CORVINUS UNIVERSITY OF BUDAPEST

Investigation of competitive interactions between lactic acid bacteria and food-borne spoilage and pathogenic bacteria

PhD. THESES

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1. INTRODUCTION

Production of foods fermented with lactic acid bacteria (LAB) looks back to several thousand years' tradition because – due to the fermentation process – these products have better storability, have a higher nutritional value and contain more aroma compounds, and at the same time less antinutritive substances than the raw materials. Besides, these foods are considered safer because LAB produce different types of antimicrobial compounds (like organic acids, hydrogen peroxide and bacteriocins) which inhibit the growth of pathogenic and food spoilage microorganisms in the product. To exploit these characteristics of LAB, special protective cultures are developed and applied during the fermentation. This approach is in accordance with the new consumer claims that demand products in a "more natural" form and at the same time containing less chemical preservatives. Moreover, LAB can be used safely in food, as they are GRAS (generally recognised as safe) microorganisms.

The hygienic condition of food processing plants has an elementary importance in safe food production. Harmful bacteria present in the raw material and/or in the air can attach to the plant surfaces and develop bacterial coating (biofilm). The biofilm bacteria therefore become continuous sources of contamination that result in product loss and food-safety problems in the processed products. A possible solution to this problem can be the impact of the "house flora". In this case the colonisation of pathogenic bacteria is inhibited by the autochton microbe species (i.e. LAB). Inhibition exerted by competitive microbes is competition for adhesion sites and nutrients, production of antimicrobial metabolites and inhibitory extracellular polymeric substance (EPS).

Researches of the last few decades aimed at to reveal the physiological-biochemical mechanisms behind the health protective impact of the gut microbiota. The role of the beneficial gut bacteria – such as lactic acid and bifidobacteria – is multiple. Among others they take part in the inhibition of pathogenic bacteria by the steric hindrance of the binding receptors on the intestinal epithelial cells and/or by production of antimicrobial metabolites, which are just as efficient in the gastrointestinal tract as in the food matrices.

2. AIMS

1. Investigation of the interaction between LAB and food pathogenic and/or spoilage bacteria on stainless steel surface
2. Selection of LAB strain(s) with good adhesion abilities to Caco-2 human intestinal epithelial cell line, and investigation of adhesion as a function of initial cell count
3. Investigation of interaction between LAB and pathogenic bacteria on epithelial cell surfaces
4. Investigation of LAB and food pathogenic/spoilage bacteria in synthetic media and in liquid foods

3. MATERIALS AND METHODS

3.1. Surfaces and media

Adhesion and interactions of LAB and food pathogenic/spoilage bacteria were investigated in three different environments:

1. On stainless steel (type 304, 2B finish, 1.5x1.5 cm coupons)
2. On intestinal epithelial cells (Caco-2, ATCC HTB 37): 14 days old cells (shows both structural and functional similarity to the normal human small intestinal epithelial cells)
3. Liquid media: PCB (Plate Count Broth), skim milk, Jerusalem artichoke juice

3.2. Microorganisms

On stainless steel:

- *Lactobacillus delbrueckii* subsp. *bulgaricus*, originating from dairy product
- *Pseudomonas fluorescens* III P 13a, isolated from food plant surface
- *Listeria monocytogenes* LM6, of milk origin.

On intestinal epithelial cells:

- *Lactobacillus* strains: *L. plantarum* 2142, *L. casei* subsp. *pseudoplantarum* 2749*, L. casei* subsp. *pseudoplantarum* 2750, *L. casei* subsp. *casei* 2752, *L. casei* subsp. *casei* 2756, *L. casei* subsp. *casei* 2763, *L. curvatus* 2768, *L. curvatus* 2770, *L. curvatus* 2771, *L. curvatus* 2775, *L. sakei* DSM20017 of food origin
- *Bifidobacterium bifidum* B3.2 and
- *Escherichia coli* Bay 100 of stool origin.

In liquid media:

- *Lactococcus lactis* subsp. *lactis* CCM1881, *L. casei* subsp. *pseudoplantarum* 2749 of food origin
- *Bacillus cereus* T vegetative cells and spores
- *Escherichia coli* Bay100.

3.3. Methods

Stainless steel:

- Bacterial adhesion was investigated on vertically and horizontally inserted coupons in single and in mixed cultures after incubating them for 24 and 3 hours, respectively. For the experiments microbes were suspended in phosphate buffer saline.

- Adhered bacteria were either detached from the surface by vortexing and bacterial counts were determined on selective media (*Lactobacillus* – MRS agar, *Pseudomonas* – Pseudomonas selective agar, *Listeria* – Oxford agar), or
- The surfaces of the coupons were stained with fluorescent dyes (hexidium iodide/SYTO 9 or acridine orange) and investigated under epifluorescent microscope. The number of adherent bacteria was determined by counting and percent coverage was also obtained by an image analysing software (Scion Image).

Intestinal epithelial cells:

- For adherence tests bacteria were suspended in the cell culture medium then were placed onto the Caco-2 cells, incubated at 37°C for 1 hour statically in a humidified atmosphere of 5% CO_2 in air.
- Detection methods for bacterial adhesion (plating, fluorescent staining, Gram-staining) were compared with the help of three test LAB strains.
- For plating MRS agar (*Lactobacillus*), TPY (Trypticase-Phytone-Yeast extract) agar (*Bifidobacterium*) and ChromoBio Coliform agar (*E. coli*) were used. Adherent bacteria were either Gram-stained or labelled fluorescently with hexidium iodide then investigated under microscope. Quantification was carried out by counting and ratio of bacterial coverage was also determined.
- *Lactobacillus* strain with the best adhesion ability was selected and competitive adhesion of the selected *Lactobacillus* strain and *E. coli* was investigated.
- The adhesion of the selected *Lactobacillus* strain as a function of the initial bacterial cell count was determined.

Liquid media:

- Interaction between *L. lactis* subsp. *lactis* CCM1881 and *B. cereus* T was investigated in PCB and in skim milk. Cultures were incubated at 30°C for 72 hours. Bacterial counts were determined at certain intervals on selective media (MRS agar for *Lactococcus* and blood agar for *Bacillus*). In the case of PCB the number of *Bacillus* spores was also determined after killing the vegetative cells using heat treatment at 60°C for 30 min.
- Interaction between *L. casei* subsp. *pseudoplantarum* 2749 and *E. coli* was investigated in Jerusalem artichoke juice (7.5% dry material content). Bacterium cultures were incubated at 25°C for 48 hours, then at 12°C for 120 hours. Bacterial count was determined by plating on MRS agar (*Lactobacillus*) and on ChromoBio Coliform agart (*E. coli*).
- pH was also measured (Physitemp).

Statistical analysis was carried out using Poisson-distribution, ANOVA, Mann-Whitney and Welch-tests.

4. RESULTS

The aim of this study was to investigate the interaction between different *Lactobacillus* and *Lactococcus* strains of food origin and pathogenic/food spoilage bacteria on inert (stainless steel) and live (intestinal epithelial cells) surfaces and in liquid culture media (synthetic media and liquid foods). The research was based on the well-known fact that lactic acid bacteria exert an antimicrobial impact on several harmful microorganisms, therefore, with the help of LAB the colonisation and growth of these bacteria might be inhibited.

In the biofilm experiments bacterial adhesion was investigated on vertically and horizontally inserted stainless steel coupons. It was found that appr. 10% of *Pseudomonas* cells, 1% of *Listeria* cells and 0.1% of the *Lactobacillus* cells adhered to the vertical coupons. However, in case of lactobacilli the result was not reproducible in each replication of the experiment – it was traceable by both of the detection methods. Presumably for this reason the adhesion of harmful bacteria did not differ in single and in mixed cultures. On the horizontally inserted coupons the adhesion of LAB was reproducible and quantifiable. It was found that both *Pseudomonas* and *Listeria* showed greater adhesion in the presence of LAB than alone. Microscopic images showed that *Pseudomonas* and *Listeria* cells attached not only to the steel surface but to the lactic acid bacteria themselves; this way *Lactobacillus* probably aided the adhesion of food pathogenic/spoilage bacteria. The *Lactobacillus* count, however, did not show differences in single and mixed cultures.

As a result of the comparison of different detection methods in case of intestinal epithelial cells it was found that there was a good correlation between the results obtained by plating and the microscopic counting of Gram-stained cells except when the bacterium strain showed strong auto-aggregating ability. In this case one colony would grow from a cell aggregate (instead of from one cell only); therefore this method may underestimate the cell count. On microscopic images stained with fluorescent dye intestinal epithelial cell components (mainly nuclei) appeared as well along with the bacteria and this artefact hampered the quantification. The fluorescent dye applied binds to nucleic acids in a non-specific way so it also attached to the nucleic acids of Caco-2 cells despite the thorough washing. This staining method, therefore, was not found satisfactory in this model system. Finally the correlation between bacterial cell count and percentage coverage was determined: it was found to be linear in cases of all the three strains and regression coefficients showed close correlations. It was concluded that percentage coverage is also a suitable index to compare the adhesion ability of the candidate strains. However, the cell sizes have to be similar. It was found that *Lb. casei* subsp. *pseudoplantarum* 2749 showed the highest

adhesion rate to the Caco-2 cells among the tested strains; therefore this strain was chosen for further investigations. Results showed that the *Lactobacillus* strain investigated attached in a concentration-dependent manner: increasing number of added bacteria resulted in a proportionally increasing number of adherent bacteria. In the concentration range investigated there was no plato stage. This implies that binding sites on the Caco-2 cell surface were not saturated even at the highest added bacterium count. Microscopic images of adherent bacteria, however, showed that the bacterium cells attached to each other and made layers on top of each other and many of the cells were not able to reach the specific receptor molecules. Investigation of the competitive adhesion of the selected *Lactobacillus* strain and *E. coli* showed contradictory results. In spite of this it was possible to draw the following conclusions based on the data: the presence of LAB presumably did not influence the adhesion of the pathogen, however, a certain part of the *E. coli* cells might have died or injured (became VBNC) in the mixed culture as an effect of the metabolites of lactic acid bacteria. At the same time the presence of *E. coli* aided the adhesion of *Lactobacillus*. In consideration of the inconsistencies further investigations are needed to reveal the interactions between the two bacteria on Caco-2 cells.

In liquid media the results showed that LAB strains inhibited the growth of harmful microorganisms in every set of experiments: inhibition of *B. cereus* was 93.5%-99.1% in PCB and 38.3% in skim milk, while inhibition of *E. coli* was 99.9% in Jerusalem artichoke juice. The main factors of inhibition were the low pH, the impact of acids and nutrient depletion. At the same time pathogenic/spoilage bacteria did not have any impact on the activity of LAB. In case of *B. cereus* the growth of the bacterium was not influenced by the growth state (vegetative cells or spores) of the bacterium. Under unfavourable conditions *B. cereus* started to form spores already a few hours after inoculation. By the end of the incubation period almost all the *B. cereus* cells were present as spores. The lactic acid bacteria might have a bactericidal impact on the vegetative cells of *B. cereus*, but complete elimination did not occur as cells "escaped" to spore state as growing conditions became unfavourable. Unlike in PCB, *B. cereus* could grow well in skim milk, which implies that neither LAB, nor *B. cereus* hindered the growth of the other. Lack of inhibition might have occurred because of the good buffer capacity and rich nutrient content of the milk. The most significant inhibition was observed in Jerusalem artichoke juice in case of *E. coli*, being the inhibition real regarding that *E. coli* does not form spores. Besides the bactericidal impact of LAB, suboptimal growth temperature was also responsible for the inhibition of *E. coli* as being an enteric bacterium its optimal growth temperature is about 37°C.

NEW SCIENTIFIC RESULTS

1. Adhesion ability of 3 bacterium strains (*Lb. delbrueckii* subsp. *bulgaricus*, *P. fluorescens* III P 13a, *L. monocytogenes* LM6) was determined on vertically inserted stainless steel surface and significant differences (0.1-10%) were found between them. Position of the steel surface influenced the adhesion; the extent of adherence of *Lb. delbrueckii* subsp. *bulgaricus* was smaller on the vertical surface than on horizontal one.

2. *Lb. delbrueckii* subsp. *bulgaricus* aided the adhesion of pathogenic/spoilage bacterium strains investigated to the horizontally inserted steel surface in mixed cultures.

3. In case of adhesion to intestinal epithelial cells it was found that *Lb. casei* subsp. *pseudoplantarum* 2749 strain did not – or not significantly – inhibit the adhesion of *E. coli* Bay100, however it showed bactericidal impact on the adherent bacterium cells.

4. The results indicated that methods applied generally to detect adherent bacteria are not sensitive enough for exact, quantitative comparisons. In order to get a real picture parallel application of more than one detection method is suggested.

5. In liquid media *Lc. lactis* subsp. *lactis* CCM1881 exerted inhibition against *B. cereus* T vegetative cells, but did not hinder the spore germination and spore forming. Inhibitory impact of *Lc. lactis* was smaller in the rich culture medium (milk).

6. It was found that *Lb. casei* subsp. *pseudoplantarum* 2749 inhibits *E. coli* Bay100 well in Jerusalem artichoke juice; inhibition remained constant at chill temperature.

5. CONCLUSIONS

Adhesion ability of *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Pseudomonas fluorescens* and *Listeria monocytogenes* was investigated on vertically and horizontally inserted stainless steel surface (the first stage of biofilm formation) and it was found that the position of the surface influenced the extent of the adhesion: the adherence and investigation of the adherence was more difficult on the vertically inserted surface and the results were difficult to reproduce. The reason for this phenomenon was the fast sedimentation of the large, non-motile *Lactobacillus* cells. In nature both vertical and horizontal surfaces occur. Therefore – according to my observation – it is suggested that the bacterial colonisation should be studied on both vertical and horizontal surfaces in order to gain a comprehensive view about the biofilm formation.

In case of competitive adhesion on stainless steel it was found that lactobacilli did not hinder but aided the adherence of *P. fluorescens* and *L. monocytogenes* by offering attachment sites for them. In the nutrient-free environment used in the experiments lactic acid bacteria could not grow therefore they did not produce antimicrobial metabolites, which could have inhibited the harmful bacteria. Environments poor in nutrients occur, albeit transiently, on the food processing plant surfaces too, therefore lactic acid bacteria, which have complex nutritional requirements, may be at a disadvantage and may not be able to take part in the biocontrol. For this reason it is suggested that experiments should use strains isolated from food processing plant surfaces that are already adapted to that specific environment and select the potential biocontrol organisms among them.

Based on the adhesion tests of three bacterium strains to intestinal epithelial cells it was found that methods applied to detect adherent bacteria (plating and microscopic observation) gave – more or less – different results. The difference can be explained by the auto-aggregating ability of the bacteria. In order to get a real picture it is suggested that more than one detection method should be used in parallel. It would be advisable to develop suitable in situ methods to model the biofilm formation and bacterial cmpetition in food processing plants.

In liquid media results indicated that *Lc. lactis* subsp. *lactis* exerted inhibitory activity against *B. cereus* T vegetative cells but hindered neither spore germination nor spore formation. As a consequence of the unfavourable conditions due to LAB growth *B. cereus* “withdrew” or “escaped” into spore state therefore the complete elimination of the pathogen did not occur. *B.*

cereus can cause significant problems in the dairy industry because pasteurization can not kill the spores, which germinate and multiply quickly among favourable conditions in milk. Results indicate that metabolites formed during the fermentation maintain the unfavourable conditions among which spores are not able to germinate. From this aspect, the fast fermentation process in the raw material is of elementary importance.

In liquid media results indicated that *Lb. casei* subsp. *pseudoplantarum,* though inhibited *E. coli* Bay100 strain efficiently, did not cause complete elimination on the time scale investigated. The extent of the inhibition remained unchanged at chill temperature. These results indicated that using LAB alone does not ensure against these pathogens. In order to reduce risk it is important to keep to the stringent sanitary practices and to pasteurize the raw material.

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