Biokar) at 37 °C for 18-22 hours; *Listeria spp*. were grown in Tryptone Soy Broth (TSB; Biokar) supplemented with 0.6% (w/v) of yeast extract (LabM) (TSBYE) at 37 °C for 18-22 hours. All strains were stored at 20 °C in appropriate culture media containing of 15% (v/v) glycerol. All bacterial strains were subcultured twice under appropriate conditions before use in experiments.

4.2.3. Preliminary examination of growth under different stress conditions

The effect of pH and salt concentration on the growth of each LAB was examined in preliminary trials to establish threshold conditions for further experimentation. Each strain was grown in MRS broth at 30 °C adjusted to different pH with 10% HCl or 1 M NaOH (3.5, 4.0, 4.5, 5.0, 8.0, 8.5, 9.0, 9.5), different NaCl concentrations (2.5, 5.0, 7.5 10.0%) and at 5, 10, 15, 40, 42, 50, 55 °C.

Table 4.1. Growth of *P. acidilactici* HA6111-2 and *Lb. plantarum* ST202Ch under different environmental conditions after 48 hours

	pH							
	3,5	4	4,5	5	8	8,5	9	9,5
P. acidilactici HA6111-2	++*	++	+++	+++	+++	++*	+	no growth
Lb. plantarum ST202Ch	++*	++	+++	+++	+++	++*	+	no growth

	NaCl concentration			
	2,5%	5%	7,5%	10%
P. acidilactici HA6111-2	+++	++	++*	no growth
Lb. plantarum ST202Ch	+++	++	++*	no growth

	Temperatures							
	5 °C	10 °C	15 °C	40 °C	42 °C	45 °C	50 °C	55 °C
P. acidilactici HA6111-2	+	++*	++	+++	+++	+++	++*	no growth
Lb. plantarum ST202Ch	no growth	+	++*	+++	++*	no growth	no growth	no growth

+: poor growth
++: weak growth
+++: good growth
*: selected conditions for further investigations

10.14751/SZIE.2016.040

Table 4.1. shows the different pH, NaCl and temperature concentrations tested and the results obtained by visual inspection of turbidity where "+" denoted weak growth; "++", moderate growth, and "+++" good growth. The most extreme stress conditions where moderate growth was observed after 48 h were selected for further experiments.

4.2.4. Antilisterial activity during growth under stress conditions

Modified MRS broth (Table 4.1.) was inoculated with 1% (v/v) of an overnight culture of each LAB and incubated at 30 °C for 48 hours. Samples were collected every 3 hour for 48 hours and LAB were enumerated on MRS agar incubated at 30 °C for 24 hours. Changes in pH and optical density at 600 nm were also recorded every hour for 48 hours. Antilisterial activity of neutralized cell-free supernatants (hereafter referred as supernatants) was measured every 3 hour for 48 hours, as described by Van Reenen et al. (1998). *L. monocytogenes* 1486/1, *L. monocytogenes* 1604/2, *L. monocytogenes* 971 and *L. innocua* NCTC 11288 were used as target strains.

Antilisterial activity measured in supernatants from cultures grown in MRS adjusted to pH 6.5 incubated at 30 °C was used as a control. All assays were carried out in duplicate.

Viable bacterial counts were expressed as logN and antilisterial activity was expressed as arbitrary units (AU) per ml. One AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition.

4.3. Results and discussion

Results of preliminary growth experiments under stress conditions are presented in Table 4.1. Antilisterial activity was examined where moderate growth was observed after 48 hours under the most extreme stress conditions. The following stress conditions were selected: for *P. acidilactici* HA6111-2, pH 3.5, pH 8.5, 7.5% NaCl, 10 ºC, 50 ºC and for *Lb. plantarum* ESB 202, pH 3.5, pH 8.5, 7.5 % NaCl, 15 ºC, 42 ºC (Table 4.1.).

When *Lb. plantarum* ST202Ch was grown at 30 °C in MRS, the highest level of antilisterial activity (25600 AU/ml) was recorded after 15 h against *L. monocytogenes* 1486/1 and after 18 h against *L. innocua* NCTC 11288. For *P. acidilactici* HA 6111-2, the same level of activity was recorded after 18 h against both Listeria species (Fig. 4.1.).

10.14751/SZIE.2016.040

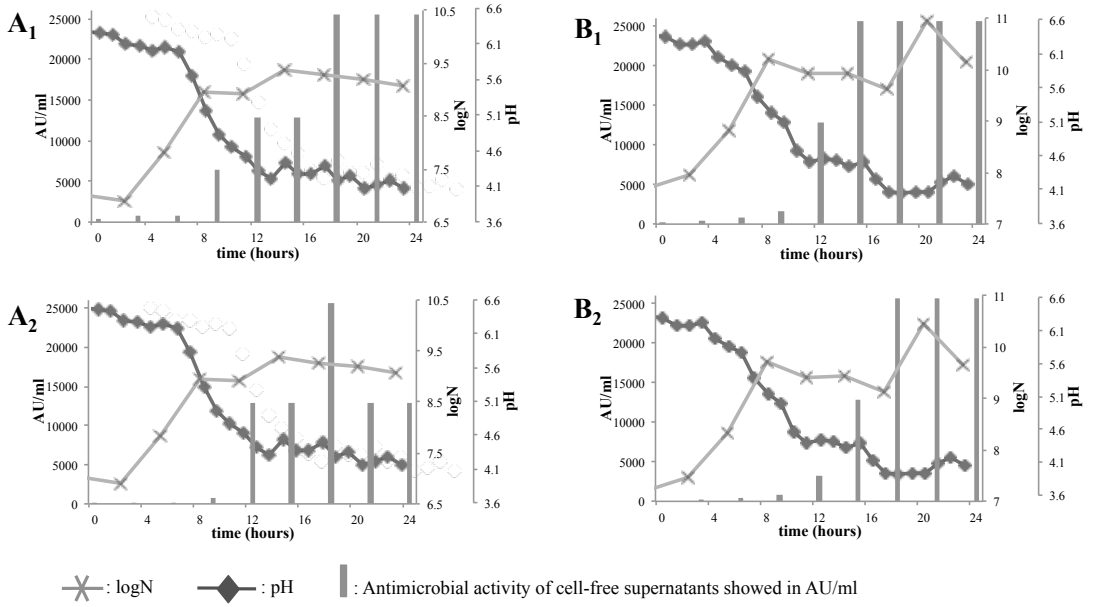


Figure 4.1. Antilisterial activity of neutralized cell-free supernatant produced by *P. acidilactici* HA 6111-2 (graph A) and by *Lb. plantarum* ST202Ch (graph B) during growth in MRS broth (pH 6.5) at 30 °C. 1: Indicator strain *L. monocytogenes* 1486/1, 2: Indicator strain *L. innocua* NCTC 11288. Viable bacterial counts are expressed as logN and antilisterial activity is expressed as arbitrary units (AU) per ml.

Growth of *P. acidilactici* HA 6111-2 and *Lb. plantarum* ST202Ch at pH 8.5 and antilisterial activity are shown in Figs. 4.2. and 4.3., respectively.

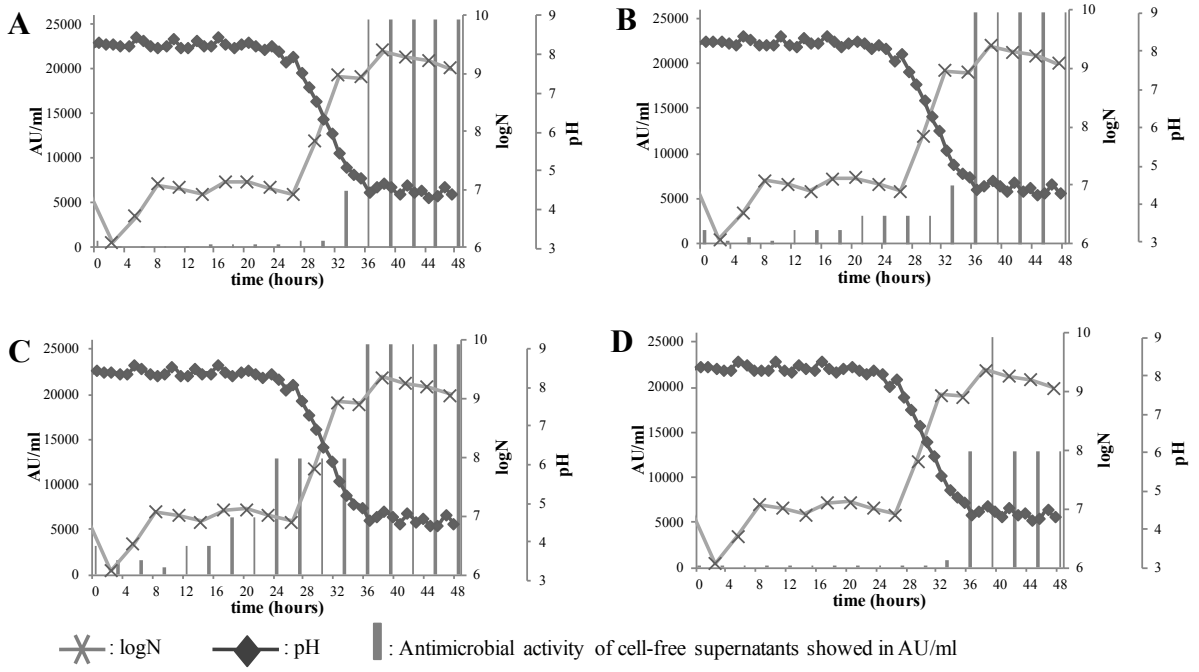


Figure 4.2. Antilisterial activity of neutralized cell-free supernatant produced by *P. acidilactici* HA 6111-2 during growth in MRS broth (pH 8.5) at 30 °C using *Listeria spp*. as indicator strains. Indicator strains: *L. monocytogenes* 1486/1 (A), *L. monocytogenes* 1604/2 (B), *L. monocytogenes* 971 (C), *L. innocua* NCTC 11288 (D). Viable bacterial counts are expressed as logN and antilisterial activity is expressed as arbitrary units (AU) per ml.

10.14751/SZIE.2016.040

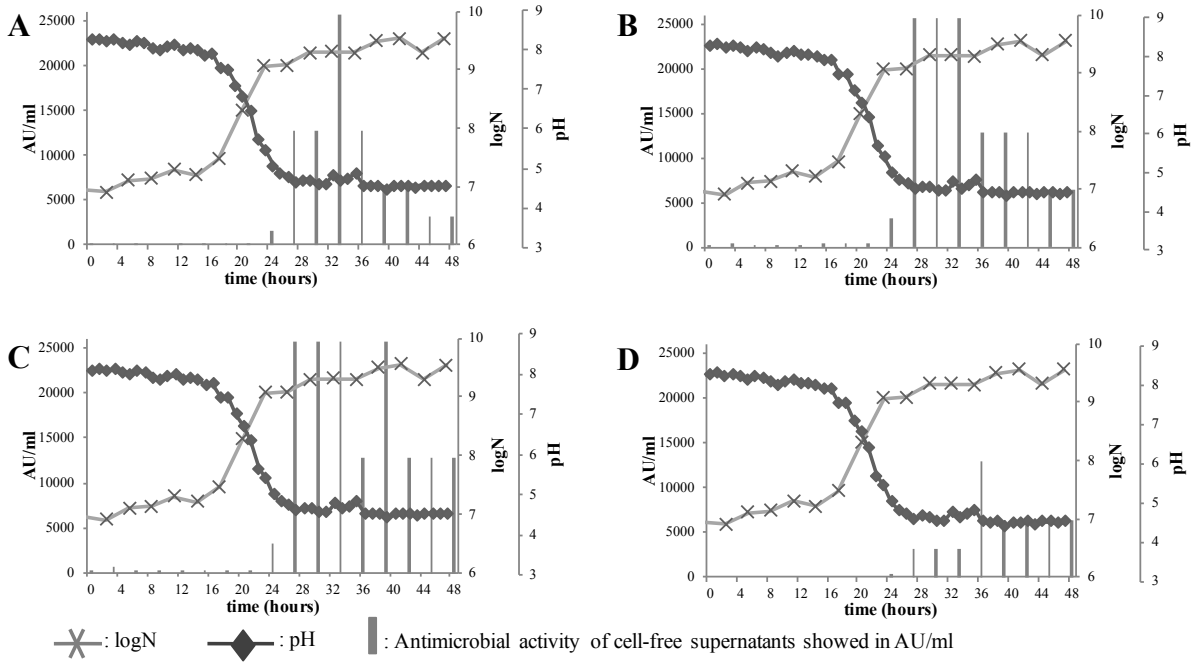


Figure 4.3. Antilisterial activity of neutralized cell-free supernatant produced by *Lb. plantarum* ST202Ch during growth in MRS broth (pH 8.5) at 30 °C using *Listeria* spp. as indicator strains. Indicator strains: *L. monocytogenes* 1486/1 (A), *L. monocytogenes* 1604/2 (B), *L. monocytogenes* 971 (C), *L. innocua* NCTC 11288 (D). Viable bacterial counts are expressed as logN and antilisterial activity is expressed as arbitrary units (AU) per ml.

For both LAB, an extended lag phase was observed as well as a delay in antilisterial activity. LAB are able to produce hyaluronic acid instead of lactic acid under alkaline stress (Liu et al., 2008). In this way cells are able to decrease the pH to levels appropriate for growth. This can be an explanation for the adaptation of *P. acidilactici* and *Lb. plantarum* to the imposed alkaline stress.

The maximum level of activity against *L. monocytogenes* strains and *L. innocua* was 25600 AU/ml for *P. acidilactici* and 25600 AU/ ml and 12800 AU/ml, respectively, against *L. monocytogenes* strains and *L. innocua*, for *Lb. plantarum*. Maximum antilisterial activity was clearly observed in the stationary phase, suggesting that bacteriocins may be secondary metabolites (Powell et al., 2007). Nevertheless, Parente et al. (1994) found bacteriocin was produced as a primary metabolite at different pH values.

The growth and antlisterial activity in cell free supernatants of *P. acidilactici* and *Lb. plantarum* at pH 3.5 are shown in Figs. 4.4. and 4.5., respectively.

10.14751/SZIE.2016.040

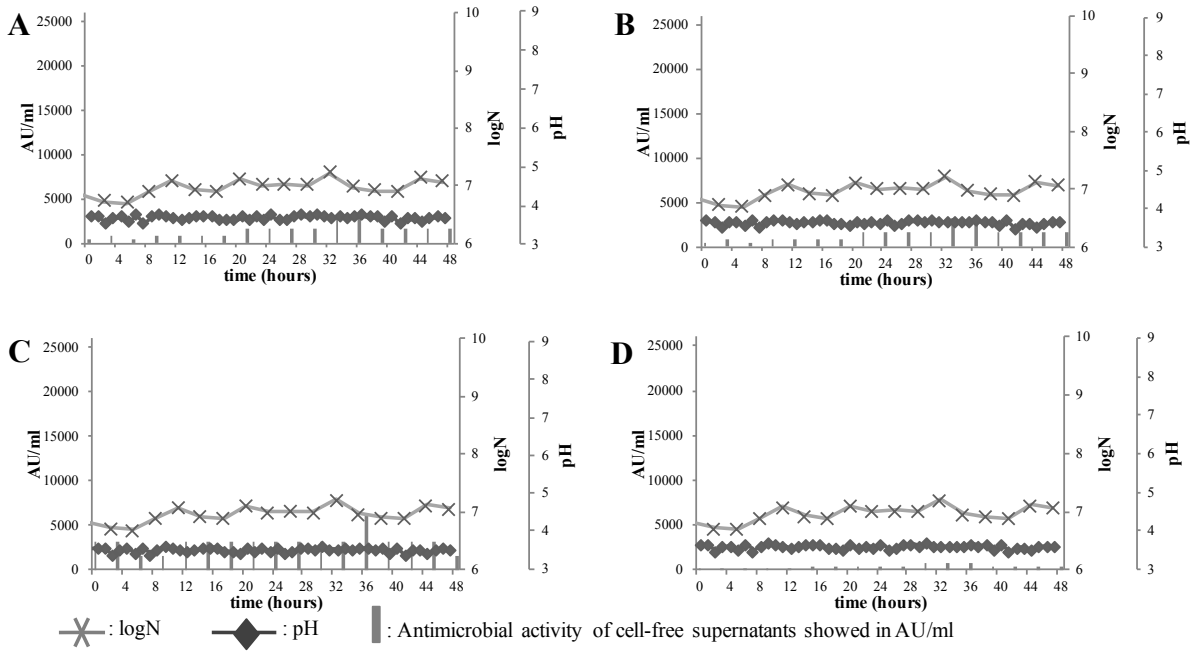


Figure 4.4. Antilisterial activity of neutralized cell-free supernatant produced by *P. acidilactici* HA 6111-2 during growth in MRS broth (pH 3.5) at 30 °C using *Listeria* spp. as indicator strains. Indicator strains: *L. monocytogenes* 1486/1 (A), *L. monocytogenes* 1604/2 (B), *L. monocytogenes* 971 (C), *L. innocua* NCTC 11288 (D). Viable bacterial counts are expressed as logN and antilisterial activity is expressed as arbitrary units (AU) per ml.

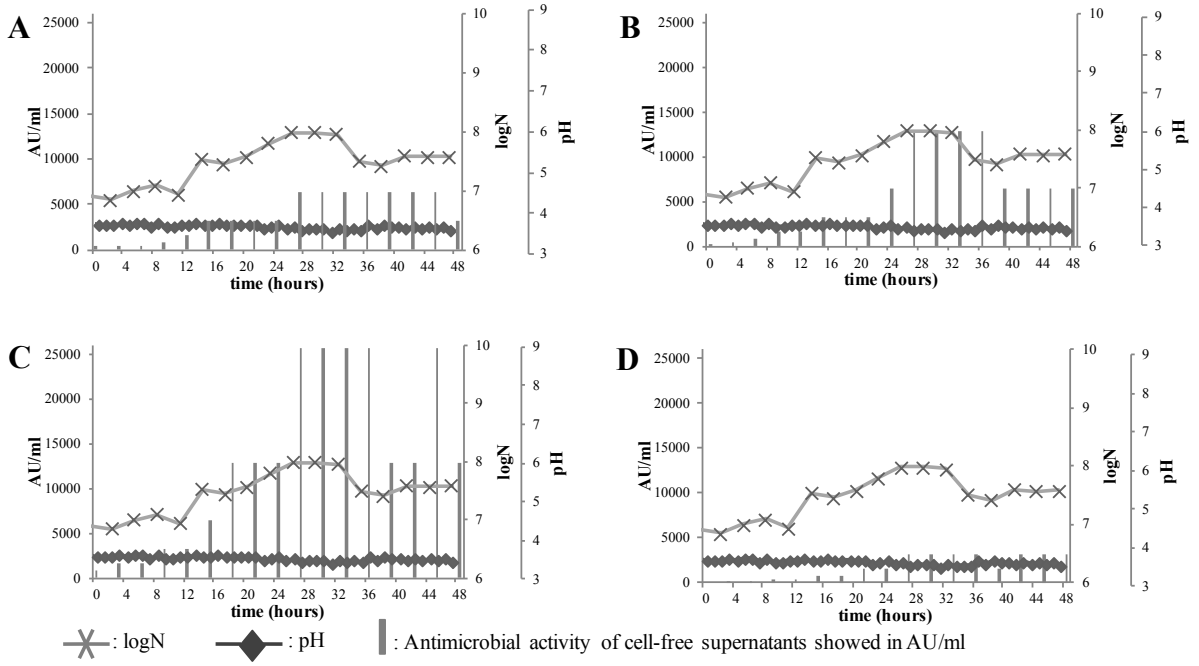


Figure 4.5. Antilisterial activity of neutralized cell-free supernatant produced by *Lb. plantarum* ST202Ch during growth in MRS broth (pH 3.5) at 30 °C using *Listeria* spp. as indicator strains. Indicator strains: *L. monocytogenes* 1486/1 (A), *L. monocytogenes* 1604/2 (B), *L. monocytogenes* 971 (C), *L. innocua* NCTC 11288 (D). Viable bacterial counts are expressed as log N and antilisterial activity is expressed as arbitrary units (AU) per ml.

10.14751/SZIE.2016.040

Although populations of *P. acidilactici* (Fig. 4.4.) did not increase and those of *Lb. plantarum* (Fig. 4.5.) increased only slightly from log 6.9 to 7.6 after 48 h, both supernatants demonstrated activity (Figs. 4.4. and 4.5.). For *P. acidilactici*, maximum antilisterial activity was observed against *L. monocytogenes* 971 (6400 AU/ml; Fig. 4.4C). Lower activities were observed against the other *L. monocytogenes* strains (Fig. 4.4A and B) and against *L. innocua* (Fig. 4.4D). Higher antilisterial activity was observed for *Lb. plantarum* (Fig. 4.5A-C), although activity was species and strain specific. It has been reported that *Lb. plantarum* can adapt to acidic conditions by amine accumulation into the cells equilibrating the acidic environment (Champomier-Verges et al., 2010). This could explain higher antilisterial activity by *Lb. plantarum*.

Under 7.5% NaCl, although populations of *P. acidilactici* increased slightly after 20-24 h (6.9-7.7 log CFU/ml, Fig. 4.6.), growth of *Lb. plantarum* was severely curtailed under these conditions (Fig. 4.7.).

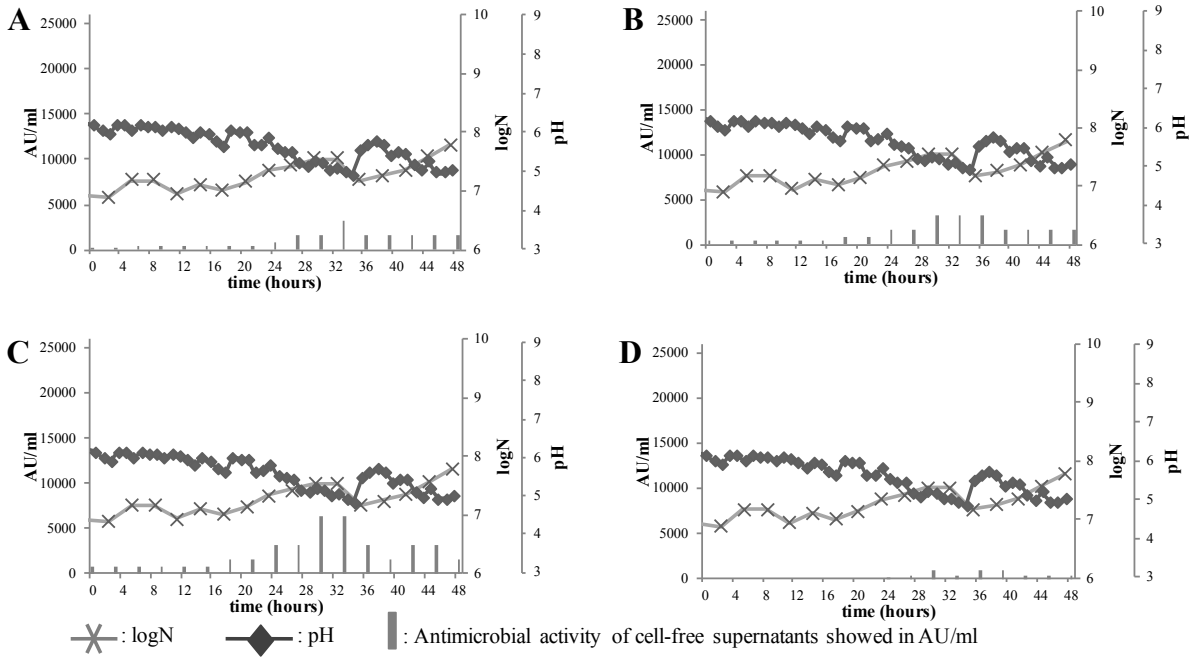


Figure 4.6. Antilisterial activity of neutralized cell-free supernatant produced by *P. acidilactici* HA 6111-2 during growth in MRS broth supplemented with 7.5% NaCl at 30 °C using *Listeria* spp. as indicator strains. Indicator strains: *L. monocytogenes* 1486/1 (A), *L. monocytogenes* 1604/2 (B), *L. monocytogenes* 971 (C), *L. innocua* NCTC 11288 (D). Viable bacterial counts are expressed as logN and antilisterial activity is expressed as arbitrary units (AU) per ml.

10.14751/SZIE.2016.040

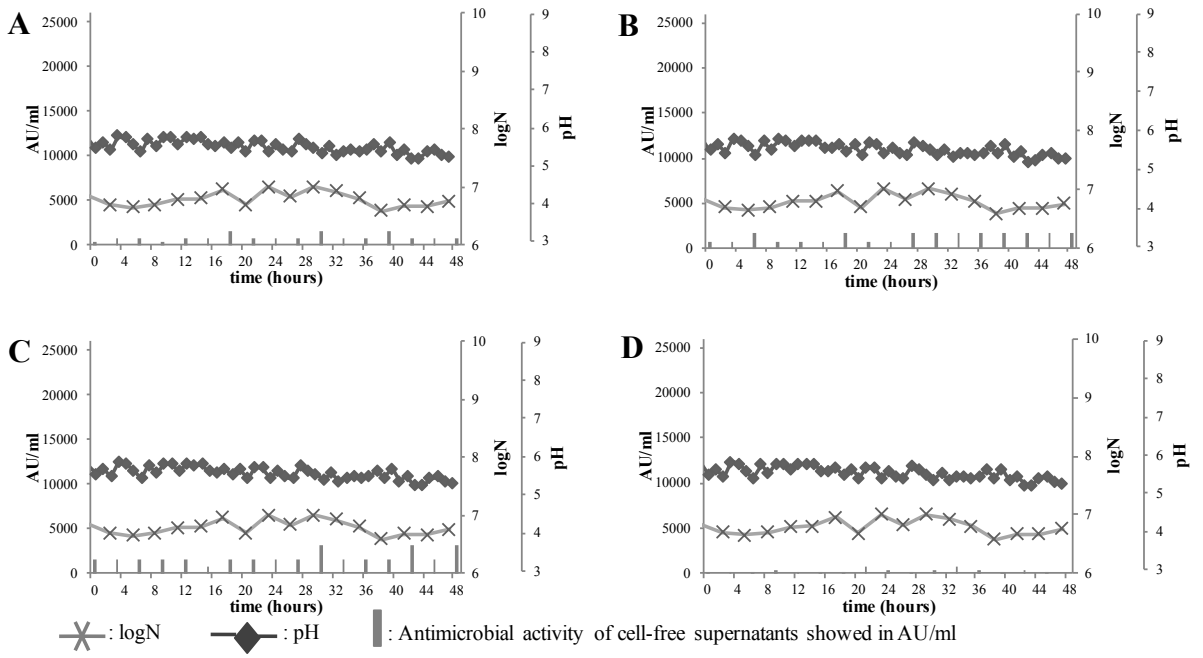


Figure 4.7. Antilisterial activity of neutralized cell-free supernatant produced by *Lb. plantarum* ST202Ch during growth in MRS broth supplemented with 7.5% NaCl at 30 °C using *Listeria spp*. as indicator strains. Indicator strains: *L. monocytogenes* 1486/1 (A), *L. monocytogenes* 1604/2 (B), *L. monocytogenes* 971 (C), *L. innocua* NCTC 11288 (D). Viable bacterial counts are expressed as logN and antilisterial activity is expressed as arbitrary units (AU) per ml. Error bars represent standard deviation of the mean.

However, antilisterial activity against all the *L. monocytogenes* strains was detected, even if at low level (800-3200 AU/ml for *Lb. plantarum* and 800-6400 for *P. acidilactici*; Figs. 4.6. and 4.7.). Similar results were described for *Lb. amylovorus* by De Vuyst et al. (1996), who concluded that sodium chloride inhibited cell growth and bacteriocin production. Glaasker and co-workers (1998) reported that *Lb. plantarum* was unable to repair under osmotic stress. It is important to highlight that centrifugation of cells of *Lb. plantarum* following exposure to the osmotic resulted in a pellet with a metallic-shine and difficult to resuspend when compared to control cells. These phenomena may indicate that the cell membrane is damaged under high osmotic stress.

During cold stress, the LAB showed differences in growth and bacteriocin production (Fig 4.8. and 4.9.). During the 48 h growth of *P. acidilactici* (Fig 4.8.), the level of pH (from 6.5 to 6.2) and the number of colony forming unit (from 6.8 to 6.2) changed very little. Contrary to *P. acidilactici*, the strain of *Lb. plantarum* (Fig 4.9.) decreased the pH (from 6.3 to 5.3) and increased the cell numbers (from 6.9 to 8.4). Neither *P. acidilactici*, nor *Lb. plantarum* could produce notable amounts of bacteriocins (the maximum activity was 1600 AU/ml against *L. monocytogenes* 971). According to these results, *P. acidilactici*

10.14751/SZIE.2016.040

could not adapt to or repair under cold stress, while *Lb. plantarum* was able to adapt/repair after 20 hours. Several LAB strains can rapidly adapt to temperature downshift to about 20 °C below the optimal growth temperature (Van de Guchte et al., 2002). However, even though the cells adapted/repaired (Fig. 4.9.), *Lb. plantarum* was not able to produce a notable amount of bacteriocin. During cold stress response, the cells undergo different physiological changes e.g. stabilization of secondary structures of RNA and DNA resulting in a reduced efficiency of translation (Phadtare et al., 1999). To survive cold shock, bacteria have developed the so-called cold-induced proteins, which play an important role in cold stress response (Van de Guchte et al., 2002). It could be an explanation why these LAB were not able to produce ribosomally synthesized bacteriocins under cold stress.

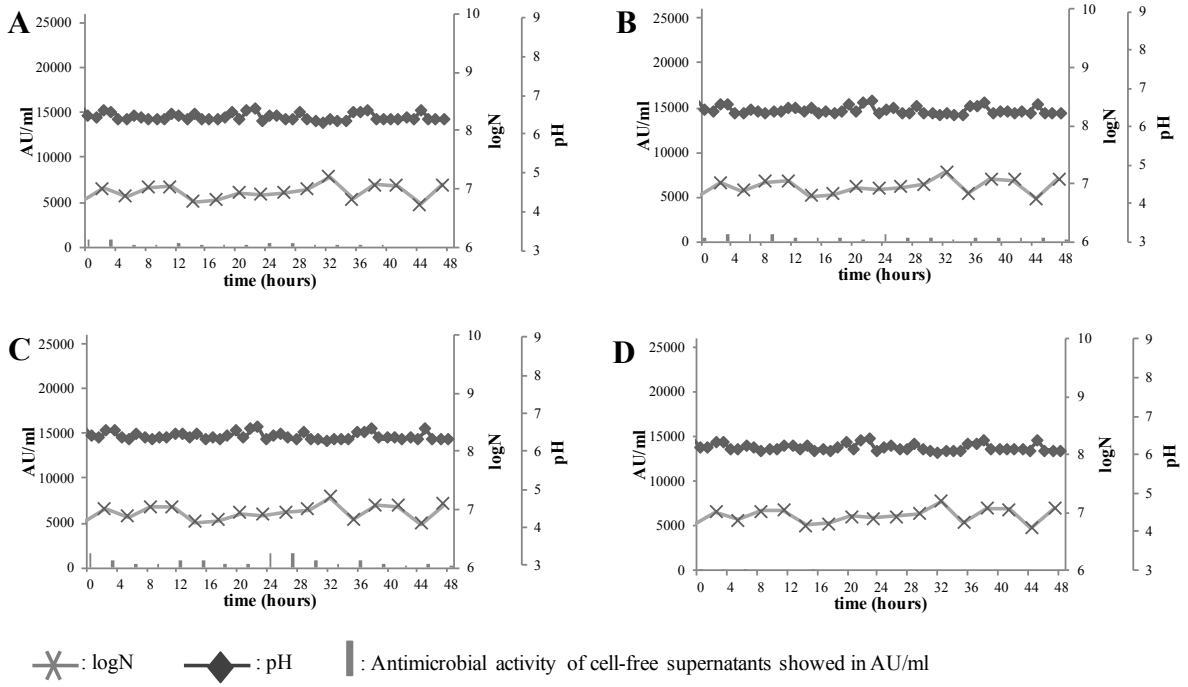


Figure 4.8. Antilisterial activity of neutralized cell-free supernatant produced by *P. acidilactici* HA 6111-2 during growth in MRS broth incubated at 10 °C using *Listeria spp*. as indicator strains. Indicator strains: *L. monocytogenes* 1486/1 (A), *L. monocytogenes* 1604/2 (B), *L. monocytogenes* 971 (C), *L. innocua* NCTC 11288 (D). Viable bacterial counts are expressed as logN and antilisterial activity is expressed as arbitrary units (AU) per ml.

10.14751/SZIE.2016.040

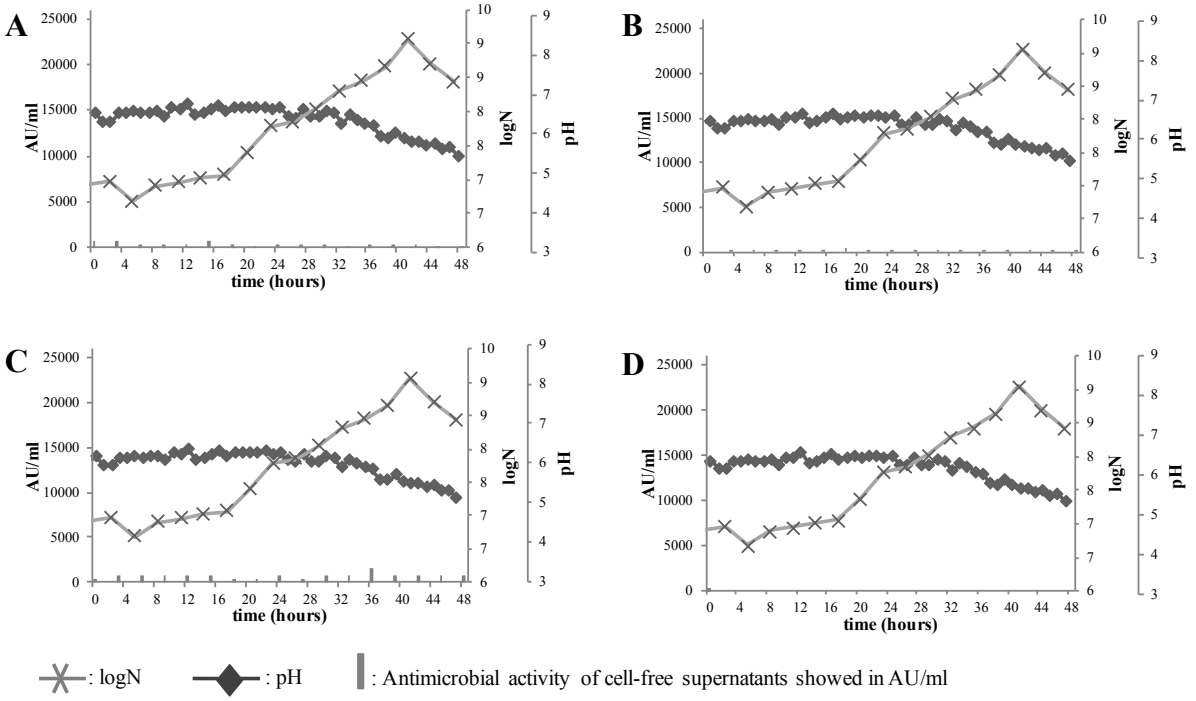


Figure 4.9. Antilisterial activity of neutralized cell-free supernatant produced by *Lb. plantarum* ST202Ch during growth in MRS broth incubated at 15 °C using *Listeria spp*. as indicator strains. Indicator strains: *L. monocytogenes* 1486/1 (A), *L. monocytogenes* 1604/2 (B), *L. monocytogenes* 971 (C), *L. innocua* NCTC 11288 (D). Viable bacterial counts are expressed as logN and antilisterial activity is expressed as arbitrary units (AU) per ml.

Figs. 4.10. and 4.11. show the survival of *P. acidilactici* and *Lb. plantarum* strains under thermal stress. Cells of *P. acidilactici* were not detectable after 24 hours and the level of pH did not change (from 6.4 to 6.3), although, low PA-1 bacteriocin activity against *L. monocytogenes* strains was observed until 39 hours. However, *Lb. plantarum* was less sensitive to heat shock than *P. acidilactici*. As can be seen in Figs. 4.4. and 4.5., *P. acidilactici* could not adapt/repair, while *Lb. plantarum* could adapt/repair in an acidic environment, as in thermal stress. De Angelis et al. (2001) found that heat response of *Lb. plantarum* is also related to cold shock, and my study showed *Lb. plantarum* could repair and produce bacteriocins under cold and heat stress conditions (Fig. 4.11.). However, the results from the heat shock experiments are doubtful. It can be explained with experimental fault, the constant temperature could not be provided, because the sampling time points were frequent, thus the temperature was constantly changing.

10.14751/SZIE.2016.040

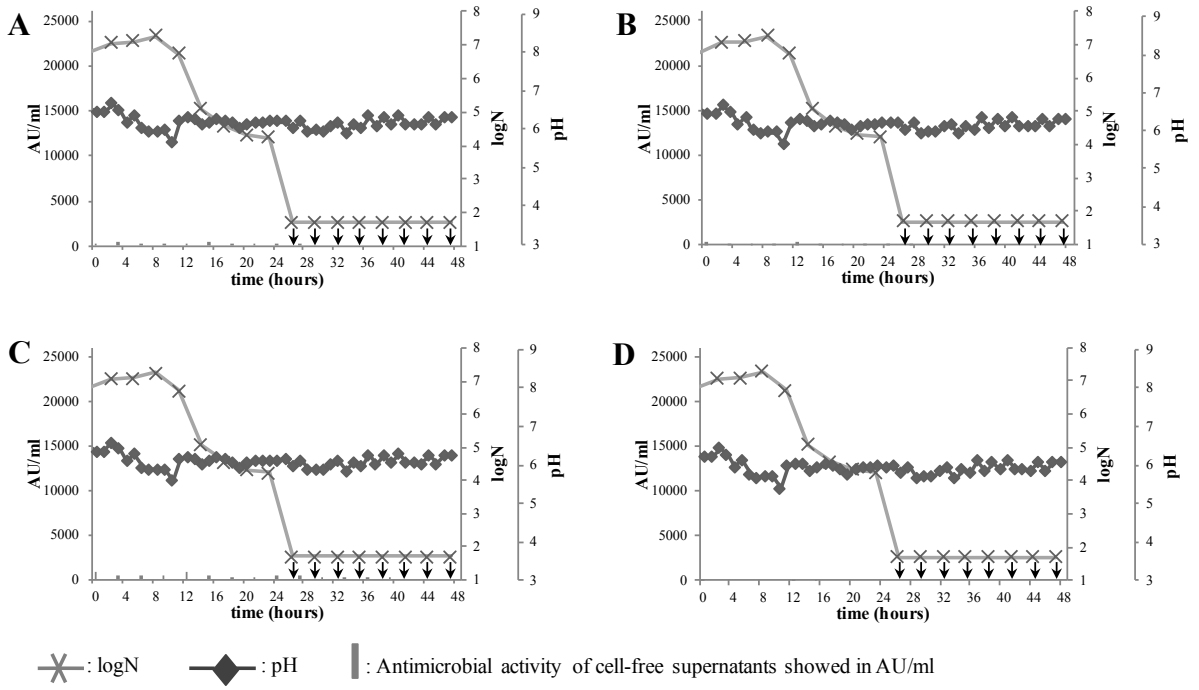


Figure 4.10. Antilisterial activity of neutralized cell-free supernatant produced by *P. acidilactici* HA 6111-2 during growth in MRS broth incubated at 50 °C using *Listeria spp*. as indicator strains. Indicator strains: *L. monocytogenes* 1486/1 (A), *L. monocytogenes* 1604/2 (B), *L. monocytogenes* 971 (C), *L. innocua* NCTC 11288 (D). Viable bacterial counts are expressed as logN and antilisterial activity is expressed as arbitrary units (AU) per ml.

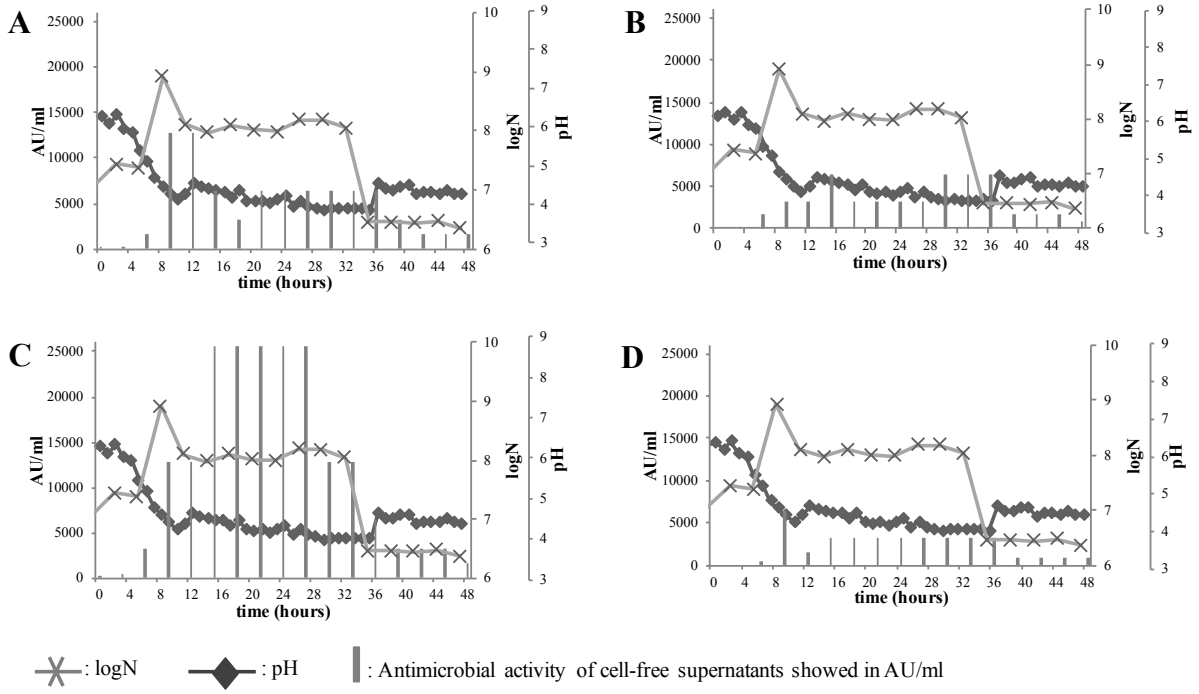


Figure 4.11. Antilisterial activity of neutralized cell-free supernatant produced by *Lb. plantarum* ST202Ch during growth in MRS incubated at 42 °C using *Listeria spp*. as indicator strains. Indicator strains: *L. monocytogenes* 1486/1 (A), *L. monocytogenes* 1604/2 (B), *L. monocytogenes* 971 (C), *L. innocua* NCTC 11288 (D). Viable bacterial counts are expressed as logN and antilisterial activity is expressed as arbitrary units (AU) per ml.

10.14751/SZIE.2016.040

L. monocytogenes strains showed different sensitivity to supernatants (*P. acidilactici* or *Lb. plantarum*) (Figs. 4.1. - 4.11.). For most of the conditions investigated, responses of *L. monocytogenes* 1486/1 and *L. monocytogenes* 1604/2 (both strains isolated from cheese) to *P. acidilactici* and *Lb. plantarum* supernatants were quite similar, while *L. monocytogenes* 971 (isolated from ground beef) demonstrated the highest sensitivity. Arguedas-Villa et al. (2010) suggested that the response of *L. monocytogenes* strains was dependent on their origin, which could explain differences observed in the current study. Similar results were obtained by Bērziņš et al. (2009), who found that *L. monocytogenes* strains showed differences in environmental stress tolerances. In all cases, *L. innocua* NCTC 11288 (Figs. 4.1D-4.11D) was found to be the most resistant to both supernatants. Furthermore, Al-Holy et al. (2012) reported that *L. innocua* was more resistant to nisin than *L. monocytogenes*. Scollard et al. (2009) also noted that *L. innocua* NCTC 11288 was more resistant to essential oil than *L. monocytogenes*. These results should be taken into consideration when *L. innocua* is used as a surrogate for *L. monocytogenes*.

10.14751/SZIE.2016.040

CHAPTER 5 - COMBINED EFFECT OF NACL AND LOW TEMPERATURE ON ANTILISTERIAL BACTERIOCIN PRODUCTION OF *LACTOBACILLUS PLANTARUM*

5.1. Introduction

The occurrence of *Listeria monocytogenes* in fermented foods is quite frequent in the European Union (EFSA, 2015). For fermentation of several types of foods bacteriocin producer lactic acid bacteria are used. *Lactobacillus plantarum* is a well-known lactic acid bacterium, which produces plantaricin (class II) bacteriocin (Collins et al., 2010). Studies can be found on the characterization of antilisterial bacteriocin produced by *Lb. plantarum* (Hata et al., 2010; Martinez et al., 2013; Todorov et al., 2010). The bacteriocin produced by *Lb. plantarum* is stable under different environmental conditions (Barbosa et al., 2016; Martinez et al., 2013; Todorov et al., 2010). Traditionally, the activity of a bacteriocin determined as described by Barefoot and Klaenhammer (1983). Bacteriocin activity was assayed by an adaptation of the critical dilution method used for the assay of bacteriocins. Portions of serial twofold dilutions of culture extracts were spotted onto the surface of agar plates, which contain the indicator strains. Antimicrobial activity is expressed as arbitrary units (AU) per ml. AU is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition. With that methodology the value of AU is a discrete variable, which may cause information loss. As a result of the twofold dilution assay used, at high bacteriocin activities the error bars on the bacteriocin data are larger, thus the uncertainty increase (Verluyten et al., 2004). To determine the antimicrobial activity of different substrates several methods are available (López-Malo et al., 2005).

The redox potential is one of the most complex indicators of the physiological state of microbial cultures and its measurement could be a useful tool for the qualitative and quantitative determination of the microbial activity. As the bacteria grow in a test cell, the redox potential of the medium starts to decrease. From the rate of the redox potential decrease the initial viable count of the medium can be calculated The MicroTester apparatus, using the redox potential method is suitable for qualitative and quantitative determination of bacteria (Reichart et al., 2007). Erdősi et al. (2010) developed the redox potential method to determine the total plate count and *Enterobacteriaceae* counts in less than 16 h for hygiene control in the food industry. Szakmár et al. (2014) used the method successfully for determination the effect of doxycycline on the microbial activity.

Limited numbers of publications are available in the literature on bacteriocin production under different environmental circumstances. The fermented food industry has great interest

in reliable, and well-defined, stable starter cultures. To fulfill this demand, the characterization of lactic acid bacteria and the bacteriocin production have to be performed. Verluyten et al. (2004) described the effect of NaCl on the growth and bacteriocin production of *Lb. curvatus*. They found that NaCl concentration highly affected the bacteriocin production. The combined effect of pH and temperature on bacteriocin production and growth of *Lb. plantarum* was examined by Zhou et al. (2015) using a response surface model. The authors set up a logistic model to predict the cell number and the rate of bacteriocin activity under different temperature and pH.

The aim of our study was to examine the combined effect of NaCl and low temperature on antilisterial bacteriocin production of *Lactobacillus plantarum* ST202Ch (hereafter *Lb. plantarum*).

5.2. Materials and methods

5.2.1. Origins of bacteria and storage conditions

Bacteriocin producer *Lb. plantarum*ST202Ch (Todorov et al., 2010), previously isolated from fermented meat sausage and deposited in the culture collection of Escola Superior de Biotecnologia (ESB), Porto, Portugal was selected for this study. The neutralized cell-free supernatant containing antilisterial bacteriocin produced by *Lb. plantarum* was characterized earlier in Chapter 3.

L. monocytogenes from the culture collection of the LRCESB 1486/1, serogroup IIb, isolated from cheese was selected as target strain. Test strains were stored at -20 °C in appropriate culture media containing of 15% (v/v) glycerol. *Lb. plantarum* was cultured in de Man, Rogosa and Sharpe (MRS) broth (Biokar) at 37 °C for 18-22 hours; *Listeria monocytogenes* was grown in Tryptone Soy Broth (TSB; Biokar) at 37 °C for 18-22 hours. All bacterial strains were subcultured twice under appropriate conditions before use in the experiments.

5.2.2. Examination the effect of NaCl and temperature on the bacteriocin production

In the selection of the appropriate parameters, the results of previous publication (Engelhardt et al., 2015, Chapter 4) were taken into account. Modified (0, 2, 4, 6% NaCl) MRS broth was inoculated with 1% (v/v) of an overnight *Lb. plantarum* culture and incubated at 20, 25 and 30 °C. Changes in cell number and bacteriocin activity in the cell-free supernatant were recorded in every 3 hours for 48 h. with the exception of at 20 °C with 4 and 6% NaCl concentration, where the time duration of the measurement was 60 hours long.

10.14751/SZIE.2016.040

Neutralized cell-free supernatant with antilisterial activity (hereafter referred as supernatant) was prepared as described by Van Reenen et al. (1998).

5.2.2.1. Determination of growth parameters

The cell number was counted on MRS agar, incubated at 30 °C for 48 hours.
The length of the lag phase was determined visually from the logarithmic growth curve.
The specific growth rate (μ) was calculated from the slope of the linear section of logarithmic growth curve by regression analysis (Microsoft Excel):

$$\mu\ (1/h) = 2{,}303 \cdot \text{slope}$$

Generation time:

$$GT\ (h) = \ln 2/\mu = 0.693/\mu$$

5.2.2.2. Principle of the measuring method

Antilisterial activity, using *L. monocytogenes* 1486/1 as target microorganism, was determined applying a MicroTester apparatus (Microtest Ltd, Hungary), which operates on the principle of the new measuring method introduced by Reichart et al. (2007).

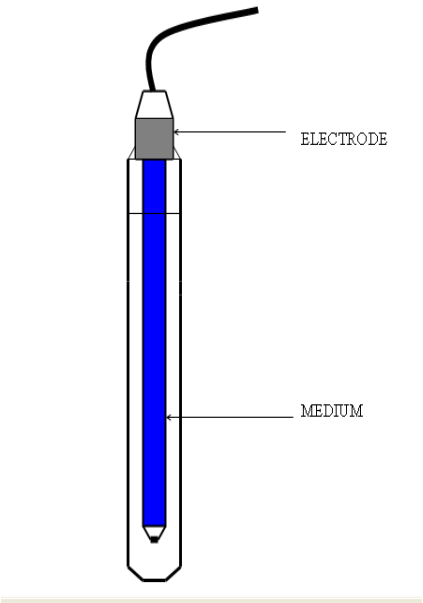


Figure 5.1. The test cell (microtest.co.hu)

10.14751/SZIE.2016.040

In the test cell (Fig. 5.1.) during the growth of microorganisms as a consequence of the metabolic activity the redox potential of the medium decreases. The system records the redox curve, calculates the first differential of the redox potential (ΔE/Δt) and determines the TTD (time to detection) value. TTD means the time taken to reach the detection threshold when the rate of redox potential decrease, ΔE/Δt overcomes the detection criterion (DC). (In case of *L. monocytogenes* DC=-0.5 mV/min). The critical viable count belonging to the detection criterion (N_{DC}) is usually 10^6-10^7 cfu/ml. The essentials of the method are demonstrated in Fig 5.2.

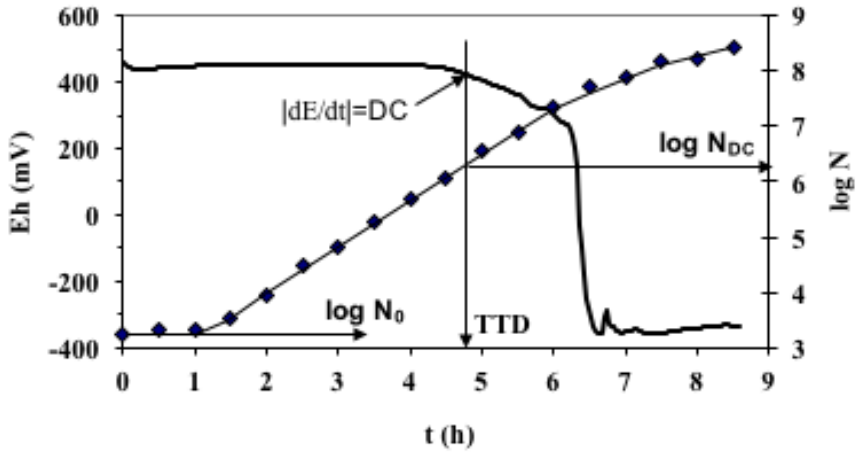


Figure 5.2. Principles of TTD detection in redox potential measurement method. Eh redox potential of the medium; N: viable cell concentration; N_0 initial viable cell concentration in the medium; DC: detection criterium; N_{DC} the threshold value of the viable cell concentration at time of TTD

The TTD is a strictly linear function of the initial log viable count of the medium. This relationship makes possible to estimate the initial viable count, $logN_0$ on the base of the measured TTD value by using calibration curve:

$$logN_0 = a \cdot TTD\ (h) + b, \qquad (1)$$

where *a:* the slope

b: the intercept of the calibration curve.

One of the advantages of the application of calibration curves is that the microbial count could directly be estimated from the sample without any dilution. The other benefit is that to get a microbial count takes only 4 – 12 hours (depending on the microbial concentration) in contrast to the 24 - 48 hours time requirements of the classical plate counting.

10.14751/SZIE.2016.040

The applied MicroTester apparatus was a computer-controlled 16-channels measuring system developed by Reichart et al. (2007).

The growth medium (9.0 ml of ½ concentration TSB broth) in the test cells (incubated at 37 °C) were inoculated with 0.1 ml of the 2nd decimal dilution of an overnight *L. monocytogenes* 1486/1 culture and 1 ml of sample (*Lb. plantarum* supernatant containing bacteriocin with antilisterial activity). In case of positive control supernatant was not added.

5.2.2.3. Determination of calibration curve

Tenfold dilution series of an overnight *L. monocytogenes* 1486/1 culture were prepared with peptone solution (1 g/l). Test tubes were inoculated with 1 ml of the members of dilution series and the TTD values were determined automatically by the MicroTester system. The viable counts of the dilutions were calculated by plate counting on TSB agar.

5.2.2.4. Determination of the antilisterial activity

The measure of the antilisterial activity was expressed as **Δ**TTD, which means the difference between the detection times of the inhibited and the control (non-inhibited) *L. monocytogenes* 1486/1 suspensions, TTD(i) and TTD(c), respectively.

$$\Delta\text{TTD} = \text{TTD(i)} - \text{TTD(c)} \qquad (2)$$

where TTD(i): inhibited *L. monocytogenes* 1486/1

TTD(c): control (non-inhibited) *L. monocytogenes* 1486/1

The N values in the calibration curve (1) mean viable cell concentrations, determined by standard plate counting.

In case of samples containing antilisterial supernatant it is not known whether the TTD increase was caused by the partial destruction of the viable *L. monocytogenes* 1486/1 inoculum, or by the inhibition of metabolic activity. Due to this uncertainty ΔTTD was interpreted as a virtual decrease in viable cell concentration of the initial number of *L. monocytogenes* 1486/1. Applying the calibration curve (1) it could be deducted:

$$\log (N_0\text{i}/N_0\text{c}) = a \cdot \Delta\text{TTD} \qquad (3)$$

where N_0i: viable cell number of the inhibited *L. monocytogenes* 1486/1

N_0c: viable cell number of the control (non-inhibited) *L. monocytogenes* 1486/1

10.14751/SZIE.2016.040

As the virtual viable counts were determined by the redox potential measurement technique, hereafter we omit the 0 index of N:

$$\log (Ni/Nc) = a \cdot \Delta TTD, \quad (4)$$

where Ni: viable count in the inhibited *L. monocytogenes* 1486/1 suspension inoculated with antilisterial supernatant of *Lb. plantarum*,

Nc: viable count in the control *L. monocytogenes* 1486/1 suspension without inoculation.

a: the slope of the calibration curve

For the quantification of the antilisterial activity of the supernatants the virtual decrease (log (Ni/Nc)) was used. The efficiency of the bacteriocin production of *Lb. plantarum* was characterized by the essential fermentation time (*te*), which means the elapsed fermentation time until the supernatant results in 2.5 log unit virtual decrease in *L. monocytogenes* 1486/1. Reason for choosing 2.5 log unit virtual decrease is that this value belongs to the linear section of logNi/Nc reduction at all of 3 temperatures (see Figs 5.5.-5.7. in section 5.3.2.). Determination of the essential fermentation time is demonstrated in Fig.5.3.

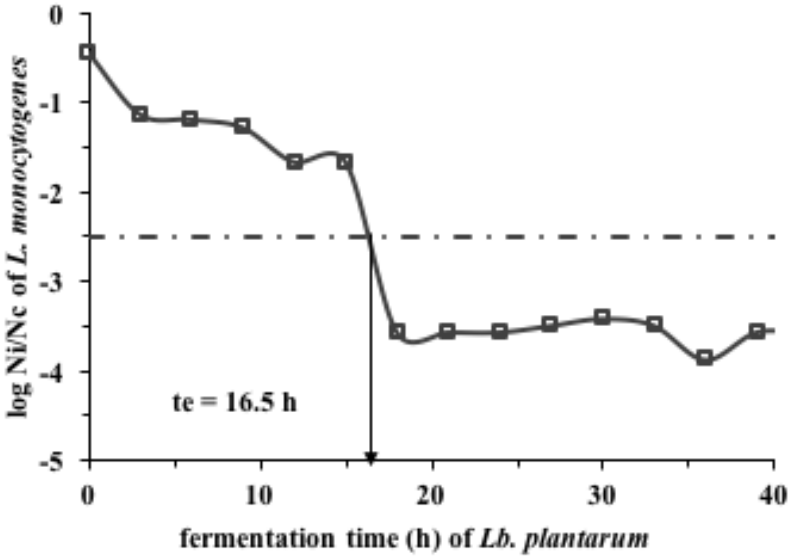


Figure 5.3. Determination of the essential fermentation time (*te*) of *Lb. plantarum* ST202Ch (T = 25 °C, NaCl = 0%). The antilisterial activity of the supernatant results in 2.5 log unit virtual destruction of *L. monocytogenes* 1486/1.

The *te* values in the function of NaCl concentrations and temperature were evaluated by Multiple Regression program of the STATGRAPHICS Centurion software package.

10.14751/SZIE.2016.040

5.3. Results and discussion

5.3.1. *Listeria monocytogenes* calibration curve

Calibration curve of *L. monocytogenes* could be seen in Fig 5.4.

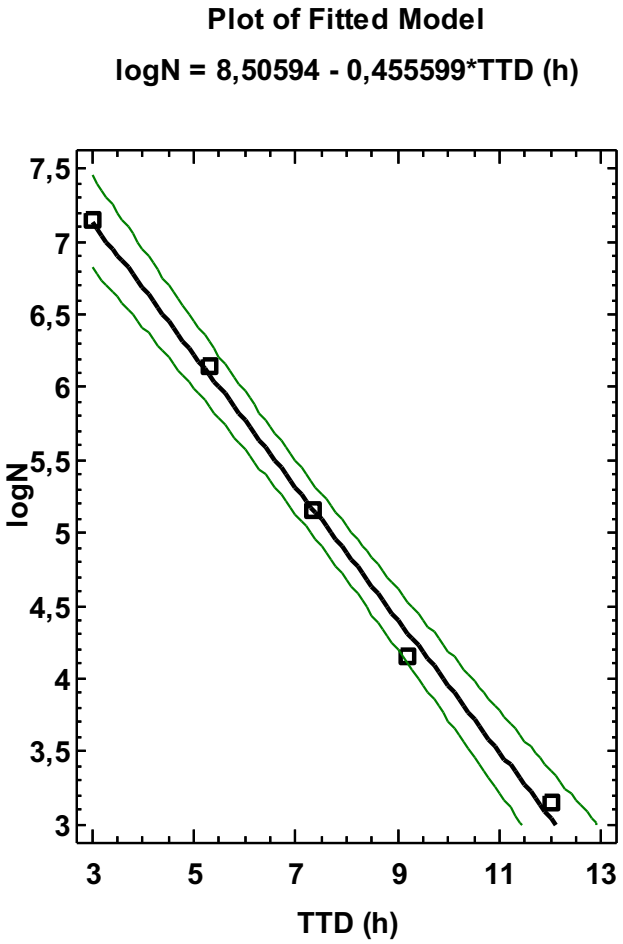


Figure 5.4. Calibration curve of *L. monocytogenes* 1486/1 with 95% confidence interval of means in ½ TSB broth at 37 °C
N: initial viable concentration in the test cell.
Statistical parameters: n = 5, R^2 = 0.9950, SD($logN_0$) = 0.1287

Equation calculated by regression analysis:

$$logN = -0.4556 \cdot TTD(h) + 8.5059$$

where *N:* the initial *L. monocytogenes* concentration in the test cell

TTD: time to detection.

Statistical parameters:

number of data-pairs	n = 5
determination coefficient	$R^2 = 0.9950$
standard error of estimation	SD(logN) = 0.1287

The virtual decrease can be calculated by substituting the slope into the equation (4): log(Ni/Nc) = -0.4556·ΔTTD. Thus, 1 log unit decrease in viable count of *L. monocytogenes* results in 1/0.4556 = 2.19 h increase in TTD.

Taking into account that log(Ni/Nc) = logNi – logNc, the standard error of the virtual decrease is:

$$SD(logNi/Nc) = \sqrt{2} \cdot SD(logN) = 0.1820$$

10.14751/SZIE.2016.040

5.3.2. Effect of the temperature and NaCl on the growth of *Lb. plantarum* and antilisterial activity of the supernatant

The logarithmic growth curves of *Lb. plantarum* ST202Ch and the antilisterial activity could be seen in Figs. 5.5A-5.7A.

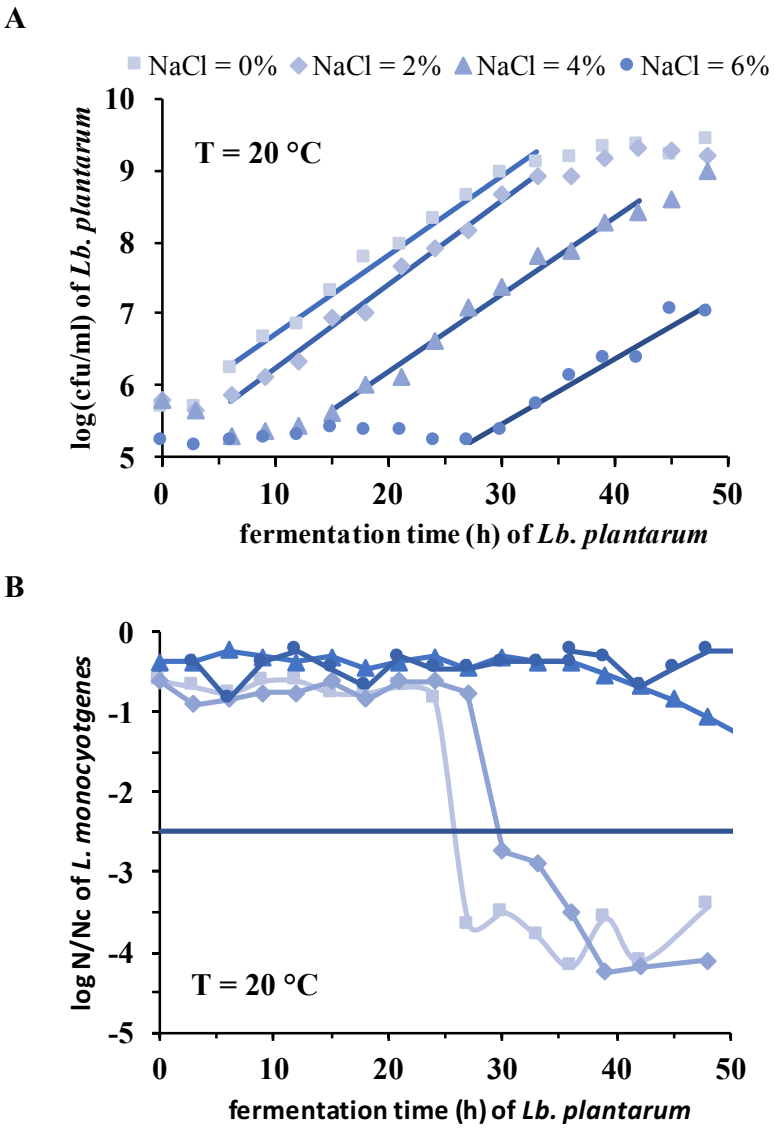
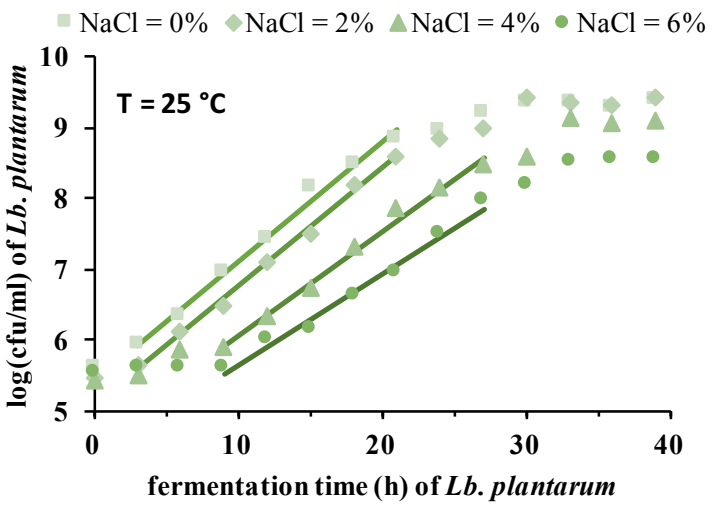


Figure 5.5. Logarithmic growth curves of *Lb. plantarum* ST202Ch with respect to the NaCl concentration (A) and the antilisterial activities of the supernatant samples determined by redox potential measurement method (B) at 20 °C.

10.14751/SZIE.2016.040

A



B

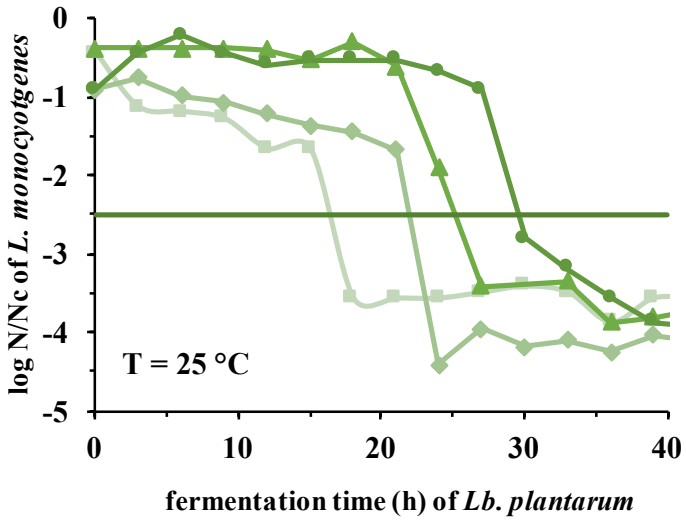
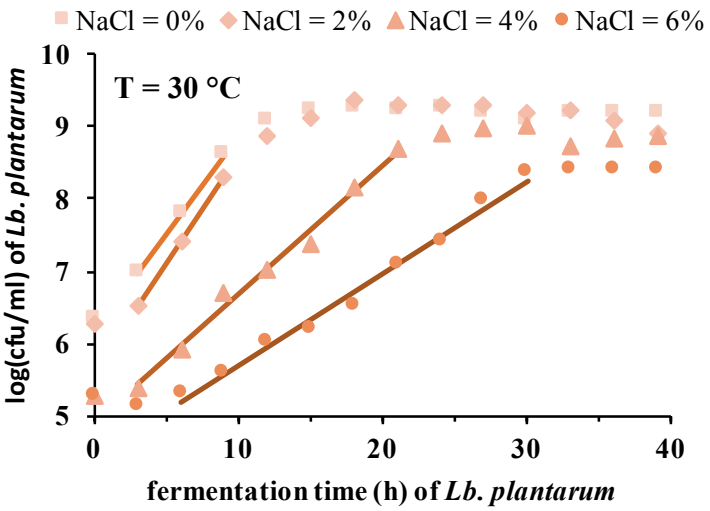


Figure 5.6. Logarithmic growth curves of *Lb. plantarum* ST202Ch with respect to the NaCl concentration (A) and the antilisterial activities of the supernatants determined by redox potential measurement method (B) at 25 °C.

10.14751/SZIE.2016.040

A



B

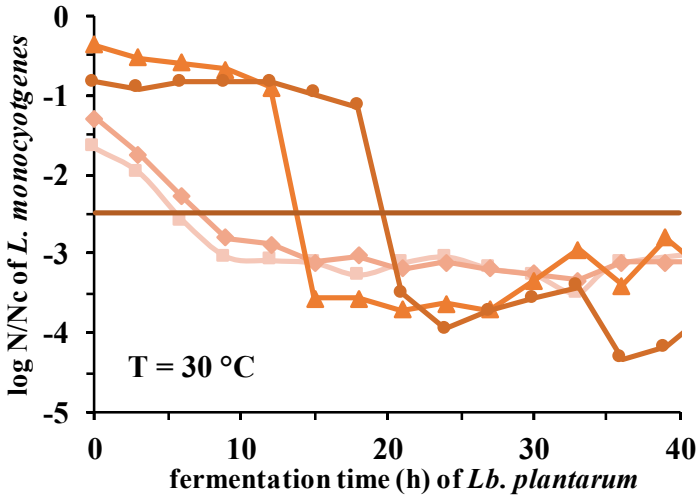


Figure 5.7. Logarithmic growth curves of *Lb. plantarum* with respect to the NaCl concentration (A) and the antilisterial activities of the supernatants determined by redox potential measurement method (B) at 30 °C.

The growth parameters (lag phase and specific growth rate) calculated from the linear section (Appendix A) of the logarithmic growth curves and the essential fermentation time (*te*), belonging to different temperatures and NaCl concentrations are summarized in Table 5.1.

10.14751/SZIE.2016.040

Table 5.1. Growth parameters of *Lactobacillus plantarum* (lag period, specific growth rate, μ, and generation time, tg) against temperature (T) and NaCl concentration (NaCl %)

T (°C)	NaCl (%)	lag (h)	μ (h^{-1})	μ/μ_0 [a]	μ 95% conf. interv.		te [b] (h)
					μ lower	μ upper	
20	0	6	0.253	1.000	0.234	0.272	27
	2	6	0.270	1.064	0.248	0.291	30
	4	15	0.249	0.984	0.225	0.274	50<
	6	27	0.211	0.835	0.172	0.251	60<
25	0	3	0.391	1.000	0.349	0.432	16.5
	2	3	0.384	0.983	0.352	0.416	22
	4	9	0.340	0.871	0.304	0.377	25
	6	9	0.297	0.760	0.254	0.339	29.5
30	0	3	0.629	1.000	0.537	0.721	5.5
	2	3	0.685	1.090	0.600	0.771	7.5
	4	3	0.413	0.657	0.361	0.465	14
	6	6	0.295	0.470	0.265	0.326	20

[a] μ_0 means the specific growth rate belonging to NaCl = 0%
[b] te means the essential fermentation time of *Lb. plantarum* ST202Ch needed to reach 2.5 log unit virtual destruction of *L. monocytogenes* 1486/1.

As it can be seen, the lag phase became longer and the specific growth rate decreased with increasing NaCl concentration and decreasing temperature. Taking into account the 95% confidence intervals the reduction of the μ values over 2% of NaCl proved to be significant. In addition, comparing the μ/μ_0 values in Table 5.1. I obtained an interaction between the temperature and NaCl concentration. At a reduced temperature the measure of the inhibitory effect of the NaCl addition decreases. Higher growth rate reduction has been observed at 30 °C than at 20 °C.

The effect of NaCl and temperature on microbial behavior and growth was well studied and different mathematical models were developed to estimate the effects. Romero-Gil and co-workers (2013) examined the effect of NaCl and temperature individually, and they found that the *Lactobacillus* species could complete the fermentation at low temperature, but were not able to grow at NaCl concentrations higher than 4%. In present study, the *Lb. plantarum* test strain was able to grow even at 6% NaCl concentration.

The results of antilisterial activity of *Lb. plantarum* supernatant at 20, 25, 30 °C in MRS broth containing 0, 2, 4, 6% NaCl are shown in figures 5.4B-5.6B. The antilisterial activity of neutralized cell-free supernatant also decreased with increasing NaCl concentration and decreasing temperature. Since the bacteriocins are secondary metabolites, higher amount of

10.14751/SZIE.2016.040

the antilisterial bacteriocin was produced as the *Lb. plantarum* entered into the stationary phase (Powell et al., 2007).

According to my measurements the antilisterial activity well detectably increases from the middle of the exponential phase of the growth curves, and reaches its maximum ($logN/N_0$ of *L. monocytogenes* is minimum) at the beginning of the stationary phase as it is demonstrated by Figs 5.5.-5.7.

The essential *Lb. plantarum* fermentation time (*te* values presented in Table 5.1.) which characterize the effectiveness of the production of antilisterial agents were determined by the intersections of the $logN/N_0$ curves and the straight line of $logN/N_0 = -2.5$.

As it could be seen in the figures 5.5B – 5.7B, the increasing concentration of NaCl in the *Lactobacillus plantarum* fermentation resulted in decreased antilisterial activity of the supernatant. Similarly, clear effect was observed in case of temperature. The lower temperature resulted in lower antilisterial effect of the supernatant. At the lowest temperature (20 °C) the *Lb. plantarum* test strain was not able to produce significant amount of antilisterial bacteriocin at 4 and 6% NaCl (Fig. 1B.), thus no calculated data were available under these parameters.

Verluyten et al. (2004) found similar response to NaCl concentrations in case of *Lb. curvatus* in their study. The NaCl concentration did not affect the μ as significantly as affected the production of antilisterial bacteriocin. When *Lb. amylovorus* grew at 4% NaCl concentration the μ and the cell number also decreased and bacteriocin actvitiy could not be determined (Neysens et al., 2003).

As both factors (temperature and NaCl) had clear effect on the antilisterial activity of the supernatant, I applied a simple two independent variables multiple regression model to describe the combined effect of these factors on the essential fermentation time (te).

$$te\ (h) = a + b1 \cdot T(°C) + b2 \cdot NaCl\ (\%) \qquad (5)$$

The results of the regression analysis are summarized in Table 5.2.

10.14751/SZIE.2016.040

Table 5.2. Mathematical modeling of the combined effect of temperature and NaCl% on the essential Lactobacillus fermentation time (te)
Analysis of Variance

Source	Sum of Squares	Df	Mean Square	F-ratio	P-value
Model	671.366	2	335.683	218.9	0.0000
Residual	10.7339	7	1.5334		
Total	682.1	9			

Parameters

Parameters	Estimate	Stand. error	t-statistic	P-value
CONSTANT	69.8548	2.7707	25.2117	0.0000
T (°C)	-2.15968	0.10941	-19.7394	
NaCl (%)	2.30645	0.18608	12.395	

Equation te (h) = 69,855 - 2,160·T (°C) + 2,306·NaCl (%)
Determination coefficient R^2 =0.98426
Standard error of Estimation SD(te) =1.238 h

As a result of the regression-analysis for the prediction of the antilisterial activity of *Lb. plantarum* fermentation the following relationship was established.

$$te(h) = 69.855 - 2.160 \cdot T\ (°C) + 2.306 \cdot NaCl\%$$

n = 10, $R^2 = 0.9843$

The statistical parameters summarized in Table 5.2 refer to a very strict fitting as it is demonstrated in Fig. 5.8.

10.14751/SZIE.2016.040

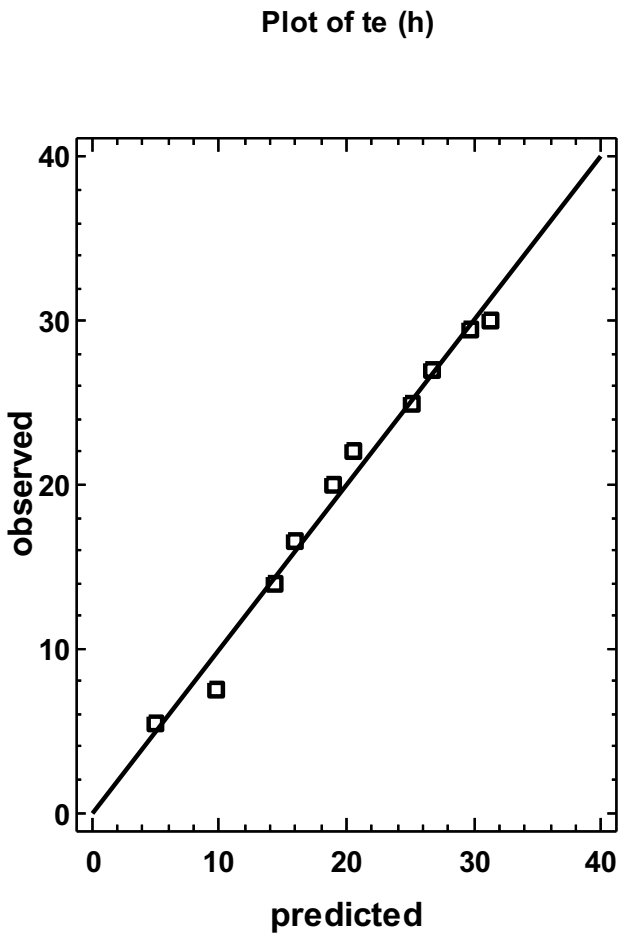


Figure 5.8. Model-fitting of the essential *Lb. plantarum* fermentation time (*te*)
te (h) = 69.85 – 2.160·T (°C) + 2.306·NaCl (%), $R^2 = 0.9843$

In the past decade *L. monocytogenes* in ready-to-eat and fermented foods became an issue due to food-borne infections (Ducic et al., 2016; Luber et al., 2011), and more studies focused on the LAB starter cultures. As it was mentioned above, the antilisterial substrates produced by LAB are well characterized and found to be stable under different environmental circumstances but still there is no information about the production of bacteriocins. The model shows the effect of the temperature and NaCl and can predict the amount of the antilisterial bacteriocin produced. The model can be useful in the fermented food industry for a better control of *L. monocytogenes*.

Verluyten et al. (2004) found similar response to NaCl concentrations in case of *Lb. curvatus* in their study. The NaCl concentration did not affect the μ as significantly as affected the production of antilisterial bacteriocin. When *Lb. amylovorus* grew at 4% NaCl concentration

the μ and the cell number also decreased and bacteriocin actvitiy could not be determined (Neysens et al., 2003).

It can be concluded that the bacteriocin production under lower temperature and higher NaCl concentration may not be sufficient to inhibit *L. monocytogenes*. Several data can be found in the literature for the growth kinetics parameters of *L. monocytogenes*. Using the ComBase (www.combase.cc) predictor, the maximum growth rate is 0.173 1/h, the generation time is 1.743 h at 20 °C, 4% NaCl. Considering these data, the *L. monocytogenes* can grow and cause food safety problems in fermented foods. Uhlich and co-workers (2006) studied the growth of *L. monocytogenes* at 20 °C in cheese in mixed culture of lactic acid bacteria and the calculated growth rate of *L. monocytogenes* was 0.0849, which can lead serious food safety problems.

Summarizing, the results showed that prediction of the bacteriocin production is possible, but when *Lb. plantarum* is used in a fermented food industry, additional hurdle strategy is needed to control *L. monocytogenes*. Furthermore, more experiments are necessary in food systems to validate the model and it will be also useful to make experiments to determine the behaviour of *Lb. plantarum* and *L. monocytogenes* in co-culture.

10.14751/SZIE.2016.040

CHAPTER 6 – EXAMINATION OF BIOFILM FORMATION OF *LISTERIA* SPECIES AND LACTIC ACID BACTERIA

6.1. Introduction

Bacterial biofilms are adequate substances for human infections and for the contamination of foods with spoilage and pathogenic organisms. *Listeria monocytogenes* can exist in biofilms in food production environment. *L. monocytogenes* can occurre in fermented food industry (EFSA, 2015), fresh-cut vegetable (Oliveria et al., 2015), dairy (Latorre et al., 2010) and fish industry (Rajkowski, 2009) and can cause listeriosis and economical losses. *L. monocytogenes* is able to form biofilms on different surfaces (polystyrene, polypropylene, glass, stainless steel, quartz, marble, and granite) in the food industry (da Silva and de Martinis, 2013). In food products, together with *L. monocytogenes*, several other microorganisms can also be found such as, lactic acid bacteria or other *Listeria* species. LAB are able to reduce the biofilm formation of *L. monocytogenes*. Zhao et al. (2013) described that *Lactococcus lactis* and *Enterococcus durans* decreased the population of *L. monocytogenes* at 4 °C in poultry processing environment. *Lb. plantarum* is able to control the biofilm formation of different pathogen and spoilage microorganisms under different environmental conditions (Jalilsood et al., 2015). Speranza et al. (2009) reported that biofilm of *Lb. plantarum*, *Lb. casei*, *Lb. curvatus* and *Lb. paracasei* can delay the growth of *L. monocytogenes* in cheese products; they found one log cycle differences in the maximum cell load attained at the stationary phase of *L. monocytogenes*. Guerrieri et al. (2009) found that, to control *L. monocytogenes* in biofilm by LAB, bacteriocin producer LAB strains are needed. When the LAB cultures were not able to produce bacteriocin, only slight influence on *L. monocytogenes* survival was observed. In food matrices, the presence of *L. monocytogenes* is frequently accompanied with the presence of *L. innocua* (Bouayad et al., 2015), which may have an inhibitory effect on *L. monocytogenes* (Cornu et al., 2002; Gnanou Besse et al., 2005; Yokoyama et al., 2005). To my knowledge, there are no reports on the formation of *L. monocytogenes* and *L. innocua* biofilm in mixed culture.

The food manufacturers are interested in natural antimicrobial substances like bacteriocins produced by lactic acid bacteria. It is also necessary to know how *L. monocytogenes* and *L. innocua* influence each other in different biofilms. The aim of my study was to examine the co-growth of *Listeria* species and lactic acid bacteria during biofilm formation in different model- and natural-media.

6.2. Materials and methods

6.2.1. Origins of bacteria and storage conditions

L. monocytogenes L4 (isolated from cheese products) and T3 (isolated from food industry) provided by the Department of Microbiology and Biotechnology (Corvinus University of Budapest, Hungary); *L. monocytogenes* 1486/1 (from cheese), *L. monocytogenes* 971 (from hamburger) provided by LRCESB, *L. innocua* CCM 4030 (C6) obtained from the Czech Collection of Microorganisms, *Pediococcus acidilactici* HA 6111-2 (isolated form "alheira") provided by ESB were investigated.

Lactic acid bacteria were cultured in de Man, Rogosa and Sharpe (MRS) broth (Biokar) at 37 °C for 18-22 hours; *Listeria spp*. was grown in Tryptone Soy Broth (TSB; Biokar) supplemented with 0.6% (w/v) of yeast extract (LabM) (TSBYE) at 37 °C for 18-22 hours. All strains were stored at -20 °C in appropriate culture media containing of 15% (v/v) glycerol. All bacterial strains were subcultured twice under appropriate conditions before use in experiments.

6.2.2. Examination of biofilm formation

Sterile stainless steel (SS) coupons (10 x 30 mm) were used as substrates for static biofilm formation, since this material is frequently used for manufacturing food processing equipments. Different matrices (model media: BH and MRS broth; natural media: milk and MRS broth supplemented with minced meet) were selected for the examination the biofilm formation. Iceberg lettuce leaves (LL) (10 x 30 mm) were also selected as substrates for biofilm formation (Table 6.1.). These food matrices are able to support the growth of *Listeria* strains and lactic acid bacteria.

10.14751/SZIE.2016.040

Table 6.1. The experimental setup for biofilm examination in different media with different microorganism

Examined surface	Examined media	Biofilm formation of strains	Incubation time	Sampling time points
Stainless steel coupons	TSB broth (Biokar)	*Listeria spp.* in mono-culture	168 hours	0, 48, 120 168 hours
	MRS broth (Biokar)	*P. acidilactici* in monoculture; mixed culture of *P. acidilactici* and *L. monocytogenes*	144 hours	0, 48, 144 hours
	Pasteurized milk	mixed culture of *P. acidilactici* and *L. monocytogenes*	144 hours	0, 48, 144 hours
	Sterilized (by gamma irradiation) minced meat supplemented with MRS broth (ratio was 1:1)	mixed culture of *P. acidilactici* and *L. monocytogenes*	144 hours	0, 48, 144 hours
Iceberg lettuce leaves	Peptone (0.1%) - NaCl (0.85%) buffer	*Listeria spp.* in mono-culture and co-culture	48 hours	0, 24, 48 hours

In every experimental setup the experimental media was inoculated with 1% (v/v) of an overnight *Listeria spp.* or *P. acidilactici* culture, thus the initial cell concentration was 10^6 CFU/ml. The SS coupons or lettuce leaves were dipped into the inoculated media and incubated for 1 hour at 30 °C. After 1-hour incubation both sides of the coupons were rinsed with 10-10 ml of sterile distilled water to eliminate planktonic and reversibly attached bacteria. The washed coupons were dipped into sterile respective media and incubated for the appropriate time interval (Table 6.1.) at 30 °C. The attached cell number was also determined after 1-hour incubation. The biofilm formation was monitored by determining the attached cell number at the sampling time points as it could be seen in Table 6.1. Both sides of the SS and LL surfaces were washed with 10-10 ml of sterile distilled water to eliminate planktonic and reversibly attached bacteria. This was followed by mechanical removal of the attached cells. In case of SS coupons sampling tampons, in case of LL glass beads were used for this purpose. Serial ten-fold dilutions were done and the cell number was determined by spread plate technique. Table 6.2. demonstrates the media which were selected to determine the cell number.

Table 6.2. The media used to determine the cell number in different experiments of biofilm formation

Biofilm formation of strains	**Media**
Listeria spp. in monoculture	TSA (Biokar)
Listeria spp. in co-culture	ALOA (Merck)
P. acidilactici in monoculture	MRS agar (Biokar)
mixed culture of *P. acidilactici* and *L. monocytogenes*	mMRS-BPB agar (Lee and Lee, 2008)

The mMRS-BPB (modified MRS supplemented with bromophenol blue) agar was selected for determination the cell number of *P. acidilactici* when it is co-cultured with *Listeria spp.* Lee and Lee (2008) found that MRS agar supplemented with 0.05% cysteine-HCl and 0.002% BPB have a better selectivity for LAB strains compared with MRS agar.
All of the experiments were done in triplicate.

6.2.3. Antilisterial activity assay

In case of *L. monocytogenes* 1486/1 or 971 grown in presence of *P. acidilactici* HA6111-2, examination of antilisterial bacteriocin produced by *P. acidilactici* was carried out. From the liquid phase of the biofilm system one ml broth was removed and examined according to the protocol by Van Reenen et al. (1998). When *P. acidilactici* grew with *L. monocytogenes* 1486/1 or *L. monocytogenes* 971, the indicator strains were *L. monocytogenes* 1486/1 or *L. monocytogenes* 971, respectively.

6.3. Results and discussion

The results of biofilm formation are shown in Fig. 6.1. - 6.9. *L. innocua* C6, *L. monocytogenes* L4 and T3 could produce biofilm equally in TSB broth when they grew individually (Fig. 6.1.). When the strains were grown in mono-culture, $10^3 – 10^4$ cell/3 cm^2

10.14751/SZIE.2016.040

attached during 1-hour incubation to the surface of the coupons. After at least two days the *Listeria* strains reached the maximum cell number in the biofilm (10^6 – 10^7 CFU/3 cm^2) in mono-culture. According to Doijad et al. (2015) observations, if a strain is able to produce approx. 10^5 CFU/cm^2, it is a strong biofilm former. Based on their work all of the three *Listeria* strains are strong biofilm formers.

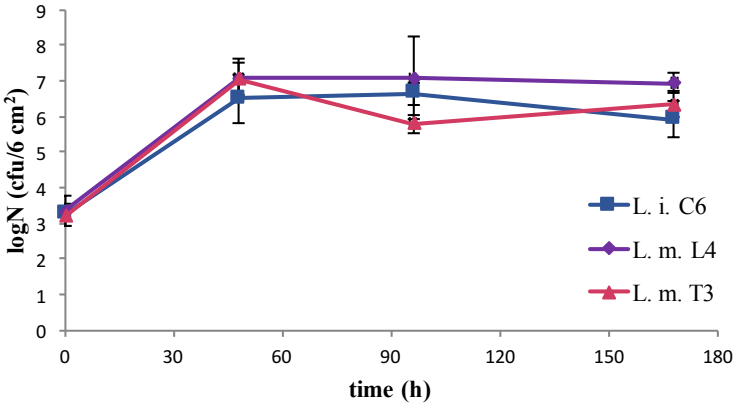


Figure 6.1. Individual biofilm formation of *L. innocua* C6, *L. monocytogenes* L4 and T3 in TSB broth on SS coupon

Based on the previous results, since both *L. monocytogenes* strains had the same biofilm producing capability, *L. monocytogenes* L4 was randomly selected to examine its behavior in mixed culture with *L. innocua* C6. The same amount of the cells of both strains was attached after the 1-hour incubation (Fig. 6.2.) and the number of attached cells increased until 48 hours ($logN_{L.m\ L4}$: 5.4 CFU/3cm^2, $logN_{L.i.\ C6}$: 6.4 CFU/3cm^2). After 120 hours *L. monocytogenes* L4 could not be detected from the biofilm and the number of *L. innocua* C6 decreased to 5.85 and 5.13 log CFU/3cm^2. This can be explained by the better growth capability of *L. innocua* which was previously described in several publications (Beumer et al., 1996; Cornu et al., 2002). In those publications the growth kinetics of mixed cultures was studied in broth, not in surface models. The kinetics of biofilm formation in this study proved to be similar to the planctonic growth kinetics.

10.14751/SZIE.2016.040

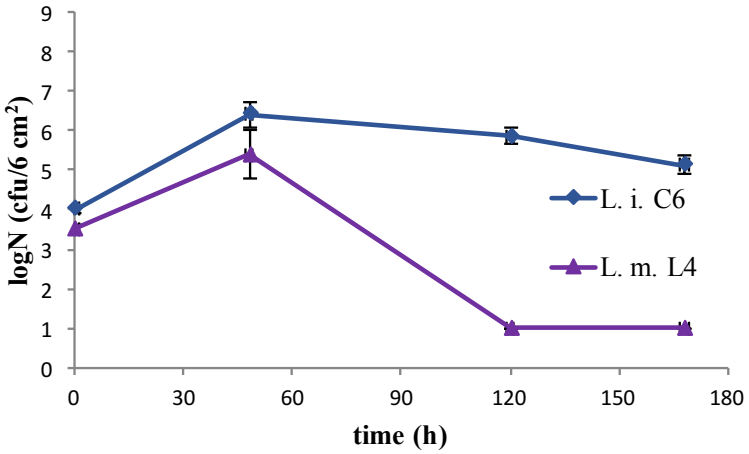


Figure 6.2. Biofilm formation in mixed culture of *L. innocua* C6 and *L. monocytogenes* L4 in TSB broth on SS coupon. *: no detectable *L. monocytogenes* L4

Biofilm formation of *L. monocytogenes* was also examined in presence of lactic acid bacteria. Firstly, it had to be proved that the selected strains were able to attach to the surface in MRS broth. The MRS broth was selected to examine the co-growth of *P. acidilactici* and *L. monocytogenes* 1486/1 and 971. All of the strains could attach to the SS surface were able to produce biofilm (Fig. 6.3.). It has to be highlighted, that *P. acidilactici* have a great capability to attach onto the SS surface and produce biofilm. It was previously reported that, *P. acidilactici* is able to produce strong biofilms in the food industry even, if it is not applied as a starter culture (Agarwal et al., 2006; Gunduz and Tuncel, 2006). *L. monocytogenes* 1486/1 and 971 produce biofilm in the same tendency as produced biofilm by *L. monocytogenes* T3 and L4, however the attachment of *L. monocytogenes* 1486/1 and 971 was higher with one log value.

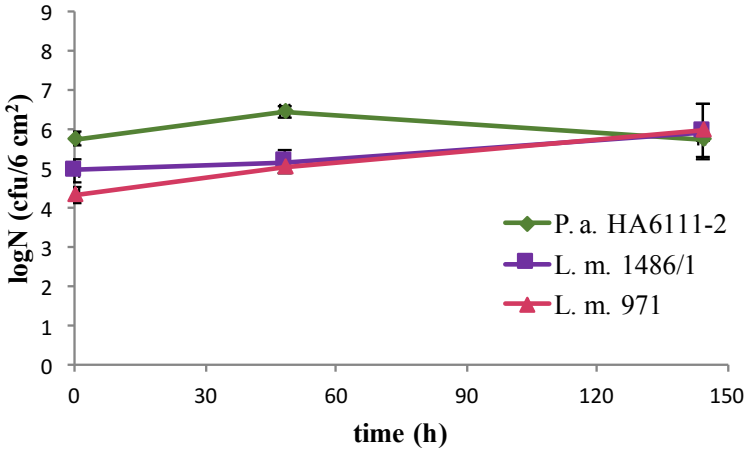


Figure 6.3. Individual biofilm formation of *P. acidilactici* HA 6111-2, *L. monocytogenes* 1486/1 and 971 in MRS broth on SS coupon

10.14751/SZIE.2016.040

When *L. monocytogenes* 1486/1 was inoculated with *P. acidilactici* in mixed culture to produce biofilm (Fig. 6.4.) the *L. monocytogenes* was not able to produce biofilm, its present was not detectable during the experiment. The antilisterial bacteriocin activity was measured after 48 and 144 hours and the activity was found 6400 AU/ml and 1600 AU/ml, respectively. The adherent cells of *P. acidilactici* were 7.9 log CFU/3 cm^2 and 5.84 log CFU/3 cm^2 after 48 and 144 hours. In Chapter 4 at Fig 4.1. A1. the growth and bacteriocin production of *P. acidilactici* can be seen. This strain could produce bacteriocin under normal condition after 3-6 hours, thus the bacteriocin activity at the first measurement point (after 1-hour incubation) was not checked. It was also found that for the 6400 AU/ml activity approx. 9 hours and 8.9 log CFU/3 cm^2cell concentration was necessary. During biofilm formation one log cycle less cell was enough to produce the same amount of bacteriocin and as the number of the attached cells decreased the activity of the bacteriocin became weaker.

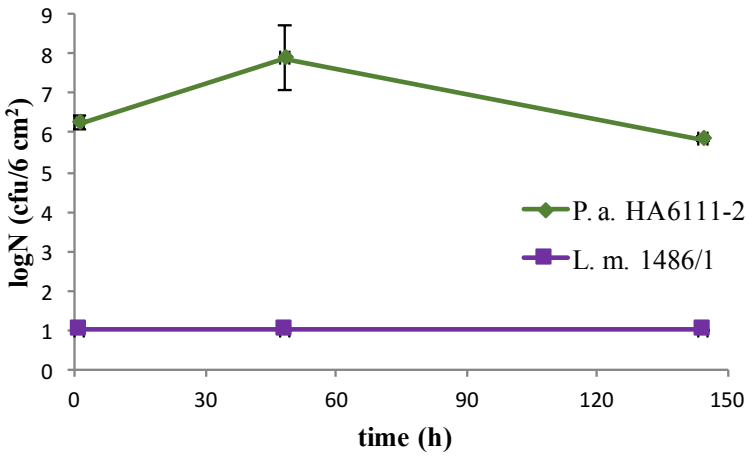


Figure 6.4. Biofilm formation in mixed culture of *P. acidilactici* HA 6111-2 and *L. monocytogenes* 1486/1 in MRS broth on SS coupon. *: no detectable *L. monocytogenes* 1486/1

In contrary, when *L. monocytogenes* 971 was co-cultured with *P. acidilactici* (Fig. 6.5.), the concentration of the adherent cells was different. The adherence of *P. acidilactici* was significantly smaller (3.4 log CFU/3 cm^2) than when it grew in mono-culture. Furthermore, the *L. monocytogenes* 971 was able to attach on to the surface and the adhered cells were 2.8 log CFU/3 cm^2. During biofilm formation *P. acidilactici* reduced the numbered of adhered cells of *L. monocytogenes* below the detection limit. The bacteriocin activity was 12800 AU/ml after 48 and 144 hours. That activity is higher than previously measured against *L. monocytogenes* 1486/1. The *L. monocytogenes* 971 is probably more sensitive to the bacteriocin compared to *L. monocytogenes* 1486/1. In Chapter 4 similar phenomena were

10.14751/SZIE.2016.040

described, *L. monocytogenes* 971 demonstrated the highest sensitivity to *P. acidilactici* supernatants.

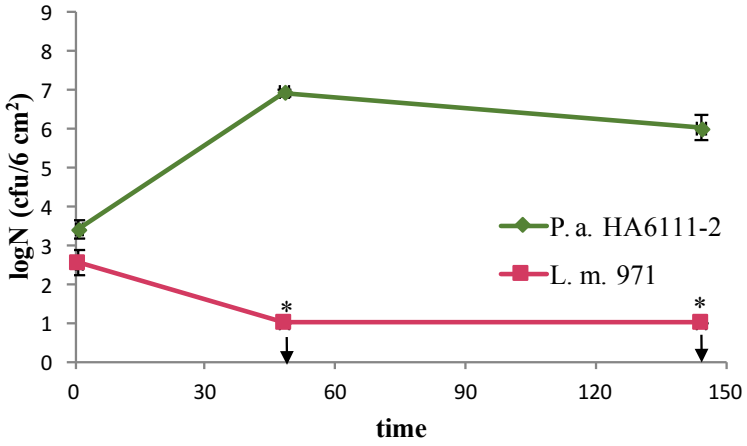


Figure 6.5. Biofilm formation in mixed culture of *P. acidilactici* HA 6111-2 and *L. monocytogenes* 971 in MRS broth on SS coupon.
*: no detectable *L. monocytogenes* 971

In pasteurized milk *P. acidilactici* and *L. monocytogenes* could adhere to the surface as well (Fig 6.6.). However, *P. acidilactici* reduced the number of *L. monocytogenes* 1486/1 cells, no bacteriocin activity was detected during the biofilm formation. Speranza et al. (2009) studied the biofilm formation of *L. monocytogenes* and LAB in cheese and they observed that the presence of LAB could delay the growth of *L. monocytogenes*. It is also significant observation that when LAB strain did not produce bacteriocin, *L. monocytogenes* was able to attach onto the SS coupons.

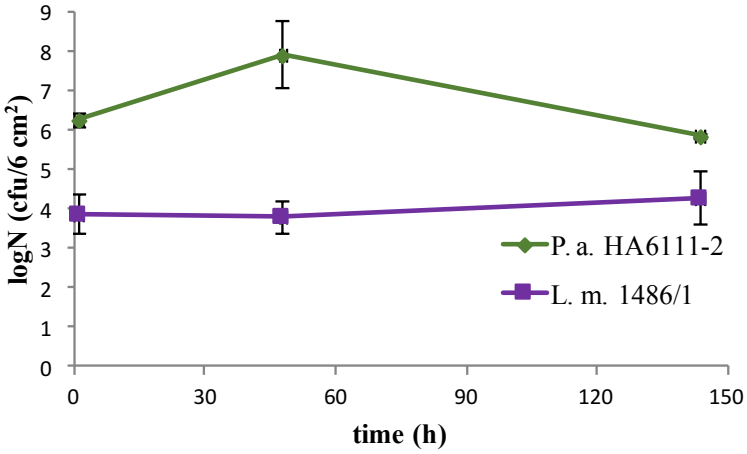


Figure 6.6. Biofilm formation in mixed culture of *P. acidilactici* HA 6111-2 and *L. monocytogenes* 1486/1 in pasteurized milk on SS coupon.

10.14751/SZIE.2016.040

Interestingly, when *P. acidilactici* was co-cultured with *L. monocytogenes* 971 in pasteurized milk the LAB strain was overgrown by *L. monocytogenes* 971 (Fig. 6.7.). In this case no bacteriocin activity was observed. The poor growth could be explained by the weak lactose uptake ability of *P. acidilactici.* Caldwell et al. (1998) studied the lactose uptake of different *P. acidilactici* and other LAB strains. They noticed differences in the lactose uptake ability of LAB strains. Some *P. acidilactici* strains do not have good lactose uptake ability.

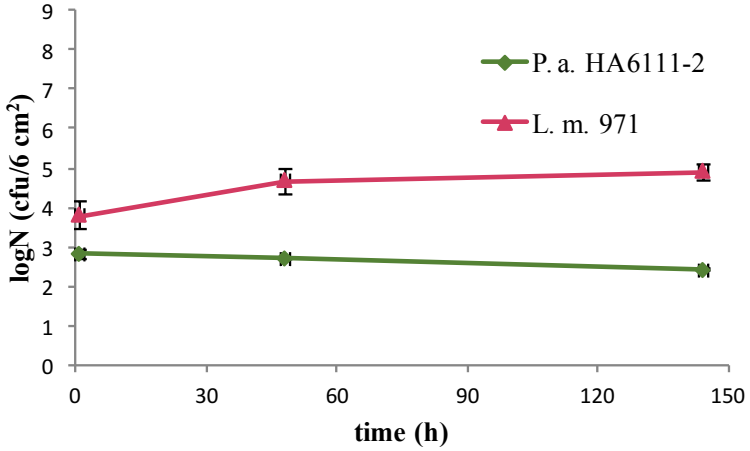


Figure 6.7. Biofilm formation in mixed culture of *P. acidilactici* HA 6111-2 and *L. monocytogenes* 971 in pasteurized milk on SS coupon

Based on the results of mixed culture experiments the *L. monocytogenes* 971 was selected to examine the biofilm formation ability in minced meat (Fig. 6.8.). In minced meat model media, the *P. acidilactici* was able to reduce the number of *L. monocytogenes* 971 below the detection limit. The highest bacteriocin activity was observed in that case, 12800 AU/ml and 25600 AU/ml after 48 and 144 hours, respectively. It can be concluded that the LAB strains do not have better growth capability to produce biofilm than *L. monocytogenes*. To control *L. monocytogenes* it is necessary to produce bacteriocins. It was clearly demonstrated that when the LAB strain was able to produce bacteriocin during the biofilm formation the growth of *L. monocytogenes* was controlled.

10.14751/SZIE.2016.040

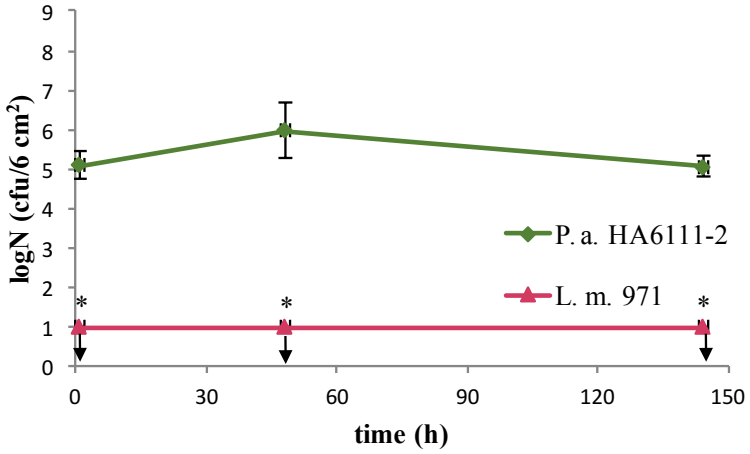


Figure 6.8. Biofilm formation in mixed culture of *P. acidilactici* HA 6111-2 and *L. monocytogenes* 971 in minced meat supplemented with MRS broth on SS coupon. *: no detectable *L. monocytogenes* 971

The biofilm formation of *L. monocytogenes* L4, T3 and *L. innocua* C6 was examined in mono-culture (Fig. 6.1. and 6.2.). The biofilm formation of *L. monocytogenes* L4 and *L. innocua* C6 in mixed culture was examined on LL surface (Fig. 6.9.). After 1-hour incubation the number of *L. innocua* C6 was almost 2 log cycle lower than the adhered cells of *L. monocytogenes* L4. After 24-hours incubation the number of *L. innocua* cells increased while at the same time the number of *L. monocytogenes* L4 cells decreased. After two days the number of adhered cells of both *Listeria* strains on the surface of LL was in the same range taken the standard deviations into consideration. These results indicate that maybe the *L. innocua* has a better growth capability than *L. monocytogenes* as described before by Beumer et al. (1996) and Cornu et al. (2002).

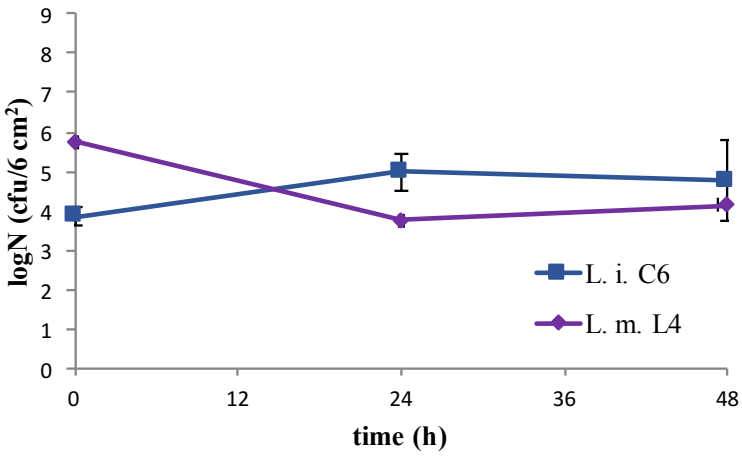


Figure 6.9. Biofilm formation in mixed culture of *L. innocua* C6 and *L. monocytogenes* L4 on lettuce leaves

10.14751/SZIE.2016.040

Results showed that *L. innocua* C6 could overgrow *L. monocytogenes* L4 during biofilm formation under specific circumstances. This phenomenon could be beneficial for the food industry, since *L innocua* is not human pathogen bacterium. When *L. monocytogenes* was inoculated together with lactic acid bacteria it was clearly demonstrated that *P. acidilactici* HA6111-2 inhibited *L. monocytogenes* 1486/1 and 971 strains in model media and minced meat biofilms. In these systems *P. acidilactici* was able to produce high amount of antilisterial bacteriocin in biofilm. That could be an explanation why *P. acidilactici* HA 6111-2 could inhibit the growth of *L. monocytogenes* strains. It was also demonstrated that *P. acidilactici* HA6111-2 was not able to produce bacteriocin in milk when *L. monocytogenes* was also present. Consequently, *L. monocytogenes* 971 strains could overgrow the *P. acidilactici* HA 6111-2 in this natural media.

My data suggest that biofilm formation of *L. monocytogenes* could be inhibited by lactic acid bacteria, but the inhibition depends on the food matrices and the origin of strains. *L. monocytogenes* was inhibited only in presence of bacteriocin produced by *P. acidilactici* HA6111-2. Other antimicrobial substances produced by this strain were not able to control *L. monocytogenes*. Further examinations are needed to extend this conclusion to other lactic acid bacteria and to define what is the exact reason for the inhibition of *L. monocytogenes* by *L. innocua*.

10.14751/SZIE.2016.040

CHAPTER 7 - THE SUITABILITY OF THE ISO 11290-1 METHOD FOR THE DETECTION OF LISTERIA MONOCYTOGENES

7.1. Introduction

Listeria monocytogenes is an ubiquitous food-borne human pathogenic bacterium that present a significant public health risk (Gelbíčová and Karpíšková, 2012). Nowadays there is an increasing consumer demand for minimally treated food products that may allow the growth of *L. monocytogenes*. Hence, reliable detection methods of *L. monocytogenes* are needed. Several detection methods have been developed in the last century, which contain enrichment steps and apply different selective media. The International Organization for Standardization published the 11290 standard family for enumeration and detection of *L. monocytogenes* in 1996 (ISO, 1996). Scientific publications (Al-Zeyara et al., 2011; Beumer, et al., 1996; Capita, et al., 2000) discuss the problems of the ISO 11290 standard methods. The use of Fraser enrichment broth (FB) is the most important step of the method. FB is suitable to inhibit Gram-negative microbes, since this broth contains nalidixic acid, acriflavine and lithium chloride (Al-Zeyara et al., 2011). Capita and co-workers (2000) studied the presence of *L. monocytogenes* in raw chicken using FB. Most of the samples that contained *L. monocytogenes* showed darkening of the growth medium. They found that FB has a very high sensitivity (98.95%) and low specificity (40%) with the aesculin-ferric ammonium citrate differential system. Other studies focused on the compounds of FB, especially acriflavine. Beumer et al. (1996) examined the effect of acriflavine on the multiplication ability of *L. monocytogenes* using different cell concentrations. Their results showed that acriflavine had inhibitory effect on the growth of *L. monocytogenes*. They also observed that the lag phase of *L. monocytogenes* lengthened when 5, 10 and 15 mg/L concentrations of acriflavine were used, with the exception of one strain of *L. monocytogenes*, which was unable to grow in presence of 15 mg/L acriflavine.

Scientific reports (Cornu et al., 2002; Gnanou Besse et al., 2005; Scotter et al., 2001; Yokoyama et al., 2005) showed that *Listeria innocua* has an inhibitory effect against *L. monocytogenes* during enrichment steps. The inhibition was related to the bacteriocin-like substances produced by *L. innocua* (Yokoyama et al., 2005). *L. monocytogenes* 1/2a was

*: Engelhardt T. et al. (2016), *LWT-Food Science and Technology* 71, 213-220.

10.14751/SZIE.2016.040

inhibited by *L. innocua* in mixed culture, which could be explained by the selective growth advantage of *L. innocua* (Cornu et al., 2002).

The generation time of *L. monocytogenes* varies widely, depending on environmental conditions. Yokoyama et al. (2005) obtained 95.5 ± 11.35 min for the generation time of *L. innocua* and 92.9 ± 8.45 min in case of *L. monocytogenes* in Difco enrichment broth incubated at 30 °C. Generation times of two *L. innocua* and *L. monocytogenes* strains in Tryptone Soy Broth complemented with Yeast Extract (TSB-YE) incubated at 30 °C proved to be 124 and 167 min, and 119 and 130 min, respectively (Carvalheira, et al., 2010). According to other investigations generation times of *L. monocytogenes* were 45 min in TSB at 30 °C, 41 min in dairy products at 35 °C, and 39.8 min in TSB at 37 °C (ICMSF, 1996).

According to ISO 11290-1:1996/A1 standard Agar Listeria Ottaviani and Agosti (ALOA) has to be used for detection of *L. monocytogenes*. The incubation time of the plates should be 24-48 h at 37 °C. Zunabovic et al. (2011) proposed to check the ALOA plates after 24 h incubation, as the size of the halo increases rapidly, thus the non-*L. monocytogenes* colonies cannot be distinguished from that of *L. monocytogenes*. However, Leclercq (2004) observed that several *L. monocytogenes* strains were able to produce halo only after 96 h. The scientific literature showed contradictory data in the field of the above mentioned detection method. Hence, in this study the problems of traditional standard detection method of *L. monocytogenes* were investigated in presence of *L. innocua*, focusing on the enrichment and agar plate steps.

7.2. Materials and methods

7.2.1. Strains

L. monocytogenes CCM 4699 (C1) and *L. innocua* CCM 4030 (C6) were obtained from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic). *L. monocytogenes* L4 and L16 (isolated from cheese products), *L. monocytogenes* T3, and *L. innocua* P6, P8, P10-11, T10, O4, O9, O14 were provided by the Department of Microbiology and Biotechnology (Corvinus University of Budapest, Hungary). The strains were stored at 80 °C in 5% glycerol. Before the experiments strains were subcultured twice on Brain Heart Agar (Merck) at 37 °C for 17-22 h.

7.2.2. Examination of growth on Agar Listeria Ottaviani and Agosti (ALOA)

10.14751/SZIE.2016.040

All *Listeria* strains were streaked onto ALOA agar (Merck) to observe the appearance of the colonies. Plates were incubated for 48 h at 37 °C and halo formation was checked after 24, 34 and 48 h, respectively. After 48 h incubation the plates were further stored at room temperature (25-30 °C) for 120 h and examined daily.

7.2.3. Examination of the inhibition ability of *L. innocua* against *L. monocytogenes*

L. innocua strains were grown in Brain Heart (BH) broth (Merck) for 17-19 h at 37 °C. Cells were harvested by centrifugation (4000 rpm, 10 min, 25 °C) and then the supernatant was filtered with 0.20 μm membrane filter. *L. monocytogenes* strains were grown in BH broth overnight at 37 °C. *L. monocytogenes* T3 and L4 strains were inoculated into the cell free supernatant of *L. innocua* strains. BH broth was used as positive control. The growth at 37 °C was monitored every 30 min for 24.5 h by Multiskan Ascent (Thermo Electron Corporation) microplate reader at 595 nm. *L. monocytogenes* T3 and L4 strains were tested against 6 different *L. innocua* cell free supernatants.

7.2.4. Culturing study of *Listeria* strains applying different cell concentrations

Different mixtures of *L. monocytogenes* strains and *L. innocua* were prepared according to Table 7.1.

Table 7.1. Combinations of L4, L16, T3 and C6 *Listeria* strains during co-culturing study

Combinations of *L. monocytogenes* L4, L16, T3, C1 and *L. innocua* C6	
L. monocytogenes ratio	*L. innocua* ratio
1	0
0	1
1	1
1	10
1	100

Half Fraser broth (hFB) and Fraser broth (FB) (Merck) were used for culturing steps (Fig. 7.1.) according to ISO 11290-1 standard.

10.14751/SZIE.2016.040

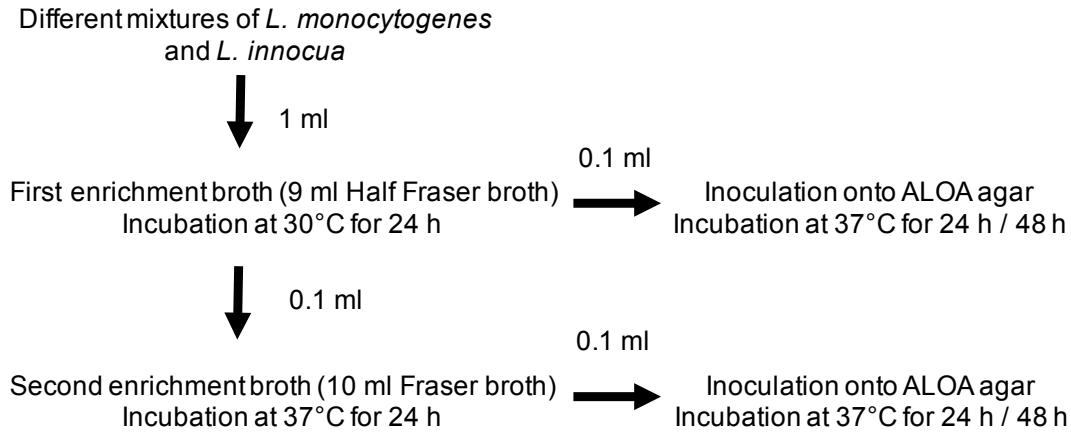


Figure 7.1. Flowchart of the enrichment steps

Individually inoculated *L. monocytogenes* strains and *L. innocua* C6 were used as controls. After culturing in hFB and FB samples were inoculated onto ALOA plates and incubated at 37 °C for 24 h. Plates were examined after an additional 24-h incubation at the same temperature.

7.2.5. Competitive growth of *L. monocytogenes* and *L. innocua* in half Fraser and Fraser enrichment broths

Growth curves of monocultures and co-cultures of *L. monocytogenes* T3 and *L. innocua* C6 in hFB and FB were determined by plating aliquots of the cultures onto ALOA plates according to steps shown in Fig. 7.1. The initial ratio of the two *Listeria* strains was 1:1 in hFB. Samples were taken from hFB and FB in every 4 h.

7.2.6. Data analysis

Data of inhibition ability of *L. innocua* against *L. monocytogenes* were analysed by Microsoft Excel and by PAST statistical software using one-way ANOVA (Hammer et al., 2001). Value of the inhibition was expressed based on the following equation:

$$inhibition\ value\ = \frac{OD_{\max C} - OD_{\max S}}{OD_{\max C}} \times 100$$

where:

$OD_{max\ C}$: the maximum optical density (OD) value of the strain without *L. innocua* supernatant

$OD_{max\ S}$: the maximum optical density (OD) value of the strain with *L. innocua* supernatant

Data of growth curves were analyzed by DMFit software available on Combase website (http://www.combase.cc). DMFit software using the Baranyi model (Baranyi & Roberts, 1994) was applied to fit growth rate (μ) and determine lag times.
Generation times (GT) were calculated from the following equation: GT = 0.693/μ (Adams & Moss, 2008).
All microbiological measurements were carried out in triplicates.

7.3. Results and discussion

7.3.1. Growth of *L. monocytogenes* strains on ALOA

L. monocytogenes L16 strain was not able to produce halo during the 168 h observation period, therefore it was excluded from further experiments. The halo around *L. monocytogenes* L4 colonies appeared after 24 h incubation at 37°C.
L. monocytogenes C1 and T3 were not able to produce halo after 24 h at 37 °C, but they gave a very typical halo around the colonies after 32 - 34 h. However, my observations showed that after two days of incubation the non-monocytogenes *Listeria* colonies may be overlapped by the halos (data are not shown). Based on my results - contrary to Leclercq (2004) and Zunabovic et al. (2011) - it is recommended to check the halo formation after 24, 34 and 48 h incubation, as well.

7.3.2. Inhibition ability of *L. innocua* against *L. monocytogenes*

When *L. monocytogenes* T3 grew in presence of the cell free supernatant of *L. innocua* strains the range of inhibition was 32 - 49% (Fig 7.2A). In case of *L. monocytogenes* L4 the range of inhibition was around 50% (Fig 7.2B), with the exception of *L. innocua* P8 (13%). Therefore, *L. innocua* C6 was selected for further examination, where the inhibition was 49% against *L. monocytogenes* T3 and 52% against *L. monocytogenes* L4, respectively. Cell free supernatant of *L. innocua* strains - except *L. innocua* P8 - caused similar effect on *L. monocytogenes* T3 and L4. These differences were not significant (data of statistical analysis are not shown). *L. innocua* C6 and other seven *L. innocua* strains could produce extracellular metabolites, which can contribute to the inhibition of *L. monocytogenes*.

10.14751/SZIE.2016.040

Nevertheless, competition can also be in the background of antagonistic interaction. These factors may influence together the growth of *L. monocytogenes* during enrichment, but for clarification further experiments are needed.

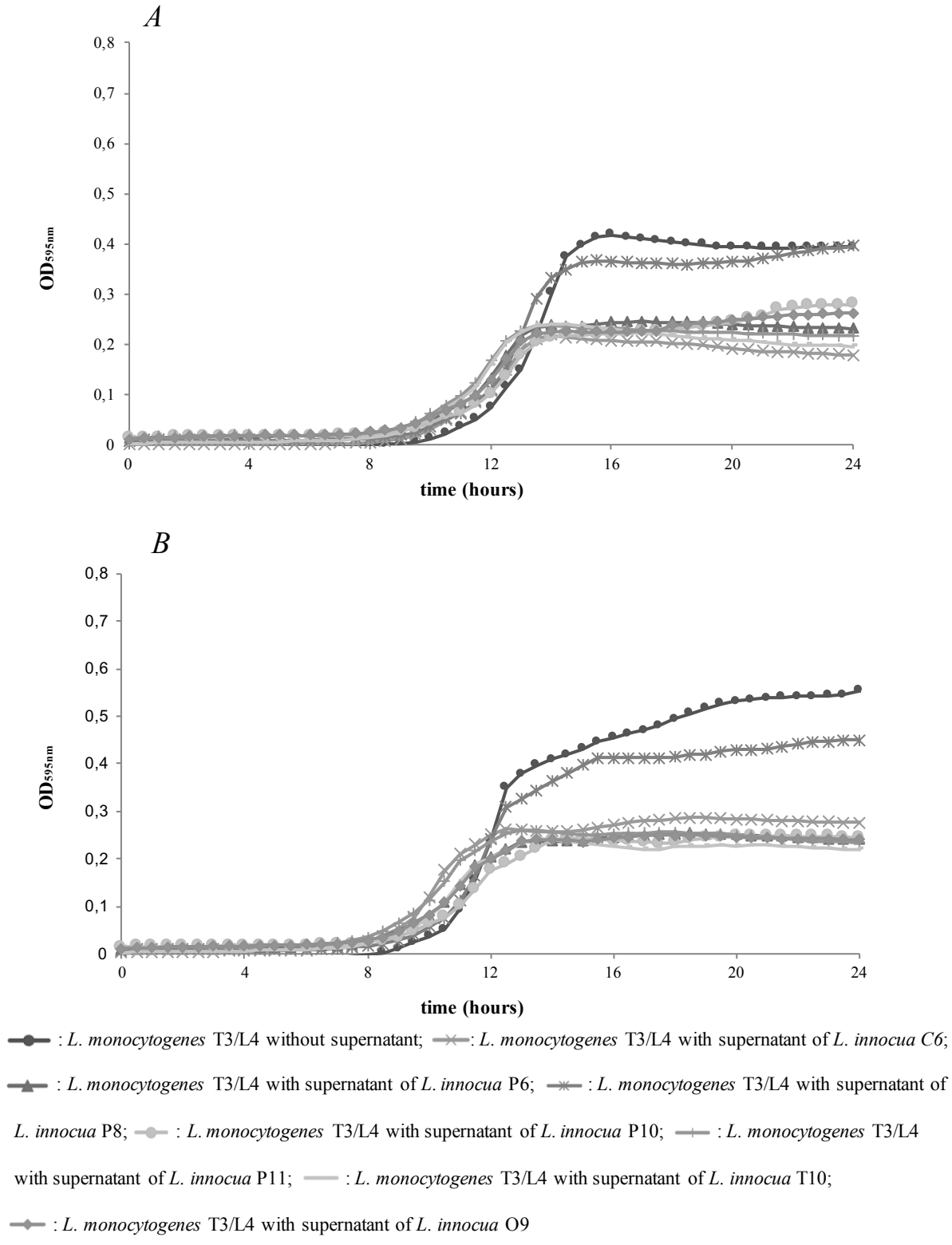


Figure 7.2. Growth of *L. monocytogenes* T3 (A) and *L. monocytogenes* L4 (B) in different *L. innocua strains* supernatants

10.14751/SZIE.2016.040

7.3.3. Co-culture growth of *L. innocua* and *L. monocytogenes* strains

The results of co-culture studies are shown in Figs. 7.3. - 7.5. The growth characteristics of the investigated *L. monocytogenes* strains in co-culture with *L. innocua* C6 depended on both the initial ratio and the strain. When the initial ratio of *L. innocua* C6 was equal with *L. monocytogenes* L4 and C1, their ratio did not change during the enrichment process.

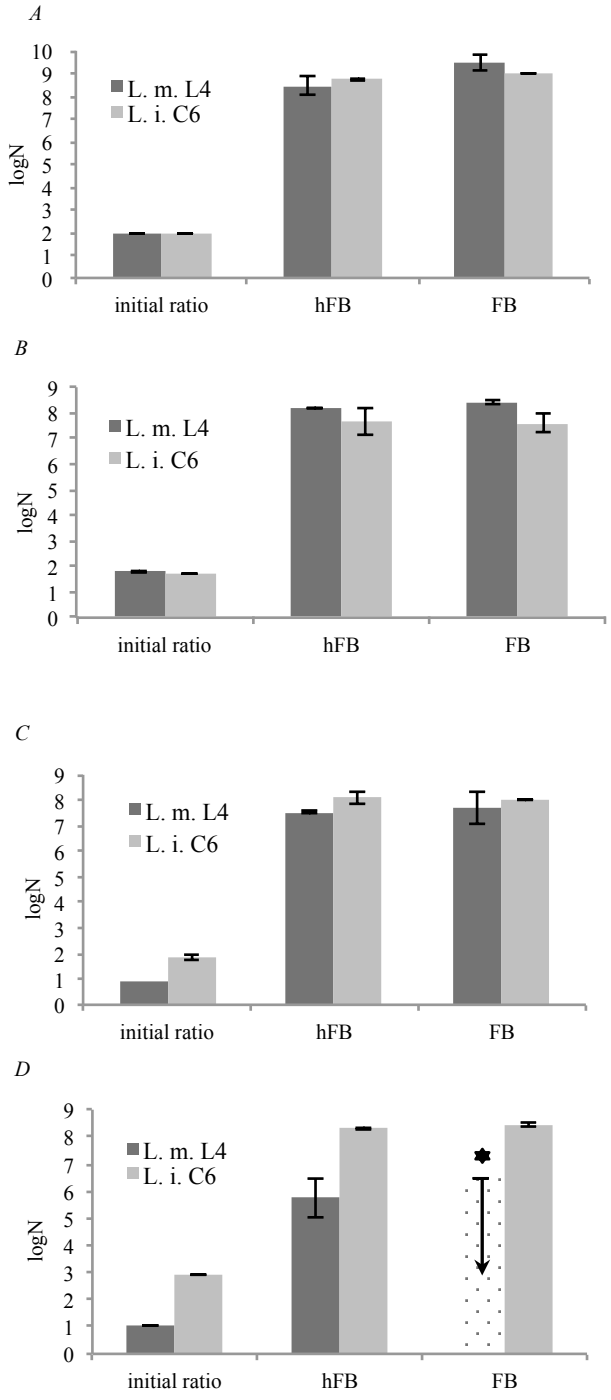


Figure 7.3. Growth of *L. monocytogenes* L4 and *L. innocua* C6 individually (A) and co-culture in 1:1 (B), 1:10 (C) and 1:100 (D) ratios; *: not detectable *L. monocytogenes* L4

10.14751/SZIE.2016.040

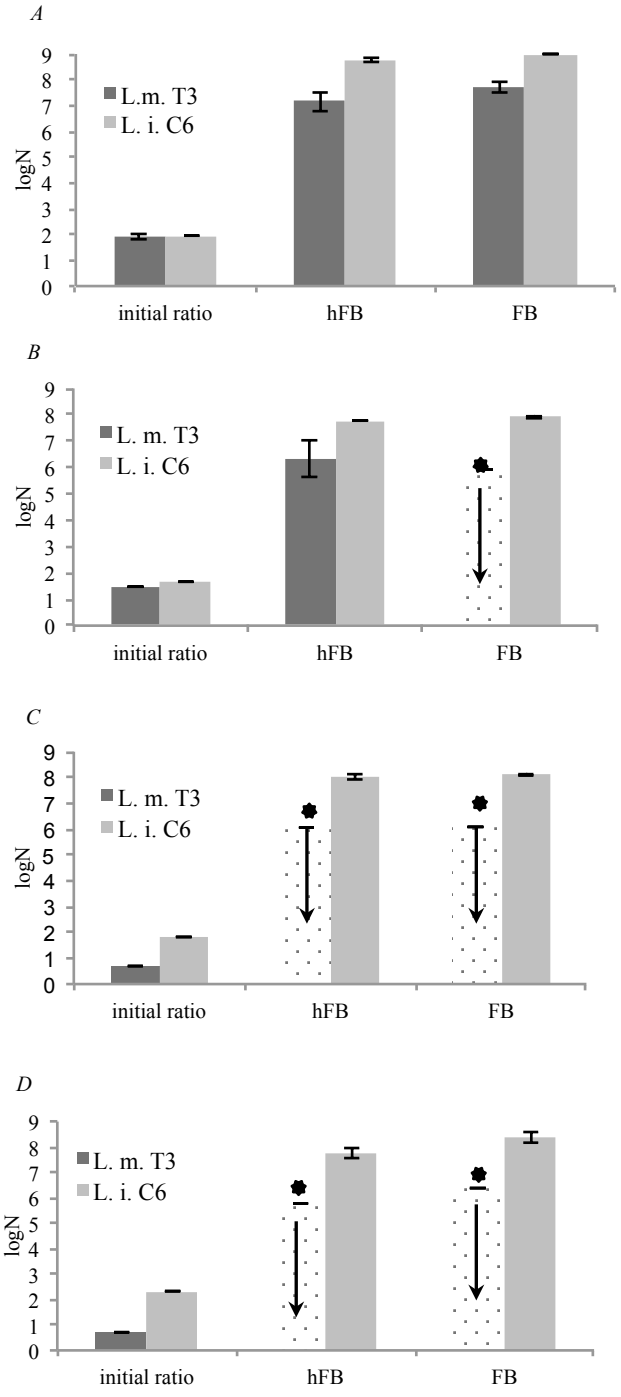


Figure 7.4. Growth of *L. monocytogenes* T3 and *L. innocua* C6 individually (A) and co-culture in 1:1 (B), 1:10 (C) and 1:100 (D) ratios; *: not detectable *L. monocytogenes* T3

10.14751/SZIE.2016.040

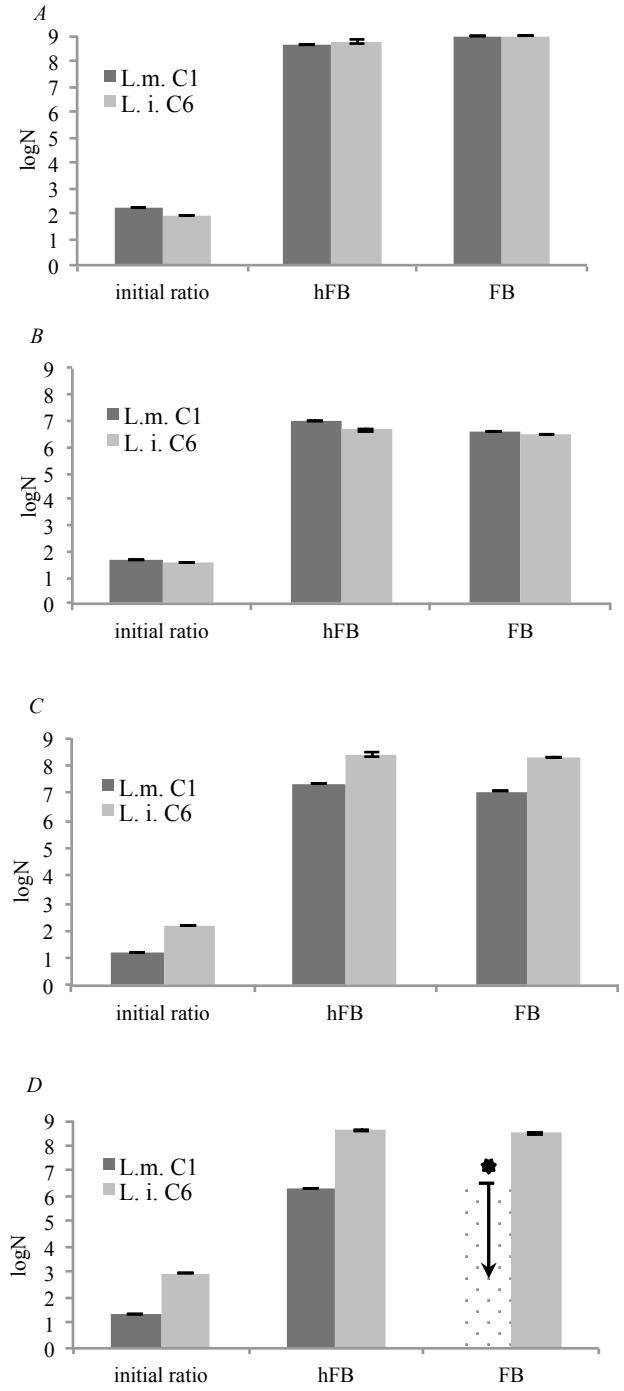


Figure 7.5. Growth of *L. monocytogenes* C1 and *L. innocua* C6 individually (A) and co-culture in 1:1 (B), 1:10 (C) and 1:100 (D) ratios; *: not detectable *L. monocytogenes* C1

However, in the case of *L. monocytogenes* T3 inhibition was observed. When 1:10 ratio was used the growth of *L. monocytogenes* C1 decreased in hFB and FB compared to mono-culture growth. In the case of *L. monocytogenes* L4 no changes were observed. Using 1:100 ratios of the test strains presence of L. monocytogenes L4 and C1 was not detectable after enrichment in FB which could be explained by the better growth ability or adaptation of *L. innocua* C6 to the selective components of the environment. Furthermore,

10.14751/SZIE.2016.040

L. monocytogenes T3 could not be detected in hFB and FB either in 1:10 or 1:100 ratios (Fig. 7.4.). In case of *L. monocytogenes* T3 ratio 1:100 was 1:50 in reality.

L. monocytogenes T3 proved to be less sensitive to the enrichment broth and/or the presence of *L. innocua* when it was inoculated in 1:1 ratio in hFB. This can be explained by the different concentration of acriflavine in hFB (6 g/L) and FB, since FB contains two times higher concentration of acriflavine than hFB. Additionally, according to Cornu et al. (2002) *L. innocua* has a growth advantage over *L. monocytogenes* in enrichment broth. Futhermore, my results demonstrate that *L. monocytogenes* T3 was more sensitive for higher acriflavine concentration in presence of *L. innocua* C6.

7.3.4. Growth kinetics of *L. monocytogenes* T3 and *L. innocua* C6 during enrichment

Results of my experiments (Fig. 7.4.) showed that the growth of *L. monocytogenes* T3 was inhibited (could not be detected) in co- culture already in 1:1 ratio after the enrichment steps in FB. Therefore, *L. monocytogenes* T3 and *L. innocua* C6 were further examined to determine the growth kinetics parameters during enrichment steps. Growth curves of mono- and co-cultures of *L. monocytogenes* T3 and *L. innocua* C6 (initial ratio 1:1 in hFB) are shown in Figs. 7.6. - 7.9., and the calculated growth parameters are given in Table 7.2. As it can be seen in Fig. 7.6. and 7.7. the growth curves of mono- and co-cultured *Listeria* strains in hFB showed similar tendency. The colony counts of *L. monocytogenes* T3 slightly decreased compared to *L. innocua* in hFB when they grew individually or in co- culture.

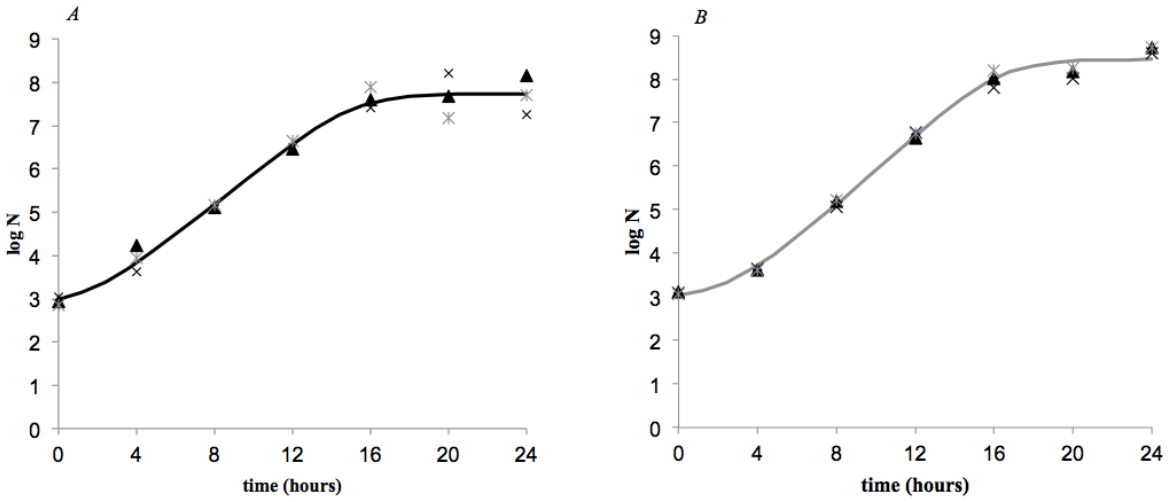


Figure 7.6. Growth curve of the mono-culture of *L. monocytogenes* T3 (A) and *L. innocua* C6 (B) in hFB

10.14751/SZIE.2016.040

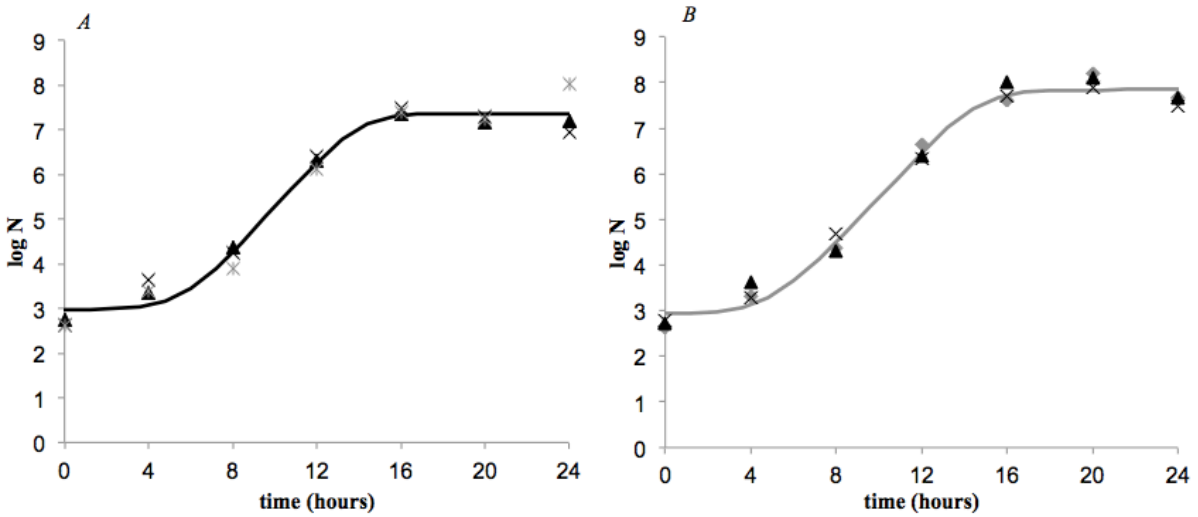


Figure 7.7. Growth curve of the co-cultured *L. monocytogenes* T3 (A) and *L. innocua* C6 (B) (1:1 ratio) in hFB

The calculated growth parameters (Table 7.2.) indicated that the lag phase was prolonged in case of *L. monocytogenes* T3 (5.1 h) when it was grown with *L. innocua* C6. However, at 6 mg/L acriflavine level the lag phase of *L. monocytogenes* T3 monoculture was shorter (1.7 h) than that of *L. innocua* C6 (3.0 h).

Table 7.2. Growth parameters of *L. monocytogenes* T3 and *L. innocua* C6 in hFB

		y_o (logN)	y_{end} (logN)	lag time (h)	growth rate (log unit h^{-1})	r^2	Generation time (min)
mono-culture	*L. monocytogenes* T3	2.97	7.73	1.7 ± 0.5	0.351 ±0.02	0.998	118
	L. innocua C6	3.06	8.44	3.0 ±1.0	0.408 ±0.04	0.991	102
co-culture	*L. monocytogenes* T3	2.94	7.37	5.1 ±1.4	0.481 ±0.10	0.978	86
	L. innocua C6	2.83	7.86	4.2 ±1.2	0.464 ±0.07	0.986	90

In my study the growth rate of individually inoculated *L. monocytogenes* proved to be 0.351 1/h, hence the generation time was 118 min, while in presence of *L. innocua* the growth rate was 0.481 1/h and the generation time was calculated as 86 min in hFB at 30 °C. The shorter generation time in co-cultured *L. monocytogenes* can be explained by production of bacteriocin- like substances or according to other investigation competitive growth between *L. monocytogenes* and *L. innocua* may lead to quorum sensing (QS) interaction (Griffiths, 2005).

The growth characteristics of the mono- and co-cultured *L. innocua* C6 and *L. monocytogenes* T3 in FB were different (Fig. 7.8. and 7.9.).

10.14751/SZIE.2016.040

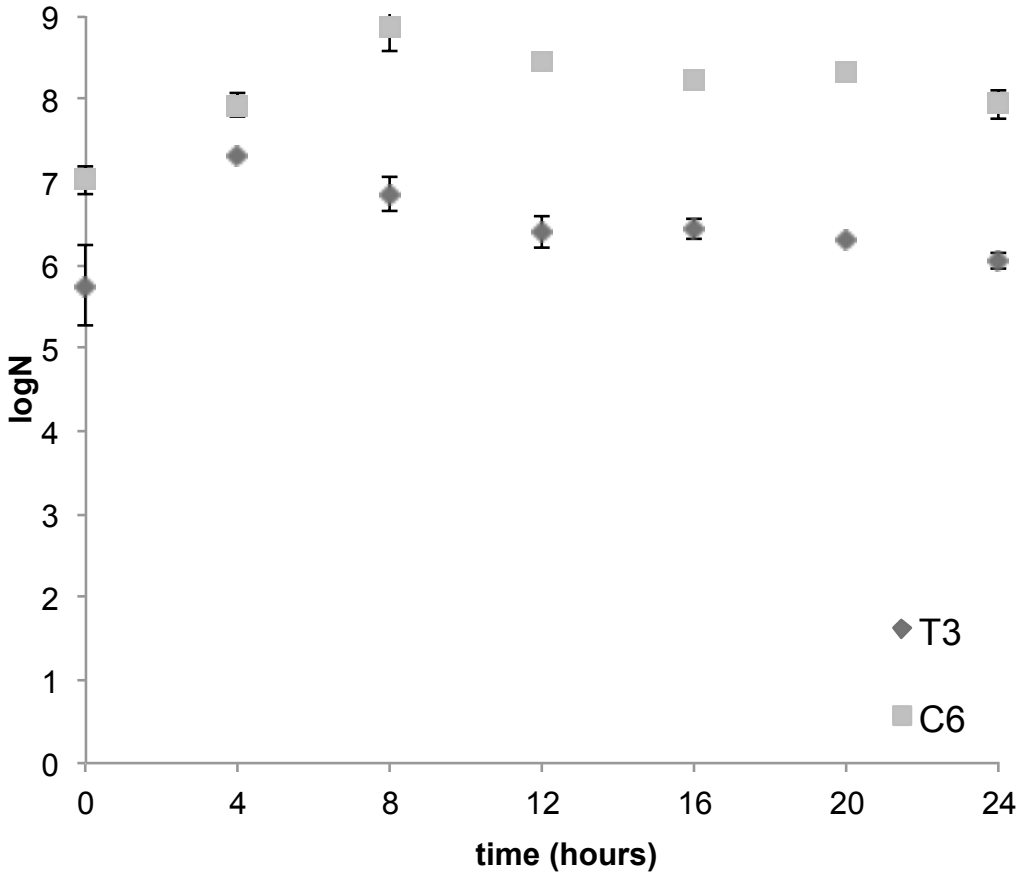


Figure 7.8. Growth of the mono-culture of *L. monocytogenes* T3 and *L. innocua* C6 in FB

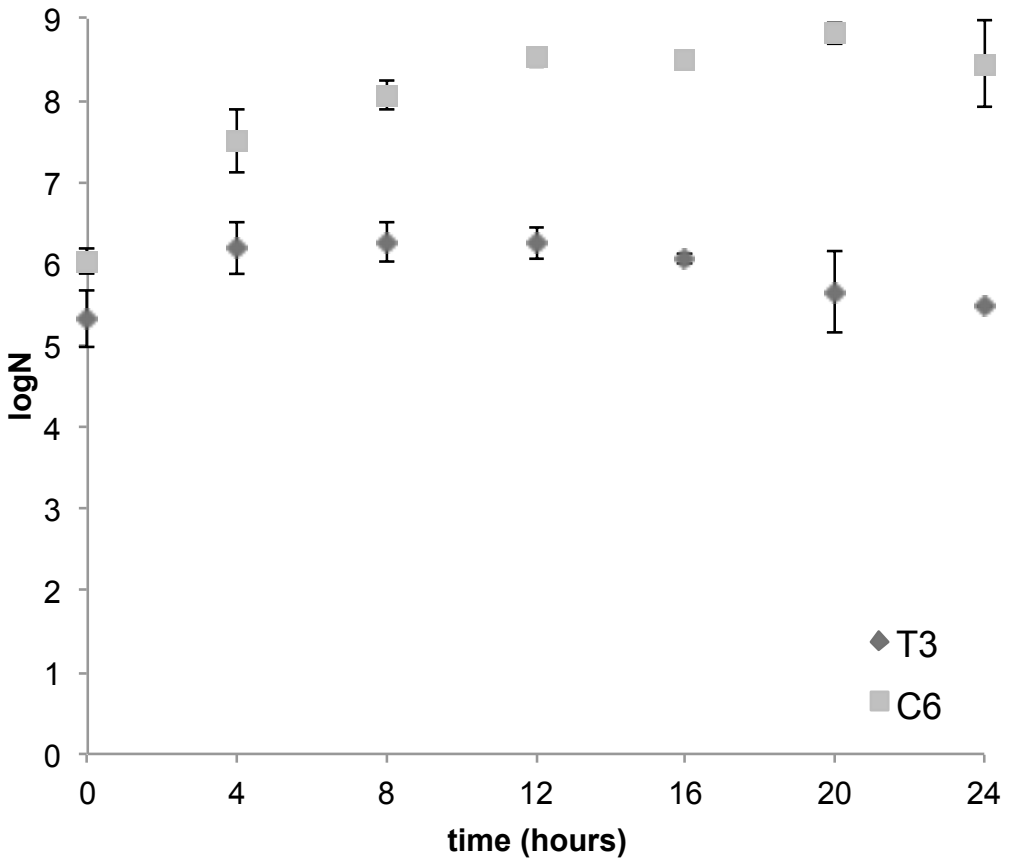


Figure 7.9. Growth of the co-cultured *L. monocytogenes* T3 and *L. innocua* C6 (1:1 ratio) in FB

Colony count of *L. innocua* C6 moderately increased within 8 h in both mono- and co-cultures. Nevertheless, after 8 h - when L. innocua C6 was grown in monoculture - the viable cell number slightly decreased at the end of the enrichment steps. When *L. innocua* C6 was grown in co-culture with *L. monocytogenes* T3, its cell number increased. In case of *L. monocytogenes* T3 decreasing cell numbers were observed when it was grown with or without *L. innocua* C6 at the end of the enrichment. When *L. monocytogenes* T3 grew individually and co-cultured, cell numbers slightly increased in the first 4 h. At the end of enrichment steps in FB two-log cycle-difference (lgN_{T3}: 6.0, lgN_{C6}: 7.9) was observed. Nevertheless, when the growth of *L. monocytogenes* and *L. innocua* was examined in co-culture (Fig. 7.9.) the initial one log cycle difference increased to almost three log cycles (lgNT3: 5.5, lgNC6: 8.4). When *L. innocua* C6 reached the stationary phase, *L. monocytogenes* T3 was not able to grow further. This phenomenon can be explained by the Jameson effect (Jameson, 1962). In 1962 J. E. Jameson published a study on the interaction of co- cultured species. The author observed that when two species are co-cultured, there is one (a) that dominates growth and the other (b) that is suppressed. When 'a' is enter in the stationary phase, growth of 'b' will be stopped. This phenomenon was later suggested to be called as Jameson effect. According to ISO 11290 standard streaking out from FB must be done after 24 ± 3 h, however – based on my results – the viable cell numbers decrease during this interval when the ratio of *L. monocytogenes* and *L. innocua* at least 1:10, depending on strains. Because of the ratio of these *Listeria* species in naturally contaminated food samples is unknown, this may lead to false negative results. Actually, no significant increase in *L. monocytogenes* T3 cell number was observed after 24 h incubation, while *L. innocua* C6 was able to grow. Based on these results the inhibition of *L. monocytogenes* T3 in co-culture can not simply be explained with the growth parameters in enrichment broth, therefore further studies are needed to elucidate the inhibition of *L. monocytogenes* T3 by *L. innocua* C6.

7.4. Conclusion

Our results show that the response of the examined *L. monocytogenes* strains was different during the enrichment protocol. It was demonstrated that growth of *L. monocytogenes* could be inhibited by *L. innocua* during Fraser enrichment steps. This inhibition is strain and ratio dependent.

The ISO 11290 series of standards recommend 24 - 48 h incubation time of ALOA at 37 °C. According to my results the optimum incubation time is 32 - 34 h, and it is nominated to

10.14751/SZIE.2016.040

check halo formation after 24, 32 and 48 h, respectively. It is also important to notice that one *L. monocytogenes* strain (L16) was not able to produce halo.

Overall, these results are in accordance with other published data, revealing the fact that the ISO 11290-1:1996 standard is not always reliable, which might lead to a major food safety problem.

10.14751/SZIE.2016.040

NEW SCIENTIFIC RESULTS

1. Two antilisterial bacteriocins produced by *Lactobacillus sakei* ST153Ch and *Lactobacillus plantarum* ST202Ch were characterized. It was demonstrated that the two bacteriocins are stable under different environmental conditions (pH, temperature, NaCl, detergents). The molecular size of the antilisterial bacteriocins is lower than 14.4 kDa.

2. The antilisterial bacteriocin production of *Pediococcus acidilactici* HA6111-2 and *Lactobacillus plantarum* ST202Ch was described under moderate stress conditions. *P. acidilactici* and *Lb. plantarum* demonstrated antilisterial activity under the stress conditions investigated (pH 3.5; pH 8.5; 7.5% NaCl). However, activity was dependent on the stress conditions applied and on the target organism.

3. A redox-potential measurement method has been adapted for measurement of bacteriocin activity of *Lb. plantarum* ST202Ch. Evaluation of bacteriocin activity was described by new parameters: ΔTTD, which means the difference between the detection times of the inhibited and the control (non-inhibited) *L. monocytogenes* suspensions; te, which means the elapsed fermentation time until the supernatant results in 2.5 log unit virtual decrease in *L. monocytogenes*.

4. A new multiple regression model was established to predict the activity of the bacteriocin produced by *Lb. plantarum* ST202Ch against *L. monocytogenes* 1486/1 under low temperature and different NaCl concentrations.

5. It was proved that, during biofilm formation the bacteriocin production of *P. acidilactici* HA6111-2 is matrix dependent.

6. *L. monocytogenes* in biofilms was inhibited only in presence of bacteriocin produced by *P. acidilactici* HA6111-2. Other antimicrobial substances produced by this strain were not able to control *L. monocytogenes*.

7. During the traditional detection method it was demonstrated that *L. monocytogenes* L4, T3 or C1 could be inhibited by *L. innocua* C6 during enrichment steps in Fraser broth. When initial ratio of *L. innocua* and *L. monocytogenes* was 100:1, *L. monocytogenes* was overgrown by *L. innocua*

10.14751/SZIE.2016.040

ÚJ TUDOMÁNYOS EREDMÉNYEK

1. Két, *Lactobacillus sakei* ST153Ch és *Lactobacillus plantarum* ST202Ch által termelt lisztéria-ellenes bacteriocint jellemeztem, amely során bizonyítást nyert, hogy a bakteriocinek ellenállnak különböző környezeti tényezőknek (pH, hőmérséklet, NaCl, detergensek). Továbbá megállapítottam, hogy a két bacteriocin mérete kisebb, mint 14,4 kDa.

2. Leírtam a *Pediococcus acidilactici* HA6111-2 és *Lactobacillus plantarum* ST202Ch törzsek *Listeria*-ellenes bakteriocin termelését enyhe stresszhatások alatt. Bakteriocin termelést pH 3,5; pH 8,5 és 7,5 % NaCl tartalom mellett figyeltem meg, valamint megállapítottam, hogy az aktivitás függ a célmikrobától.

3. Sikeresen adaptáltam egy redoxpotenciál mérésen alapuló módszert a bakteriocin aktivitás meghatározásához *Lb. plantarum* ST202Ch törzs esetében. A bakteriocin aktivitás meghatározásához két új paramétert vezettem be: a ΔTTD-t, amely a TTD-k különbséget mutatja meg a gátolt és kontroll (nem gátolt) *L. monocytogenes* szuszpenziók között; te-t, amely az az eltelt idő a fermentáció során ahol, a gátlóanyag a *L. monocytogenes* sejtszámában logN 2,5 virtuális értékű csökkenést eredményezett.

4. Új, többszörös regressziós modellt hoztam létre a kis hőmérséklet és a NaCl koncentráció közötti összefüggés megállapítására, mellyel a bakteriocin aktivitása előre jelezhető *Lb. plantarum* ST202Ch esetében *L. monocytogenes* 1486/1 törzs ellen.

5. Bebizonyítottam, hogy a biofilm képzés során a bakteriocin képzés mátrix-függő *P. acidilactici* HA6111-2 esetében.

6. Megállapítottam, hogy a biofilmet képző *L. monocytogenes* törzseket csak abban az esetben lehet teljesen gátolni, ha a *P. acidilactici* HA6111-2 törzs képes a bakteriocin termelésre. *P. acidilactici* törzs által termelt más antimikrobás anyagok nem bizonyultak ehhez elegendőnek.

7. A hagyományos tenyésztéses eljárás során megállapítottam, hogy a *L. monocytogenes* L4, T3 és C1 törzseket a *L. innocua* C6 képes gátolni a Fraser dúsítóban végzett lépések alatt. Amikor a *L. innocua* és *L. monocytogenes* kezdeti koncentrációjának aránya 100:1-hez, akkor a *L. innocua* képes túlnőni a *L. monocytogenes* törzseket.

10.14751/SZIE.2016.040

SUGGESTIONS

1. Additional research is needed to determine exactly the bacteriocin produced by *Lactobacillus sakei* ST153 Ch and *Lb. plantarum* ST202Ch.

2. My results should be taken into consideration when *L. innocua* is used as a surrogate for *L. monocytogenes*. Further examinations are needed to reveal these differences between the two strains. Overall, based on the results, it is recommended to use *L. monocytogenes* instead of *L. innocua* in the experiments.

3. Based on my results the bacteriocin production under relatively low temperature and relatively high NaCl concentration may not be sufficient to inhibit *L. monocytogenes* and additional hurdle strategy is needed to control *L. monocytogenes*. It would be necessary to validate the model in food systems, which would lead to more accurate prediction.

4. Further examinations are needed to define what is the exact reason for the inhibition of *L. monocytogenes* by *L. innocua* and lactic acid bacteria in biofilms.

5. My results suggest that *L. innocua* produce metabolites, which might be involved in the overgrowth of *L. monocytogenes*, thus more investigations are needed to identify those metabolites.

10.14751/SZIE.2016.040

SUMMARY

In the past decade a number of foodborne pathogens caused more and more illnesses and death worldwide, which lead serious economical losses for the food industry, the government as well as individuals. One of the most important emerging food-borne pathogens is the *L. monocytogenes* which causes serious disease, and its incidence in ready-to-eat, fermented, fresh food products is high. From the consumers' side foods with high nutritional value, less treated, natural, fresh products are demanded. To fulfil these requirements, food business operators are needed to develop new methodologies, new hurdles to ensure safe products, to eliminate food-borne pathogens. Applying novel preservation techniques safe products with high quality can be produced. Several novel techniques were developed in the past decades e.g. high-hydrostatic pressure, modified atmosphere packaging, addition of protective culture/antimicrobial compounds. In this work I focused on the possibilities to apply protective cultures against the pathogen *L. monocytogenes*. The overall focus of my dissertation was to better understand the interactions between the beneficial LAB strains and the pathogen *L. monocytogenes*. *L. innocua* which is commonly used as a surrogate of *L. monocytogenes*, therefore *L. innocua* was also involved in these studies. The influence of *L. innocua* on *L. monocytogenes* was also investigated.

Two antilisterial bacteriocins produced by *Lactobacillus sakei* ST153Ch and *Lactobacillus plantarum* ST202Ch were characterized. The bacteriocins have a static effect on the growth of *L. monocytogenes* serogroup IIb (from cheese) and *L. innocua* NCTC 11288. It was demonstrated that the two bacteriocins are stable under different environmental conditions (pH, temperature, NaCl, detergents). The molecular size of the antilisterial bacteriocins is lower than 14.4 kDa. Both bacteriocins have a potential to be used in the food industry as bio- preservative agents since they seem to be stable under environmental circumstances.

Inhibition of *Listeria* spp. by bacteriocin producing *Pediococcus acidilactici* and *Lactobacillus plantarum* (both isolated from fermented meats) was investigated under conditions of stress induced by pH, temperature and salt concentrations. *Listeria monocytogenes* serogroup IIb (from cheese), *L. monocytogenes* serogroup IVb (from cheese), *L. monocytogenes* serogroup IIb (from ground beef) and *Listeria innocua* NCTC 11288 were used as target strains. *P. acidilactici* and *Lb. plantarum* demonstrated antilisterial activity under the stress conditions investigated (pH 3.5; pH 8.5; 7.5% NaCl). However, the rate of activity was dependent on the stress conditions applied and on the target organism. *L. monocytogenes* serogroup IIb (from ground beef) and *L. innocua* NCTC 11288

respectively, were the most sensitive and the most resistant to the cell-free supernatants of the LAB culture investigated.

To measure the bacteriocin activity of *Lb. plantarum*, the redox-potential measurement method has been adapted. A new multiple regression model was established to predict the activity of the bacteriocin produced by *Lb. plantarum* ST202Ch against *L. monocytogenes* serogroup IIb at low temperature and different NaCl concentrations. Evaluation of bacteriocin activity was described by new parameters: ΔTTD, which means the difference between the detection times of the inhibited and the control (non-inhibited) *L. monocytogenes* suspensions; te means the essential fermentation time of *Lb. plantarum* needed to reach 2.5 log unit virtual destruction of *L. monocytogenes*. The adapted method might also be used to determine the bacteriocin activity against other species. According to my results the efficacy of the bacteriocin produced by *Lb. plantarum* ST202Ch against *L. monocytogenes* serogroup IIb can be predicted at low temperature and different NaCl concentrations. On the basis of the mathematical model it can be concluded that the bacteriocin production at lower temperature (<25 °C) and higher NaCl concentration (> 4%) may not be sufficient enough to inhibit *L. monocytogenes*, therefore and additional hurdle strategy is needed to control *L. monocytogenes*.

Laboratory studies have been performed to study the biofilm formation of *Listeria* strains and *P. acidilactici* on different matrices (stainless steel, minced meat, iceberg lettuce leaves). It was proved that during biofilm formation the bacteriocin production of *P. acidilactici* HA6111-2 depends on the matrix. *P. acidilactici* HA6111-2 was not able to produce bacteriocin in milk when *L. monocytogenes* was also present. Consequently, *L. monocytogenes* serogroup IIb (from ground beef) strains could overgrow the *P. acidilactici* HA 6111-2 in this natural media. *L. monocytogenes* was inhibited only in presence of bacteriocin produced by *P. acidilactici* HA6111-2. Other antimicrobial substances (e.g. lactic acid) produced by this strain were probably not able to control *L. monocytogenes*. Further examinations are needed to extend this conclusion to other lactic acid bacteria and to define what is the exact reason for the inhibition of *L. monocytogenes* by *L. innocua.*

Numbers of published data suggest that detection of *Listeria monocytogenes* is interfered by occurrence of other *Listeria* species in food. In this study the problems of traditional standard detection method of *L. monocytogenes* were also investigated, focusing on the enrichment steps in Fraser broth (FB). The growth of different mixtures of *L. monocytogenes* strains and *L. innocua* strain in the enrichment steps was investigated. Growth parameters were determined by DMFit software using Baranyi-model. When initial cell concentration of

10.14751/SZIE.2016.040

L. innocua was higher than that of *L. monocytogenes* inhibition of the latter was observed. Based on the results it was concluded that strain of *L. monocytogenes* was overgrown by *L. innocua* during the Fraser enrichment steps. When their growth was monitored during co-culturing in hFB (half Fraser broth) the lag phase of *L. monocytogenes* was prolonged by 3.4 hours. When *L. monocytogenes* grew in presence of *L. innocua* in 1:1 ratio three log cycles difference was observed at the end of Fraser enrichment step. All these problems might lead to false negative results.

Overall, in this research it was demonstrated that *L. monocytogenes* could be controlled by *P. acidilactici* or *Lb. plantarum*, but during the application all circumstances should be taken into account carefully. It is also important that *L. innocua* was able to inhibit the growth of *L. monocytogenes* not just in the food systems but during the detection method, too. When *L. innocua* is used as a surrogate of *L. monocytogenes* in model experiments, there is a chance to obtain inaccurate results.

REFERENCES

Adams, M. R. and Moss, M. O. (2008): *Food Microbiology*, (3rd ed.) Guildford: RSC Publishing, (Chapter 3).

Agarwal, S., Sharma, K., Swanson, B.G., Yüksel, G.Ü. and Clark, S. (2006): Nonstarter lactic acid bacteria biofilms and calcium lactate crystals in cheddar cheese. *Journal of Dairy Science,* 89, 1452-1466.

Al-Holy, M.A., Al-Nabulsi, A., Osaili, T.M., Ayyash, M.M. and Shaker, R.R. (2012): Inactivation of *Listeria innocua* in brined white cheese by a combination of nisin and heat. *Food Control* 23, 48-53.

Al-Zeyara, S. A., Jarvis, B. and Mackey, B. M. (2011): The inhibitory effect of natural microflora of food on growth of *Listeria monocytogenes* in enrichment broths. *International Journal of Food Microbiology,* 145, 98-105.

Albano, H., Todorov, S.D., van Reenen, C.A., Hogg, T., Dicks, L.M.T. and Teixeira P. (2007): Characterization of a bacteriocin produced *by Pediococcus acidilactici* isolated from "Alheira", a fermented sausage traditionally produced in Portugal. *International Journal of Food Microbiology,* 116, 239–247.

Altuntas, E.G., Cosansu, S. and Ayhan, K. (2010): Some growth parameters and antimicrobial activity of a bacteriocin-producing strain *Pediococcus acidilactici* 13. *International Journal of Food Microbiology,* 141, 28-31.

Anastasiadou, S., Papagianni, M., Filiousis, G., Ambrosiadis, I. and Koidis, P. (2008): Pediocin SA-1, an antimicrobial peptide from *Pediococcus acidilactici* NRRL B5627: Production conditions, purification and characterization. *Bioresource Technology*, 99, 5384-5390.

Arguedas-Villa, C., Stephan, R. and Tasara, T. (2010): Evaluation of cold growth and related gene transcription responses associated with *Listeria monocytogenes* strains of different origins. *Food Microbiology*, 27, 653-660.

Balciunas, E.M., Castillo Martinez, F.A., Todorov, S.D., Franco, B.D.G.D.M., Converti, A. and Oliveira, R.P.D.S. (2013): Novel biotechnological applications of bacteriocins: A review. *Food Control,* 32, 134-142.

Baranyi, J. and Roberts, T. A. (1994): A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology*, 23, 277-294.

Barbosa, M.S, Todorov, S.D., Ivanova, I.V., Belguesmia, Y., Choiset, Y., Rabesona, H., Chobert, J.-M., Haertlé, T. and Franco, B.D.G.M. (2016): Characterization of a two-peptide

10.14751/SZIE.2016.040

plantaricin produced by *Lactobacillus plantarum* MBSa4 isolated from Brazilian salami. *Food Control,* 60, 103-112.

Barefoot, S. F. and Klaenhammer, T. R. (1983): Detection and activity of Lactacin B, a bacteriocin produced by *Lactobacillus acidophilus. Applied and Environmental Microbiology, 45,* 1808-1815.

Belák, Á. and Maráz, A. (2015): Antagonistic effect of *Pseudomonas* sp. CMI-1 on foodborne pathogenic *Listeria monocytogenes. Food Technology and Biotechnology*, 53, 223-230.

Bērziņš, A., Terentjeva, M. and Korkeala, H. (2009): Prevalence and genetic diversity of *Listeria monocytogenes* in vacuum-packaged ready-to-eat meat products at retail markets in Latvia. *Journal of Food Protection,* 72, 1283-1287.

Beumer, R. R., Te Giffel, M. C., Anthonie, S. V. R. and Cox, L. J. (1996): The effect of acriflavine and nalidixic acid on the growth of *Listeria spp.* in enrichment media. *Food Microbiology,* 13, 137-148.

Bigwood, T., Hudson, J.A., Cooney, J., McIntyre, L., Billington, C., Heinemann, J.A. and Wall, F. (2012): Inhibition of *Listeria monocytogenes* by *Enterococcus mundtii* isolated from soil. *Food Microbiology,* 32, 354-360.

Bouayad, L., Hamdi, T.M., Naim, M., Leclercq, A. and Lecuit, M. (2015): Prevalence of *Listeria* spp. and molecular characterization of *Listeria monocytogenes* isolates from broilers at the abattoir. *Foodborne Pathogens and Disease*, 12, 606-611.

Bover-Cid, S., Belletti, N., Aymerich, T. and Garriga, M. (2015): Modeling the protective effect of aw and fat content on the high pressure resistance of Listeria monocytogenes in dry-cured ham. *Food Research International,* 75, 194-199.

Buchanan, R.L., Havelaar, A.H., Smith, M.A., Whiting, R.C. and Julien, E. (2009): The key events dose-response framework: Its potential for application to foodborne pathogenic microorganisms. *Critical Reviews in Food Science and Nutrition,* 49, 718-728.

Caldwell, S.L., Hutkins, R.W., McMahon, D.J., Oberg, C.J. and Broadbent, J.R. (1998): Lactose and galactose uptake by genetically engineered *Pediococcus* species. *Applied Microbiology and Biotechnology*, 49, 315-320.

Callewaert, R., Hugas, M. and De Vuyst, L. (2000): Competitiveness and bacteriocin production of *Enterococci* in the production of Spanish-style dry fermented sausages. *International Journal of Food Microbiology,* 57, 33-42.

Capita, R., Alonso-Calleja, C., García-Arias, M. T., Moreno, B. and García-Fernández, M. C. (2000): Evaluation of Fraser Broth to Isolate *Listeria* from Poultry. *LWT - Food Science and Technology*, 33, 560-563.

Carvalheira, A., Eusébio, C., Silva, J., Gibbs, P. and Teixeira, P. (2010): Influence of *Listeria innocua* on the growth of *Listeria monocytogenes*. *Food Control,* 21, 1492-1496.

Champomier-Vergés, M-C., Zagorec, M. and Fadda, S. (2010): Proteomics: A tool for understanding lactic acid bacteria adaption to stressful environments, in: Mozzi, F., Raya, R. R., Vignolo, G. M. (Eds.), *Biotechnology of Lactic Acid Bacteria Novel Applications*, Blackwell Publishing, Ames, pp. 57-72.

Collins, B., Cotter, P.D., Hill, C., and Ross R.P. (2010): Applications of lactic acid bacteria-produced bacteriocins. In: F. Mozzi, R. R. Raya, G. M. Vignolo (Eds.), *Biotechnology of lactic acid bacteria novel applications* (pp. 89-110). Ames: Blackwell Publishing.

European Commission (EC) Regulation No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs

Cornu, M., Kalmakoff, M. and Flandoris, J. P. (2002): Modelling the competitive growth of *Listeria monocytogenes* and *Listeria innocua* in enrichment broths. *International Journal of Food Microbiology*, 73, 261-274.

Cui, Y., Zhang, C., Wang, Y., Shi, J., Zhang, L., Ding, Z., Qu, X. and Cui, H. (2012): Class IIa bacteriocins: Diversity and new developments. *International Journal of Molecular Sciences,* 13, 16668-16707.

Da Silva, E.P. and De Martinis, E.C.P. (2013): Current knowledge and perspectives on biofilm formation: The case of *Listeria monocytogenes*. *Applied Microbiology and Biotechnology*, 97, 957-968

De Angelis, M., Bini, L., Pallini, V., Cocconcelli, P. S. and Gobbetti, M. (2001): The acid-stress response in *Lactobacillus sanfranciscensis* CB1. *Microbiology*, 147, 1863–1873.

De Vuyst, L., Callewaert, R. and Crabbé, K. (1996): Primary metabolite kinetics of bacteriocin biosynthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable growth conditions. *Microbiology,* 142, 817-827.

den Bakker, H.C., Warchocki, S., Wright, E.M., Allred, A.F., Ahlstrom, C., Manuel, C.S., Stasiewicz, M.J., Burrell, A., Roof, S., Strawn, L.K., Fortes, E., Nightingale, K.K., Kephart, D. and Wiedmann, M. (2014): Five new species of *Listeria* (*L. floridensis* sp. nov, *L. aquatica* sp. nov., *L. cornellensis* sp. nov. *L. riparia* sp. nov., and *L. grandensis* sp. nov.) from agricultural and natural environments in the United States. *International Journal of Systematic and Evolutionary Microbiology*, 64, 1882-1889.

10.14751/SZIE.2016.040

Devi, S.M. and Halami, P.M. (2011): Detection and characterization of pediocin PA-1/AcH like bacteriocin producing lactic acid bacteria. *Current Microbiology,* 63, 181-185.

Doijad, S.P., Barbuddhe, S.B., Garg, S., Poharkar, K.V., Kalorey, D.R., Kurkure, N.V., Rawool, D.B. and Chakraborty, T. (2015): Biofilm-forming abilities of *Listeria monocytogenes* serotypes isolated from different sources. *PLoS ONE*, 10, e0137046.

Dortu, C., Huch, M., Holzapfel, W. H., Franz, C. M. A. P and Thonart, P. (2008): Anti-listerial activity of bacteriocin-producing *Lactobacillus curvatus* CWBI-B28 and *Lactobacillus sakei* CWBI-B1365 on raw beef and poultry meat. *Letters in Applied Microbiology,* 47, 581-586.

Ducic, M., Klisara, N., Markov, S., Blagojevic, B., Vidakovic, A. and Buncic, S. (2016): The fate and pasteurization-based inactivation of *Escherichia coli* O157, *Salmonella* Typhimurium and *Listeria monocytogenes* in dry, fermented sausages. *Food Control,* 59, 400-406.

Erdősi, O., Szakmár, K., Reichart, O., Székely-Körmöczy P. and Laczay, P. (2010): Application of the redox potential measurement based rapid method in the microbial hygienic control. *Acta Alimentaria,* 41, 45-55.

Esteban, M. and Palop, A. (2011): Nisin, carvacrol and their combinations against the growth of heat-treated *Listeria monocytogenes* cells. *Food Technology and Biotechnology*, 49, 89-95.

European Food Safety Authority (EFSA) (2016): The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. *EFSA Journal* 13 (12): 4329.

Fadda, S., Anglade, P., Baraige, F., Zagorec, M., Talon, R., Vignolo, G. and Champomier-Vergès, M.C. (2010): Adaptive response of *Lactobacillus sakei* 23K during growth in the presence of meat extracts: A proteomic approach. *International Journal of Food Microbiology*, 142, 36-43.

Garrett, T.G., Bhakoo, M. and Zhang, Z. (2008): Bacterial adhesion and biofilms on surfaces. *Progress in Natural Science,* 18, 1049-1056.

Gelbíčová T. and Karpíšková R. (2012): Outdoor environment as a source of *Listeria monocytogenes* in food chain. *Czech Journal of Food Sciences,* 30, 83–88.

Geremew, T., Kebede, A. and Andualem, B. (2015): The role of spices and lactic acid bacteria as antimicrobial agent to extend the shelf life of metata ayib (traditional Ethiopian spiced fermented cottage cheese). *Journal of Food Science and Technology*, 52, 5661-5670.

Ghanbari, M., Jami, M., Domig, K.J. and Kneifel, W. (2013): Seafood biopreservation by lactic acid bacteria - A review. *LWT- Food Science and Technology,* 54, 315-324.

Giaouris, E., Chorianopoulos, N., Doulgeraki, A. and Nychas, G.J. (2013): Co-culture with *Listeria monocytogenes* within a dual-species biofilm community strongly increases resistance of *Pseudomonas putida* to benzalkonium chloride. *PLoS ONE* 8, e77276.

Glaasker, E., Tjan, F.S.B., Ter Steeg, P.F., Konings, W.N. and Poolman, B. (1998): Physiological response of *Lactobacillus plantarum* to salt and nonelectrolyte stress. *Journal of Bacteriology,* 180, 4718-4723.

Gnanou Besse, N., Audinet, N., Kérouanton, A., Colin, P. and Kalmakoff, M. (2005): Evolution of *Listeria* populations in food samples undergoing enrichment culturing. *International Journal of Food Microbiology,* 104, 123–134.

Gong, H.S., Meng, X.C. and Wang, H. (2010): Plantaricin MG active against Gram-negative bacteria produced by *Lactobacillus plantarum* KLDS1.0391 isolated from "Jiaoke", a traditional fermented cream from China. *Food Control,* 21, 89-96.

Griffiths, M. (2005): Quorum-sensing and virulence in foodborne pathogens. In M. Griffiths (Ed.), *Understanding pathogen behaviour* (pp. 549-586). Cambridge: Woodhead Publishing Limited.

Guerrieri, E., de Niederhäusern, S., Messi, P., Sabia, C., Iseppi R., Anacarso, I. and Bondi, M. (2009): Use of lactic acid bacteria (LAB) biofilms for the control of *Listeria monocytogenes* in a small-scale model. *Food Control,* 20, 861-865.

Guillet, C., Join-Lambert, O., le Monnier, A., Leclercq, A., Mechaï, F., Mamzer-Bruneel, M-F., Bielecka, M. K., Scortti, M., Disson, O, Berche, P., Vazquez-Boland, J., Lortholary, O. and Lecuit, M. (2010): Human listeriosis caused by *Listeria ivanovii*. *Emerging Infectious Diseases*, 16, 136–138.

Gunduz, G.T. and Tuncel, G. (2006): Biofilm formation in an ice cream plant. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology,* 89, 329-336.

Gupta, A. and Tiwari, S.K. (2014): Plantaricin LD1: A bacteriocin produced by food isolate of *Lactobacillus plantarum* LD1. *Applied Biochemistry and Biotechnology,* 172, 3354-3362.

Hammer, Ø., Harper, D.A.T. and Ryan, P.D. (2001): PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica,* 4, 1-9.

Hata, T., Tanaka, R. and Ohmomo, S. (2010): Isolation and characterization of plantaricin ASM1: A new bacteriocin produced by *Lactobacillus plantarum* A-1. *International Journal of Food Microbiology,* 137, 94-99.

10.14751/SZIE.2016.040

Hernández, D., Cardell, E. and Zárate, V. (2005): Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: Initial characterization of plantaricin TF711, a bacteriocin-like substance produced by *Lactobacillus plantarum* TF711. *Journal of Applied Microbiology*, 99, 77-84.

ICMSF (International Comission on Microbiological Specifications for Foods) (1996): *Listeria monocytogenes.* In T. A. Roberts, A. C. Baird-Parker, R. B. Tompkin (Eds.), *Microorganisms in Foods 5* (pp.141-182). London: Blackie Academic & Professional.

ISO (International Organization for Standardization)11290-1. (1996): Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 1: detection method.

Jalilsood, T., Baradaran, A., Song, A.A.-L., Foo, H.L., Mustafa, S., Saad, W.Z., Yusoff, K. and Rahim, R.A. (2015): Inhibition of pathogenic and spoilage bacteria by a novel biofilm-forming *Lactobacillus* isolate: A potential host for the expression of heterologous proteins. *Microbial Cell Factories*, 14, 96.

Jameson, J. E. (1962): A discussion of the dynamics of salmonella enrichment. *Journal of Hygiene,* 60, 193-207.

Jiang J., Shi B., Zhu D., Cai Q., Chen Y., Li J., Qi K. and Zhang M. (2012): Characterization of a novel bacteriocin produced by *Lactobacillus sakei* LSJ618 isolated from traditional Chinese fermented radish. *Food Control,* 23, 338-344.

Jones, R.J., Hussein, H.M., Zagorec, M., Brightwell, G., Tagg, J.R. (2008): Isolation of lactic acid bacteria with inhibitory activity against pathogens and spoilage organisms associated with fresh meat. *Food Microbiology,* 25, 228-234.

Katla, T., Møretrø, T., Aasen, I.-M., Holck, A., Axelsson, L. and Naterstad K. (2001): Inhibition of *Listeria monocytogenes* in cold smoked salmon by addition of sakacin P and/or live *Lactobacillus sakei* cultures. *Food Microbiology,* 18, 431–439.

Kjos, M., Borrero, J., Opsata, M., Birri, D. J., Holo, H., Cintas, L. M., Snipen, L., Hernández, P. E., Nes, I. F. and Diep, D. B. (2011): Target recognition, resistance, immunity and genome mining of class II bacteriocins from gram-positive bacteria. *Microbiology,* 157, 3256-3267.

Kouakou, P., Ghalfi, H., Dortu, C., Evrard, P. and Thonart, P. (2010): Combined use of bacteriocin-producing strains to control *Listeria monocytogenes* regrowth in raw pork meat. *International Journal of Food Science and Technology,* 45, 937-943.

Kumar, C.G. and Anand, S.K. (1998): Significance of microbial biofilms in food industry: a review. *International Journal of Food Microbiology*, 42, 9-27.

Latorre, A.A., Van Kessel, J.S., Karns, J.S., Zurakowski, M.J., Pradhan, A.K., Boor, K.J., Jayarao, B.M., Houser, B.A., Daugherty, C.S. and Schukken, Y.H. (2010): Biofilm in milking equipment on a dairy farm as a potential source of bulk tank milk contamination with *Listeria monocytogenes. Journal of Dairy Science,* 93, 2792-2802.

Leclercq, A. (2004): Atypical colonial morphology and low recoveries of *Listeria monocytogenes* strains on Oxford, PALCAM, Rapid'L.mono and ALOA solid media. *Journal of Microbiological Methods,* 57, 251-258.

Lee, H.M. and Lee, Y. (2008): A differential medium for lactic acid-producing bacteria in a mixed culture. *Letters in Applied Microbiology*, 46, 676-681.

Lemos, J.A.C., Chen, Y.-Y.M. and Burne, R.A. (2001): Genetic and physiologic analysis of the groE operon and role of the HrcA repressor in stress gene regulation and acid tolerance in *Streptococcus mutans*. *Journal of Bacteriology,* 183, 6074-6084.

Leroy, F., Verluyten, J. and De Vuyst, L. (2006): Functional meat starter cultures for improved sausage fermentation. *International Journal of Food Microbiology*, 106, 270-285.

Liu, L., Wang, M., Du, G. and Chen, J., 2008. Enhanced hyaluronic acid production of *Streptococcus zooepidemicus* by an intermittent alkaline-stress strategy. Lett. Appl. Microbiol. 46, 383-388.

López-Malo, A., Palou, E., Parish, M. E. and Davidson P. M. (2005): Methods for activity assay and evaluation of results. In: Davidson, P. M., John, N. and Sofos, A. (Eds*.), Antimicrobials in food* (pp. 659-680). London: Taylor and Francis Group.

Lourenço, A., Machado, H. and Brito, L. (2011): Biofilms of *Listeria monocytogenes* produced at 12 ° C either in pure culture or in co-culture with *Pseudomonas aeruginosa* showed reduced susceptibility to sanitizers. *Journal of Food Science*, 76, 142-148.

Luber, P., Crerar, S., Dufour, C., Farber, J., Datta, A. and Todd, E. C. D. (2011): Controlling *Listeria monocytogenes* in ready-to-eat foods: Working towards global scientific consensus and harmonization - Recommendations for improved prevention and control. *Food Control,* 22, 1535 – 1549.

Luo, K. and Oh, D.-H. (2016): Inactivation kinetics of *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium on fresh-cut bell pepper treated with slightly acidic electrolyzed water combined with ultrasound and mild heat. *Food Microbiology*, 53, 165-171

Makarova, K.S. and Koonin E.V. (2007): Evolutionary genomics of lactic acid bacteria. *Journal of Bacteriology*, 189, 1199-1208.

Martinez, R.C.R., Wachsman, M., Torres, N.I., LeBlanc, J.G., Todorov, S.D. and Franco,

10.14751/SZIE.2016.040

B.D.G.D.M. (2013): Biochemical, antimicrobial and molecular characterization of a noncytotoxic bacteriocin produced by *Lactobacillus plantarum* ST71KS. *Food Microbiology,* 34, 376-381.

Mayo, B., Aleksandrzak-Piekarczyk, T., Fernández, M., Kowalczyk, M., Álvarez-Martín, P. and Bardowski, J. (2010): Updates in the Metabolism of Lactic Acid Bacteria. in: Mozzi, F., Raya, R. R., Vignolo, G. M. (Eds.), *Biotechnology of Lactic Acid Bacteria Novel Applications*, Blackwell Publishing, Ames, pp. 3-34.

Melo, J., Andrew, P.W. and Faleiro, M.L. (2015): *Listeria monocytogenes* in cheese and the dairy environment remains a food safety challenge: The role of stress responses. *Food Research International*, 67, 75-90.

Milioni, C., Martínez, B., Degl'Innocenti, S., Turchi, B., Fratini, F., Cerri, D. and Fischetti, R. (2015): A novel bacteriocin produced by *Lactobacillus plantarum* LpU4 as a valuable candidate for biopreservation in artisanal raw milk cheese. *Dairy Science and Technology*, 95, 479-494.

Mills, S., Stanton, C., Hill, C. and Ross, R.P. (2011): New developments and applications of bacteriocins and peptides in foods. *Annual Review of Food Science and Technology,* 2, 299 - 329.

Mizan, M.F.R., Jahid, I.K. and Ha, S.-D. (2015): Microbial biofilms in seafood: A food-hygiene challenge. *Food Microbiology,* 49, 41-55.

Neysens, P., Messens, W., Gevers, D., Swings, J. and De Vuyst, L. (2003): Biphasic kinetics of growth and bacteriocin production with *Lactobacillus amylovorus* DCE 471 occur under stress conditions. *Microbiology,* 149, 1073-1082.

Oliveira, M., Abadias, M., Colás-Medà, P., Usall, J. and Viñas, I. (2015): Biopreservative methods to control the growth of foodborne pathogens on fresh-cut lettuce. *International Journal of Food Microbiology,* 214, 4-11.

Ollé Resa, C.P., Gerschenson, L.N. and Jagus, R.J. (2016): Starch edible film supporting natamycin and nisin for improving microbiological stability of refrigerated argentinian Port Salut cheese. *Food Control*, 59, 737-742.

Országos Epidemiológiai Központ (OEK) (2015): Fertőző betegségek adatai. Epinfo. 22, 578-581.

Pan, Y., Breidt Jr., F. and Kathariou, S. (2006): Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. *Applied and Environmental Microbiology*, 72, 7711-7717

Parente, E., Ricciardi, A. and Addario, G. (1994): Influence of pH on growth and bacteriocin production by *Lactococcus lactis* subsp. *lactis* 140NWC during batch fermentation. *Applied Microbiology and Biotechnology,* 41, 388-394.

Phadtare, S., Alsina, J. and Inouye, M. (1999): Cold-shock response and cold-shock proteins. *Current Opinion in Microbiology, 2,* 175-180.

Powell, J.E., Witthuhn, R.C., Todorov, S.D. and Dicks, L.M.T. (2007): Characterization of bacteriocin ST8KF produced by a kefir isolate *Lactobacillus plantarum* ST8KF. *International Dairy Journal,* 17, 190-198.

Rajkowski, K.T. (2009): Biofilms in fish processing. In: Fratamico, P.M., Annous, B.A. and Guenther, N.W. (Eds.), *Biofilms in the food and beverage industries,* Woodhead Publishing, Cornwall, pp. 499-516.

Reichart, O., Szakmár, K., Jóźwiak, Á., Felföldi, J. and Baranyai, L. (2007): Redox potential measurement as a rapid method for microbiological testing and its validation for coliform determination. *International Journal of Food Microbiology,* 114, 143-148.

Rocourt J., Jacquet Ch.. and Reilly J. (2000): Epidemiology of human listeriosis and seafoods. *International Journal of Food Microbiology,* 62, 197–209.

Rodríguez, E., González, B., Gaya, P., Nuñez, M. and Medina, M. (2000): Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. *International Dairy Journal,* 10, 7-15.

Rodríguez-Lázaro, D. and Hernández, M. (2014): Confirmation of isolates of *Listeria* by conventional and Real-Time PCR. in: Jordan, K., Fox, E. M., Wagner, M. (Eds.), *Listeria monocytogenes – Methods and Protocols*, Springer Science+Business Media, New York, pp. 31-40.

Romero-Gil, V., Bautista-Gallego, J., Rodríguez-Gómez, F., García-García, P., Jiménez-Díaz, R., Garrido-Fernández, A. and Arroyo-López, F.N. (2013): Evaluating the individual effects of temperature and salt on table olive related microorganisms. *Food Microbiology*, 33, 178-184.

Samelis, J., Kakouri, A., Rogga, K.J., Savvaidis, I.N. and Kontominas, M.G (2003): Nisin treatments to control *Listeria monocytogenes* post-processing contamination on Anthotyros, a traditional Greek whey cheese, stored at 4°C in vacuum packages. *Food Microbiology,* 20, 661-669.

SANCO/1628/2008 ver. 9.3 (26112008): Guidance document on *Listeria monocytogenes* shelf-life studies for ready-to-eat foods, under Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs

10.14751/SZIE.2016.040

Schägger, H. and von Jagow, G. (1987): Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analytical Biochemistry,* 166, 368-379.

Scollard, J., Francis, G.A. and O'Beirne, D. (2009): Effects of essential oil treatment, gas atmosphere, and storage temperature on *Listeria monocytogenes* in a model vegetable system. *Journal of Food Protection*, 72, 1209-1215.

Scotter, S.L., Langton, S., Lombard, B., Schulten, S., Nagelkerke N., In't Veld P.H., Rollier, P. and Lahellec, C. (2001): Validation of ISO method 11290 Part 1 — Detection of Listeria monocytogenes in foods. *International Journal of Food Microbiology*, 64, 295-306.

Sidira, M., Galanis, A., Nikolaou, A., Kanellaki, M. and Kourkoutas, Y. (2014): Evaluation of *Lactobacillus casei* ATCC 393 protective effect against spoilage of probiotic dry-fermented sausages. *Food Control,* 42, 315-320.

Smet, C., Noriega, E., Van Mierlo, J., Valdramidis, V.P. and Van Impe, J.F. (2015): Influence of the growth morphology on the behavior of *Salmonella* Typhimurium and *Listeria monocytogenes* under osmotic stress. *Food Research International*, 77, 515-526.

Smith, B., Larsson, J.T., Lisby, M., Müller, L., Madsen, S.B., Engberg, B., Bangsborg, J., Ethelberg, S. and Kemp, M. (2011): Outbreak of listeriosis caused by infected beef meat from a meals-on-wheels delivery in Denmark 2009. *Clinical Microbiology and Infection*, 17, 50-52.

Speranza, B., Sinigaglia, M. and Corbo, M.R. (2009): Non starter lactic acid bacteria biofilms: A means to control the growth of *Listeria monocytogenes* in soft cheese. *Food Control,* 20, 1063-1067.

Strydom, A., Vorster, R., Gouws, P.A. and Witthuhn, R.C. (2016): Successful management of *Listeria spp.* in an avocado processing facility. *Food Control*, 62, 208-215.

Szakmár, K., Reichart, O., Szatmári, I., Erdosi, O., Szili, Z., László, N., Székely Körmöczy, P. and Laczay, P. (2014): In vitro study on the effect of doxycycline on the microbial activity of soil determined by redox-potential measuring system. *Acta Microbiologica et Immunologica Hungarica, 61*, 617-628.

Todorov, S.D. and Dicks, L.M.T. (2004): Partial characterization of bacteriocins produced by four lactic acid bacteria isolated from regional South African barley beer. *Annals of Microbiology,* 54, 403-413.

Todorov, S.D. and Dicks, L.M.T. (2006): Screening for bacteriocin-producing lactic acid bacteria from boza, a traditional cereal beverage from Bulgaria Comparison of the bacteriocins. *Process Biochemistry,* 41, 11–19.

Todorov, S.D., Ho, P., Vaz-Velho, M. and Dicks, L.M.T. (2010): Characterization of bacteriocins produced by two strains of *Lactobacillus plantarum* isolated from Beloura and Chouriço, traditional pork products from Portugal. *Meat Science*, 84, 334-343.

Todorov, S.D., Vaz-Velho, M., de Melo Franco, B.D.G. and Holzapfel, W.H. (2013): Partial characterization of bacteriocins produced by three strains of *Lactobacillus sakei*, isolated from salpicao, a fermented meat product from North-West. *Food Control,* 30, 111-121.

Työppönen, S., Markkula, A., Petäjä, E., Suihko, M.-L. and Mattila-Sandholm, T. (2002): Survival of *Listeria monocytogenes* in North European type dry sausages fermented by bioprotective meat starter cultures. *Food Control,* 14, 181-185.
Uhlich, G.A., Luchansky, J.B., Tamplin, M.L., Molina-Corral, F.J., Anandan, S. and Porto-Fett, A.C.S. (2006): Effect of storage temperature on the growth of *Listeria monocytogenes* on Queso Blanco slices. *Journal of Food Safety,* 26, 202-214.

Upadhyay, A., Upadhyaya, I., Mooyottu, S., Kollanoor-Johny, A. and Venkitanarayanan, K. (2014): Efficacy of plant-derived compounds combined with hydrogen peroxide as antimicrobial wash and coating treatment for reducing *Listeria monocytogenes* on cantaloupes. *Food Microbiology*, 44, 47-53.

Van de Guchte, M., Serror, P., Chervaux, C., Smokvina, T., Ehrlich, S.D. and Maguin, E. (2002): Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology,* 82, 187-216.

Van Reenen, C.A., Dicks, L.M.T. and Chikindas, M.L. (1998): Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology,* 84, 1131–1137.

Verluyten, J., Messens, W. and Vuyst, D. L. (2004): Sodium chloride reduces production of curvacin A, a bacteriocin produced by *Lactobacillus curvatus* strain LTH 1174, originating from fermented sausage. *Applied and Environmental Microbiology,* 70, 2271-2278.

Vesković Moračanin, S.M., Dukić, D.A. and Memiši, N.R. (2014): Bacteriocins produced by lactic acid bacteria - A review. *Acta Periodica Technologica*, 45, 271-283.

Winkelströter, L.K. and De Martinis, E.C.P. (2013): Effect of bacteriocins and conditions that mimic food and digestive tract on biofilm formation, *in vitro* invasion of eukaryotic cells and internalin gene expression by *Listeria monocytogenes. Probiotics and Antimicrobial Proteins,* 5, 153-164.

Winkelströter, L.K., Tulini, F.L. and De Martinis, E.C.P. (2015): Identification of the bacteriocin produced by cheese isolate *Lactobacillus paraplantarum* FT259 and its potential influence on Listeria monocytogenes biofilm formation. *LWT - Food Science and Technology,* 64, 586-592.

10.14751/SZIE.2016.040

Xie, Y., An, H., Hao, Y., Qin, Q., Huang, Y., Luo, Y. and Zhang, L. (2011): Characterization of an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* LB-B1 isolated from koumiss, a traditionally fermented dairy product from China. *Food Control,* 22, 1027-1031.

Yang R., Johnson M. and Ray B. (1992): Novel method to extract large amounts of bacteriocins from lactic acid bacteria. *Applied and Environmental Microbiology,* 58, 3355-3359

Yang, S.C., Lin, C.H., Sung, C.T. and Fang J.Y. (2014): Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. *Frontiers in Microbiology*, 5, 241.

Yokoyama, E., Shibusawa, Y., Maruyama, S., Katsube, Y. and Mikami, T. (2005): Influence of bacteriocin-like substance, generation times, and genetic profiles of *Listeria innocua* on the isolation of *Listeria monocytogenes*. *Comparative Immunology, Microbiology and Infectious Diseases,* 28, 177–186.

Zacharof , M.P. and Lovitt R.W. (2012): Bacteriocins produced by lactic acid bacteria a review article. *APCBEE Procedia* 2, 50-56.

Zunabovic, M., Domig K. J. and Kneifel W. (2011): Practical relevance of methodologies for detecting and tracing of *Listeria monocytogenes* in ready-to-eat foods and manufacture environments – A review. *LWT - Food Science and Technology*, 44, 351-362.

Zhao, T., Podtburg, T.C., Zhao, P., Chen, D., Baker, D.A., Cords, B. and Doyle, M.P. (2013): Reduction by competititive bacteria of *Listeria monocytogenes* in biofilms and *Listeria* in floor drains in a ready-to-eat poultry processing plant. *Journal of Food Protection,* 74, 601-607.

Zhou, K., Zeng, Y.-T., Han, X.-F. and Liu, S.-L. (2015): Modelling growth and bacteriocin production by *Lactobacillus plantarum* BC-25 in response to temperature and pH in batch fermentation. *Applied Biochemistry and Biotechnology, 176,* 1627-1637.

Zhu, X., Zhao, Y., Sun, Y. and Gu, Q. (2014): Purification and characterisation of plantaricin ZJ008, a novel bacteriocin against *Staphylococcus* spp. from *Lactobacillus plantarum* ZJ008. *Food Chemistry,* 165, 216-223.

Electronic sources:

ComBase Predictor: <http://browser.combase.cc/ComBase_Predictor.aspx?model=1>. Downloaded: 03. 12. 2015.

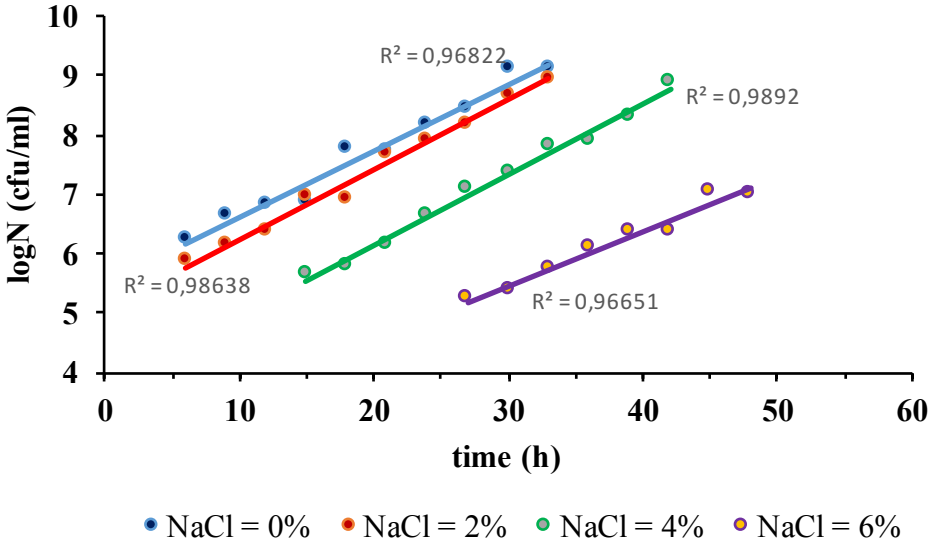
Public Health Agency of Canada (2011): *Listeria monocytogenes* – Pathogen safety data sheet – infectious substances. <http://www.phac-aspc.gc.ca/lab-bio/res/psds-ftss/listeria-monocytogenes-eng.php>. Downloaded: 05. 12. 2015.

The MicroTester test cell: <http://www.microtest.co.hu/?hu;tevekenyseg> in the presentation: Redox-potential measurement as a rapid method for microbiological testing, Budapest, 2011. Downloaded: 06. 11. 2015.

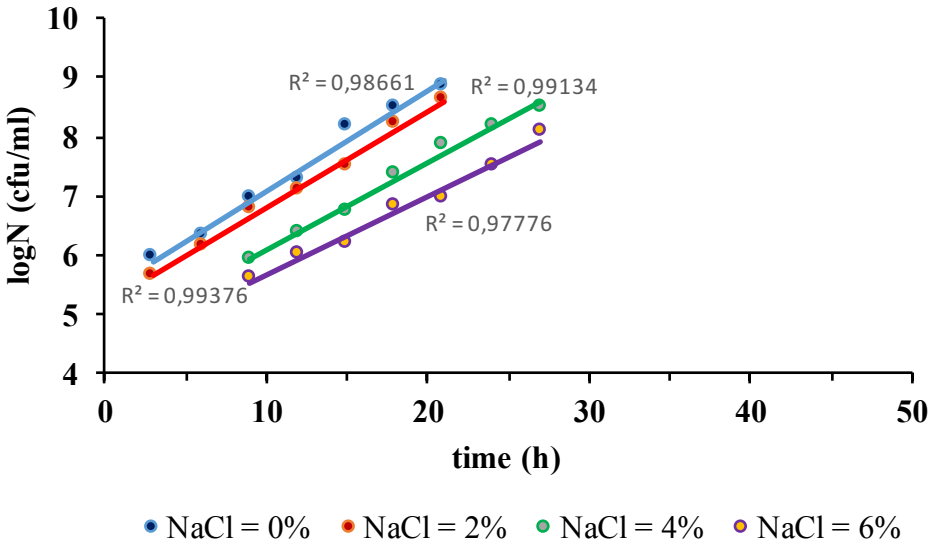
U.S. Food and Drug Administration (FDA) (2014): Bad Bug Book – *Listeria monocytogenes*.
http://www.fda.gov/downloads/Food/FoodborneIllnessContaminants/UCM297627.pdf>. Downloaded: 05. 12. 2015.

10.14751/SZIE.2016.040

APPENDIX A

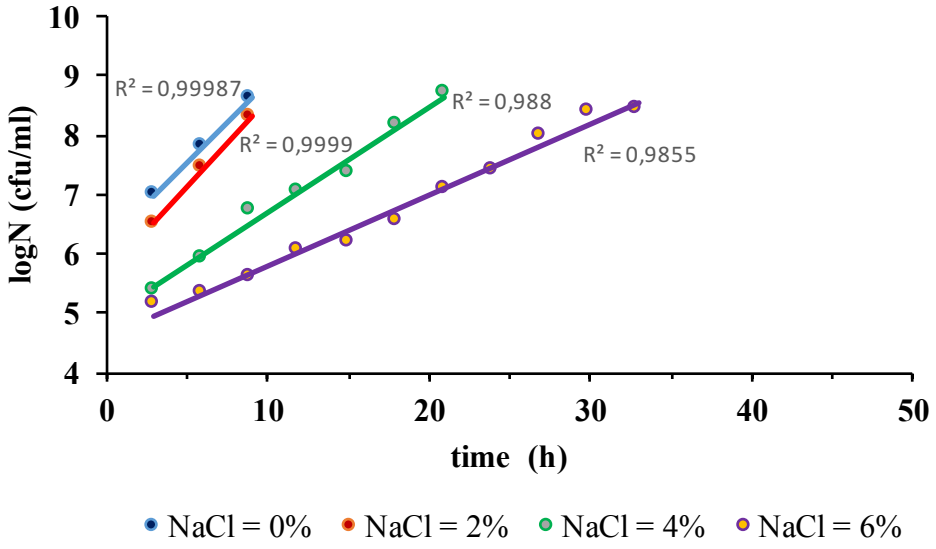


Appendix A/I: The exponential phase of *Lb. plantarum* SD202Ch at 20 °C.



Appendix A/II: The exponential phase of *Lb. plantarum* SD202Ch at 25 °C.

10.14751/SZIE.2016.040



Appendix A/III: The exponential phase of *Lb. plantarum* SD202Ch at 30 °C.

10.14751/SZIE.2016.040

ACKNOWLEDGEMENT

First of all, I would like to say thank you to my family, without their support this thesis wouldn't have be existed.

I am really grateful to my husband for his love and his patience, he always tried to understand why I did not have time for him and he accepted if I worked late at night in the lab and on weekends in front of my laptop.

I say thank you to my supervisors, Prof. Csilla Mohácsi-Farkas and Dr. Gabriella Kiskó, for their never ending support and guidance. They were always answering my questions and were helping me when I had difficulties with my work. Their attention and academic knowledge led me through all of my studies.

I would like to say thank you to all of the current and former workers, PhD students at the Department of Microbiology and Biotechnology, especially: Dr. Réka Ágoston and Dr. Ágnes Belák for their practical help in the lab and their critical remarks, which was a big help in my studies.

I am really thankful to Professor Paula Teixeira who received me in her lab in Porto. I would like to say a special thanks to Dr. Helena Albano not just for her scientific help, but for her friendship as well. I will never forget the people in the lab in Porto: Sara, Lúcia, Vania, Ana, Riccardo.

Without the knowledge and the help of Dr. Katalin Szakmár and Dr. Olivér Reichart I would have been in trouble. I am really appreciating that, they shared their opinion and helped me to finish my work.

I am beholden to all of my students, who are working hard on their thesis, thus support me and my work, especially for: Ilka Hóbor, Viktória Deli, Silvia Mottini, Judit Orgován.

I would like to say a big thanks to Adrienn Berkics, who was always at my side, and listened my "work-doubts" and she was always there for a coffee after work.

I am thankful to all of my friends - who are not listed here – for their tolerance to all of my stories about my work and I am also grateful they are still here in spite of my not having enough time for them in the past few years.

For providing the strains of *Lactobacillus plantarum* ST202Ch and *Lb. sakei* ST153Ch are highly acknowledged to Svetoslav D. Todorov PhD, Universidade Federal de Viçosa, Veterinary Department, Viçosa, Minas Gerais, Brazil.

My fellowship at Escola Superior de Biotecnologia of the Universidade Católica Portuguesa was financially supported by the Campus Hungary program in the frame of TÁMOP 4.2.4.B/1-11/1 and TÁMOP 4.2.4.B/2-11/1 project

Thank you!