



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

Thesis

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INTRODUCTION

It is estimated that millions of people suffer from food-borne diseases and 2 million death 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 producer *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. 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 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. Moreover, *L. monocytogenes* is a well-known bad competitor, thus some other microbes are able to overgrow it.

AIMS

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 *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.

MATERIALS AND METHODS

Microorganisms: *Lactobacillus plantarum* ST202Ch and *Lb. sakei* ST153Ch – both previously isolated from fermented meat sausages –, *Pediococcus acidilactici* HA6111-2 previously isolated from ‘alheira’ are deposited in the culture collection of Escola Superior de Biotecnologia, Universidade Católica Portuguesa. Three isolates of *L. monocytogenes* were 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. Three isolates from *L. monocytogenes* were provided by the Department of Microbiology and Biotechnology, Szent István University: *L. monocytogenes* L4 (isolated from cheese products), *L. monocytogenes* L16 (isolated from cheese products) , *L. monocytogenes* T3 (isolated from food industry). Further strains are: *L. innocua* NCTC 11288, *L. monocytogenes* CCM 4699 (C1), *L. innocua* CCM 4030 (C6).

Characterization of the bacteriocins produced by *Lb. plantarum* and *Lb. sakei*: MRS broth 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 every hour, for 24 h. Bacteriocin

activity (AU/ml) in the cell-free supernatant was recorded every 3 h for 24 h. The examination of effect of enzymes, temperature, pH and detergents on bacteriocin activity and mode of activity of the bacteriocin was done. Molecular size of bacteriocins was determined by tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Listeria monocytogenes 1486/1 and *L. innocua* NCTC 11288 were used as target strains in all measurements.

Examination of antilisterial activity during growth under stress conditions: Screening of different stress conditions was performed previously in order to select the adequate stress conditions for *P. acidilactici* and *Lb. plantarum* and for proceeding the further experiments. MRS broth was set up to the chosen stress conditions (pH, temperature, NaCl) and inoculated with 1% (v/v) of an overnight culture and incubated at different temperatures. Changes in pH were recorded in every hour, the cell count was determined in every 3 h for 42 h. Bacteriocin activity (AU/ml) in the cell-free supernatant was recorded in every 3 h for 48 h. *L. monocytogenes* 1486/1, *L. monocytogenes* 1604/2, *L. monocytogenes* 971 and *L. innocua* NCTC 11288 were used as target strains.

Examination the effect of NaCl and temperature on the bacteriocin production by redox potential measurement technique: 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. The growth parameters were determined. To determine the bacteriocin activity 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).

L. monocytogenes 1486/1 were used as target strains.

Examination of biofilm formation of *Listeria* species and lactic acid bacteria: *L. monocytogenes* 1486/1, 971, *L4*, *L. innocua* CCM 4030 (C6) and *P. acidilactici* were investigated for biofilm formation. Stainless steel (SS) coupons were used as substrates for biofilm formation, since this material is frequently used for the manufacture of food processing equipment. Different matrices (model media: BH and MRS; milk; and minced meat) selected to examine the biofilm formation. Iceberg lettuce leaves (LL) were also selected as substrates for biofilm formation. These food matrices are able to support the growth of *Listeria* spp. and lactic acid bacteria. In every experimental setup the experimental media was inoculated with an overnight *Listeria*

spp. or *P. acidilactici* culture, the initial cell concentration was 10^6 CFU/ml. The time duration for the irreversible attachment was 1-hour long. The biofilm formation was monitored for 48-168 hours, depending on the media and the surface.

Growth of *L. monocytogenes* in presence of *L. innocua* during the ISO 11290-1 detection method: *L. monocytogenes* C1, L4, L16, T3 strains and *L. innocua* C6 strain were used. Different mixtures of *L. monocytogenes* strains and one strain of *L. innocua* were inoculated into half Fraser Broth (hFB) and FB for enrichment steps. After enrichment in hFB and FB, samples were inoculated onto Ottaviani-Agosti (ALOA) agar. Growth curves in hFB and FB of one strain (T3) individually and in mixed culture with *L. innocua* were also determined. Growth parameters were determined by DMFit software using Baranyi-model.

RESULTS

Characterization of the bacteriocins produced by *Lb. plantarum* and *Lb. sakei*: *Lb. plantarum* produced the antilisterial bacteriocin at a highest level (25600 AU/ml) against *L. monocytogenes* 1486/1 after 15 hours and against *L. innocua* NCTC 11288 after 18 hours. In case of *Lb. sakei* the maximum level (25600 AU/ml) against *L. monocytogenes* 1486/1 and 12800 AU/ml against *L. innocua* NCTC 11288 was observed after 15 hours.

The activity of both bacteriocins was moderately reduced after treatment at different temperatures and pH, but *L. innocua* NCTC 11288 showed to be more resistant. Treatment the bacteriocin produced by *Lb. plantarum* and *Lb. sakei* with detergents were have different effect. No change in activity was observed against *L. monocytogenes* 1486/1 in case of bacteriocin produced by *Lb. sakei*. After treatment with protease K, tyrosinase, peroxidase, pepsin and trypsin complete inactivation or significant reduction in antimicrobial activity was observed. No complete inactivation was recorded when treated with catalase, which indicated that H₂O₂ was not strictly responsible for inhibition.

The addition of bacteriocin produced by *Lb. plantarum* and *Lb. sakei* to mid-log phase (3 hours old) *L. monocytogenes* 1486/1 and *L. innocua* NCTC 11288 cultures showed a clearly static effect. No effect was recorded in untreated (positive controls) samples in case of both *Listeria* species.

Bacteriocins produced by *Lb. plantarum* and *Lb. sakei* are small peptides (lower than 14.5 kDa). The bacteriocins did adhere to the surface of the *Lb. plantarum* and *Lb. sakei* cells.

Examination of antilisterial activity during growth under stress conditions: *P. acidilactici* could not grow well at low pH, the pH and the cell count did not change during 48 h. However, *P. acidilactici* could produce moderate amount of PA-1 bacteriocin. The highest activity was 6400 AU/ml. In case of pH 8.5 the alkaline adaptation was clearly observed (~ 28 hours). During the adaptation, the pH slightly decreased; at pH 7 the exponential phase started, and the pH rapidly dropped until levels of pH ~ 4.3. The highest bacteriocin production (25600 AU/ml) was observed in the stationary phase. Previous examinations show that several LAB strains can rapidly adapt to temperature downshift to about 20 °C below the optimal growth temperature. In contrast, my results show that at 10 °C, *P. acidilactici* could not recover. However, it was able to produce low amount of bacteriocin. At 50 °C the cells were damaged; after 24 hours we could not detect viable cells, although the bacteriocin activity was detected at low levels until 40 hours. The response of *P. acidilactici* to elevated temperature can be similar to cold shock response. In my study I demonstrated that *P. acidilactici* could not recover and produce only low amount of bacteriocins under both cold and heat stresses. Under 7.5% NaCl, viable cell number of *P. acidilactici* was slightly increased and the pH was poorly decreased after 20-24 hours. *P. acidilactici* was able to show the highest bacteriocin activity (6400 AU/ml) after 30-33 hours. Adaptation of bacteria to osmotic stress can be explained by accumulation, synthesis and transport of certain solutes to restore turgor.

Populations of *Lb. plantarum* at low pH increased only slightly from logN 6.9 to 7.6 after 48 h. Maximum antilisterial activity was observed against *L. monocytogenes* 971 (25600 AU/ml). It has been reported that *Lb. plantarum* can adapt to acidic conditions by amine accumulation into the cells equilibrating the acidic environment.

In case of pH 8.5 *Lb. plantarum* has an extended lag phase and delay in antilisterial activity. Maximum antilisterial activity (25600 AU/ml) was clearly observed in the stationary phase, suggesting that bacteriocins may be secondary metabolites.

At cold stress cell number of *Lb. plantarum* slightly increased after 20 hours and could not produce notable amount of bacteriocins. During cold stress response, the cells undergo different physiological changes like stabilization of secondary structures of RNA and DNA resulting in a reduced efficiency of translation. It can be an explanation why *Lb. plantarum* was not able to produce ribosomally synthesized bacteriocin under cold stress.

Lb. plantarum did not showed to be sensitive to heat shock. The maximum antilisterial (25600 AU/ml) activity was observed against *L. monocytogenes* 971.

Growth of *Lb. plantarum* was severely curtailed under 7.5 % NaCl conditions. However, low level (800-3200 AU/ml) of antilisterial activity was detected. It is important to highlight that

centrifugation of *Lb. plantarum* cells following exposure to the osmotic stress resulted in a pellet with a metallic-shine and difficult to re-suspend. These phenomena may indicate that the cell membrane is damaged under high osmotic stress.

Examination the effect of NaCl and temperature on the bacteriocin production by redox potential measurement technique: The activity of the bacteriocin (AU/ml) is defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition. With that methodology the value of AU/ml is a discrete variable, which may cause information loss. 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; t_e which means the essential fermentation time of *Lb. plantarum* needed to reach 2.5 log unit virtual destruction of *L. monocytogenes*.

During the *Lb. plantarum* fermentations the antilisterial activity proved to be the highest in the transient section between the late linear and stationer phase of the logarithmic growth curves. 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, interaction was obtained 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. 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 at the beginning of the stationary phase.

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 (t_e). 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*.

Examination of biofilm formation of *Listeria* species and lactic acid bacteria: *L. monocytogenes* L4 was randomly selected to examine its behaviour in mixed culture with *L. innocua* C6. After 120 hours *L. monocytogenes* L4 could not be detected from the biofilm and the number of *L. innocua* C6 decreased on SS coupons. When *L. monocytogenes* L4 and *L. innocua* C6 produced biofilm on lettuce leaves (LL) the number of *L. innocua* C6 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.

When *L. monocytogenes* 1486/1 and 971, was inoculated together with lactic acid bacteria it was clearly demonstrated that *P. acidilactici* inhibited *L. monocytogenes* strains in model media and minced meat biofilms on SS coupons. In these systems *P. acidilactici* was able to produce high amount of antilisterial bacteriocin in biofilm. That could be an explanation why *P. acidilactici* could inhibit the growth of *L. monocytogenes* strains. It was also demonstrated that *P. acidilactici* was not able to produce bacteriocin in milk when *L. monocytogenes* was also present. Consequently, *L. monocytogenes* 971 strain could overgrow the *P. acidilactici* in this natural media.

Growth of *L. monocytogenes* in presence of *L. innocua* during the ISO 11290-1 detection method: *L. monocytogenes* L16 strain was not able to produce halo during the 168 h observation period, therefore it was excluded from further experiments. Testing the suitability of ALOA plates for detection of *L. monocytogenes* species it was determined that halo formation has to be checked after 24, 34 and 48 h incubation.

During the enrichment steps, when initial cell concentration of *L. innocua* C6 was higher than that of *L. monocytogenes* L4, C1, T3 inhibition of the latter was observed. 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. The calculated growth parameters indicated that the lag phase was prolonged in case of *L. monocytogenes* T3 (5.1 h) when it was grown with *L. innocua* C6 (ratio 1:1). However, in hFB the lag phase of *L. monocytogenes* T3 monoculture was shorter (1.7 h) than that of *L. innocua* C6 (3.0 h). When *L. monocytogenes* T3 grew in the presence of *L. innocua* C6 in 1:1 ratio a three log cycle difference was observed at the end of Fraser culturing step. 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.

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: Δ TTD, 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*

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.

List of publications regarding the thesis

Journals

In journals with impact factor

Engelhardt, T., Albano, H., Kiskó, G., Mohácsi-Farkas, Cs., Teixeira, P.: Antilisterial activity of bacteriocinogenic *Pediococcus acidilactici* HA6111-2 and *Lactobacillus plantarum* ESB 202 grown under pH and osmotic stress conditions. *Food Microbiology* 48: pp. 109-115. (2015). doi:10.1016/j.fm.2014.11.015. IF (2014): 3,331

Engelhardt, T., Ágoston R., Belák Á., Kiskó G., Mohácsi-Farkas, Cs.: The suitability of the ISO 11290-1 method for the detection of *Listeria monocytogenes*. *LWT-Food Science and Technology* 71: pp. 213-220. (2016). doi:10.1016/j.lwt.2016.03.038. IF (2014): 2,416

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International conference proceedings

Engelhardt, T., Albano, H., Kiskó, G., Mohácsi-Farkas, Cs., Teixeira, P. (7-8. Nov. 2013): Characterization of two bacteriocins active against *Listeria* species produced by *Lactobacillus plantarum* and *Lactobacillus sakei*. In: Dalmadi I., Engelhardt T., Bogó-Tóth Zs., Baranyai L., Bús-Pap J., Mohácsi-Farkas Cs. (ed.) *Food Science Conference 2013 - With research for the success of Darányi Program: Book of proceedings*, Budapest, Hungary, ISBN 978-963-503-550-2

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Engelhardt, T., Orgován, J., Kiskó G., Mohácsi-Farkas, Cs. (15-17. May 2013.): Screening the inhibitory effect of *L. innocua* strains on several *L. monocytogenes* strains, IAFP European Symposium on Food Safety, Marseille, France, Book of Abstracts.

Engelhardt, T., Ágoston, R., Belák, Á., Kiskó G., Mohácsi-Farkas, Cs. (15-17. May 2013.): Growth of *Listeria monocytogenes* in Presence of *Listeria innocua* During Traditional Detection Method, IAFP European Symposium on Food Safety, Marseille, France, Book of Abstracts.

Engelhardt, T., Albano, H., Kiskó, G., Mohácsi-Farkas, Cs., Teixeira, P. (6-8. Dec. 2013.): Production of antilisterial bacteriocin by *Pediococcus acidilactici* HA6111-2 under different stress conditions, Microbiotec13, Aveiro, Portugal, Book of Abstracts pp. 91.

Engelhardt, T., Albano, H., Kiskó, G., Mohácsi-Farkas, Cs., Teixeira, P. (7-9. May 2014.): Characterization of two bacteriocins active against *Listeria monocytogenes* and *Listeria innocua* produced by *Lactobacillus* spp. IAFP European Symposium on Food Safety, Budapest, Hungary, Book of Abstracts.

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