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## GROWTH AND SURVIVAL OF PROBIOTIC LACTIC ACID BACTERIA IN THE PRESENCE OF HETEROCYCLIC AROMATIC AMINES

### Summary

Heterocyclic aromatic amines (HCAs) ingested with heat-processed meat can contribute to colon cancer development. Those compounds interact with human intestinal microbiota. Under the research study, the effect was analyzed of IQ, MeIQx, or PhIP amines (each of them at 5 and 25 µg/ml concentration rates) on the growth (24 h cultivation in MRS broth) and survival (incubation for max. 120 h in a phosphate buffer) of four probiotic strains of *Lactobacillus*. It was found that no concentration level of the HCAs analyzed impacted the growth of bacteria. A higher concentration level of IQ (25 µg/ml) decreased the survival of *Lb. casei* 0900 after a 24 h period of incubation. MeIQx and PhIP also decreased the survival of *Lb. paracasei* 0919 after a 24 h period of incubation. Totally, the count of living cells decreased from 10<sup>9</sup> cfu/ml to 10<sup>8</sup> cfu/ml. Three strains were absolutely resistant to PhIP during a 120 h period of incubation. The results obtained prove that the probiotic bacteria studied maintain their high viability in the presence of the amines tested and, probably, they can bind together with them in human colon and, then, the aggregated particles are removed from the human body.

**Key words:** probiotics, *Lactobacillus*, heterocyclic aromatic amines

### Introduction

2-Amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (IQ), 2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenyl-1*H*-imidazo[4,5-*b*]pyridine (PhIP) are heterocyclic aromatic amines (HCAs) formed in a heated muscle tissue, e.g. in the roasted and grilled meat and fish as a result of interactions between amino acids, sugars, and creatinine [20, 24]. Those compounds can contribute to the genotoxic or carcinogenic load within colon. Together with other factors (e.g. toxic metabolites of protein fermentation, increased colonic fat), they may account for an

increased risk of colorectal cancer (CRC) associated with the processed meat [22]. Additionally, the colonic microbiota can transiently respond to dietary intake. Meat rich diets affect the microbial composition towards a profile with more effective metabolizers of heterocyclic aromatic amines, which, in turn, may increase the risk of CRC development [3]. Probiotics are capable of transient modulation of the gut microbiota and their beneficial effects include the prevention of CRC by scavenging toxic compounds, *e.g.* HCA, and the prevention of their generation *in situ* [2].

A large number of reports exist to describe the adsorption or the binding by lactic acid bacteria (LAB) of a variety of food-borne HCAs *in vitro*; in some of those studies, a concomitant decrease in mutagenicity was reported [4, 8, 11, 16, 17, 19, 25]. Nevertheless, nothing is known about direct toxic effects of HCAs on certain species of probiotic lactic acid bacteria. In the previous research, the toxicity was assessed of major products of the bacterial aromatic amino acids metabolism in the colon (phenolic and indolic compounds) [15].

The objective of the study was to evaluate the ability of probiotic lactobacilli of human origin to grow and survive in the presence of three heterocyclic aromatic amines that occur most commonly, *i.e.*: IQ, MeIQx, and PhIP, and to determine whether or not there are differences in reactions between the LAB strains and those compounds.

## Materials and Methods

### *Strains and carcinogens*

The following probiotic *Lactobacillus* strains were analyzed: *Lb. casei* ŁOCK 0900, *Lb. casei* ŁOCK 0908, and *Lb. paracasei* ŁOCK 0919. They were acquired from a collection owned by the Institute of Fermentation Technology and Microbiology (ŁOCK 105), Lodz University of Technology, Poland. The strains are licensed (Nos. P-382760, P-382761, P-382762), their complete probiotic documentation is available, and they are kept by the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław, Poland (Nos. B/00019, B/00020, B/00021). *Lb. plantarum* ŁOCK 0945 originated from the same Institute; its complete probiotic documentation exists (the strain is under the licensing process), and it is kept by the Department of Molecular Microbiology of the National Medicines Institute in Warsaw, Poland.

To maintain the activity of the strains, 24-hour cultures in MRS broth (Merck) were frozen at -20 °C with the addition of 20 % glycerol. Prior to application, the bacteria were activated twice in a liquid MRS broth (3 % inoculum) and incubated for 24 h at 37 °C in 5 % CO<sub>2</sub> (in a New Brunswick CO<sub>2</sub> incubator). The stock cultures used in

the experiments were stored at 4 - 5 °C. The 24-hour cultures of bacteria in MRS broth were used as an inoculum (3 %); the cell density was  $10^9$  cfu/ml.

The following HCAs were used: IQ (2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline); MeIQx (2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline) and PhIP (2-amino-1-methyl-6-phenyl-1*H*-imidazo[4,5-*b*]pyridine); they were purchased from a Toronto Research Chemicals Company (Canada). To obtain stock solutions, IQ and PhIP were diluted in DMSO to receive a final concentration level of 0.1 % (for IQ) and 0.05 % (for PhIP); MeIQx was diluted in water to receive a final concentration level of 0.1 %. The stock solutions were stored at 4 - 5 °C. The concentrations levels of HCA tested were 5 and 25 µg/ml for each HCA.

#### *Cultivation of bacteria in MRS broth*

To determine the impact of IQ, MeIQx or PhIP on the growth of lactobacilli during a 24 h period, the cells (3 % inoculum) were incubated in a liquid MRS broth at 37 °C in 5 % CO<sub>2</sub> with IQ, MeIQx, and PhIP added at 5 and 25 µg/ml concentration rates of each amine separately. The control samples were bacterial cultures without HCA. To study the impact of carcinogens on the growth of bacteria, the quantity of living cells was counted using a Koch's plate method. One ml of each culture was diluted in a sterile saline (0.85 % NaCl) and the serial dilutions of the culture were transferred onto plates along with MRS broth (with 1.5 % agar). The cell number was determined at '0' time and after a 24 h period of incubation; it was expressed as cfu/ml (colony forming units/ml). Every sample of a given concentration level was 4 times plated and a standard deviation ( $\pm$  SD) was calculated for each one. The differences among the means were compared using a one-way analysis of variance (ANOVA,  $p < 0.01$ ).

#### *Incubation of bacteria in phosphate buffer*

In order to assess the survival of non-growing lactobacilli in the presence of IQ, MeIQx, or PhIP, the 24-hour cultures in MRS broth were centrifuged ( $10700 \times g$  for 10 min), washed using 20 ml of a sterile 0.2 M phosphate buffer (pH = 6.2 - 6.3), and once more centrifuged. Next, the cells were re-suspended in the buffer of a cell density being  $10^9$  -  $10^{10}$  cfu/ml using 5 and 25 µg/ml of each amine separately, and incubated for 120 h (5 days) at 37 °C in 5 % CO<sub>2</sub>. Control samples were cell suspensions of each strain with no amine added.

In order to estimate the number of living cells, a pour plate method was applied every 24 or 48 hours, i.e.: bacteria were plated using MRS broth (with addition of 1.5 % agar) and incubated for 24 h at 37 °C in 5 % CO<sub>2</sub>. The colonies were counted after every period of 48 h incubation, and, as a result, survival curves for each strain

and mutagen concentration were obtained. The differences among the means were compared using a one-way analysis of variance ( $p < 0.01$ ).

## Results

### *Impact of HCAs on growth of probiotic bacteria*

No difference was found in the number of living bacterial cells (after a period of 24h incubation with carcinogens in MRS medium) compared to the control sample (Tab. 1). Thus, all the *Lactobacillus* strains were able to grow in the presence of 5 and 25  $\mu\text{g/ml}$  of IQ, MeIQx, or PhIP. Only as regards *Lb. casei* 0900, a slight decrease in the number of living cells was reported in the presence of 25  $\mu\text{g/ml}$  of PhIP ( $p < 0.01$ ).

Table 1. Impact of IQ, MeIQx, and PhIP on growth (log CFU/ml) of probiotic *Lactobacillus* strains after a period of 24 h incubation in MRS medium.

Tabela 1. Wpływ IQ, MeIQx i PhIP na wzrost (log jtk/ml) szczepów probiotycznych z rodzaju *Lactobacillus* po 24 h inkubacji w pożywce MRS.

Strain Szczep	HCA concentration [ $\mu\text{g/ml}$ ] Stężenie HCA [ $\mu\text{g/ml}$ ]								
	IQ ( $\bar{x} \pm \text{SD} / s$ )			MeIQx ( $\bar{x} \pm \text{SD} / s$ )			PhIP ( $\bar{x} \pm \text{SD} / s$ )		
	K	5	25	K	5	25	K	5	25
0900	9.60 $\pm$ 0.09	9.59 $\pm$ 0.12	9.63 $\pm$ 0.11	9.85 $\pm$ 0.08	9.79 $\pm$ 0.09	9.71 $\pm$ 0.01	9.86 $\pm$ 0.01	9.83 $\pm$ 0.02	9.73* $\pm$ 0.01
0908	9.72 $\pm$ 0.08	9.74 $\pm$ 0.11	9.72 $\pm$ 0.08	9.79 $\pm$ 0.12	9.89 $\pm$ 0.01	9.76 $\pm$ 0.10	9.99 $\pm$ 0.05	9.97 $\pm$ 0.07	9.94 $\pm$ 0.10
0919	9.54 $\pm$ 0.12	9.54 $\pm$ 0.14	9.42 $\pm$ 0.08	9.76 $\pm$ 0.07	9.76 $\pm$ 0.09	9.79 $\pm$ 0.09	9.99 $\pm$ 0.20	9.98 $\pm$ 0.02	9.95 $\pm$ 0.01
0945	9.45 $\pm$ 0.08	9.40 $\pm$ 0.04	9.42 $\pm$ 0.03	9.70 $\pm$ 0.10	9.77 $\pm$ 0.19	9.67 $\pm$ 0.19	9.92 $\pm$ 0.05	9.82 $\pm$ 0.12	9.89 $\pm$ 0.19

Explanatory notes: / objaśnienia:

IQ – 2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline / 2-amino-3-metyloimidazo[4, 5-*f*]chinolina; MeIQx – 2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline / 2-amino-3,8-dimetyloimidazo[4,5-*f*]chinoksalina; PhIP – 2-amino-1-methyl-6-phenyl-1*H*-imidazo[4,5-*b*]pyridine / 2-amino-1-metylo-6-fenyloimidazo[4,5-*b*]pirydyna; K – próba kontrolna / control sample; 5, 25 – concentration of amines / stężenia amin;  $\bar{x} \pm \text{SD} / s$  – mean value  $\pm$  standard deviation / wartość średnia  $\pm$  odchylenie standardowe;

\* – the differences between average values of the samples and the control are statistically significant ( $p < 0.01$ ) / różnice pomiędzy wartościami średnimi badanych prób i próby kontrolnej statystycznie istotne na poziomie  $p < 0.01$ .

### *Impact of HCAs on survival of probiotic bacteria*

Compared to the control sample, 5  $\mu\text{g/ml}$  of every amine had no impact on the survival of bacteria in the phosphate buffer. As shown in Figure 1, a 5-times higher concentration of IQ, MeIQx, and PhIP (25  $\mu\text{g/ml}$ ) had a clear impact on the survival of non-growing lactobacilli during 120h incubation, and it was strain dependent.

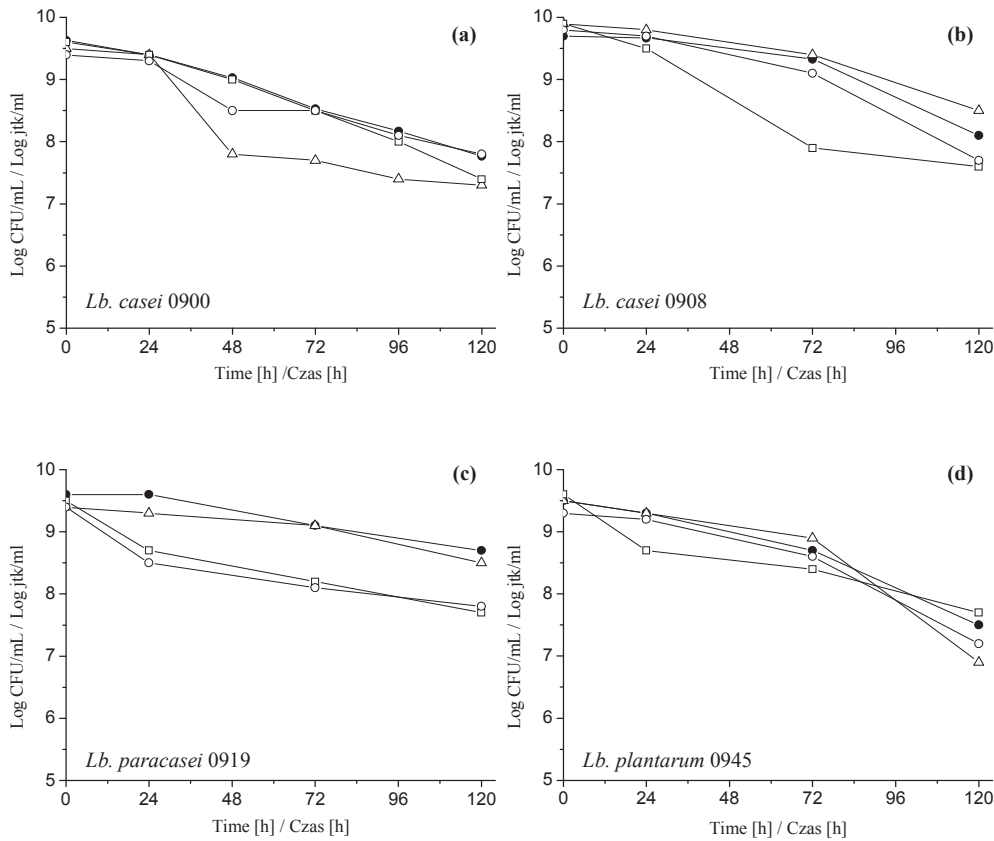


Fig. 1. Impact of IQ, MeIQx or PhIP on survival of *Lactobacillus* strains; ● control sample, Δ 25 μg/ml of IQ, □ 25 μg/ml of MeIQx, ○ 25 μg/ml of PhIP.

Rys. 1. Wpływ IQ, MeIQx i PhIP na przeżywalność szczepów probiotycznych z rodzaju *Lactobacillus*; ● kontrola, Δ 25 μg/ml IQ, □ 25 μg/ml MeIQx, ○ 25 μg/ml PhIP.

In the presence of IQ, the number of viable cells of *Lb. casei* 0900 decreased after the 24h period of incubation; the decrease continued until the incubation period was 120h, and it was significantly different from the control sample ( $p < 0.01$ ) (Fig. 1a). As for *Lb. casei* 0908 (Fig. 1b), a significant decrease in the number of living cells was reported after the 24h incubation period in the presence of MeIQx, and the ability of bacteria to survive decreased along with the increasing time of incubation. *Lb. paracasei* 0919 was significantly affected by MeIQx and PhIP from the beginning of incubation (Fig. 1c) ( $p < 0.01$ ).

So, *Lb. casei* 0908 and *Lb. paracasei* 0919 appeared to be the most sensitive to MeIQx, and the higher concentration of the compound in the environment, the less

viable cells. PhIP and IQ did not affect 3 strains at all; until the period of 120 h, the change in the number of viable cells thereof was the same as that in the control sample ( $p < 0.01$ ). *Lb. plantarum* 0945 was resistant to all the amines at the concentrations analyzed (Fig. 1d) ( $p < 0.01$ ).

## Discussion

Colorectal cancer is the third most prevalent cancer in humans. One of the recognised risk factors includes a diet rich in heat-processed meats [10]. The consumption of HCAs is very low; however, several compounds are consumed at the same time. The estimations performed show that human exposure to HCA ranges between a few ng/day and  $\mu\text{g/day}$  depending on the dietary and cooking habits. The daily intake level of IQ is 1 ng, of MeIQx between 9.8 and 135 ng, and of PhIP between 39 and 458 ng per one adult person [1, 5, 9, 12, 18]. It is important to remember that HCAs occur in wine, beer, cigarette smoke, exhaust fumes, as well as in many other products; thus, the genuine human exposition to the carcinogens is unknown.

Under the research study, IQ, MeIQx, and PhIP were analyzed at high concentration levels; in the case of IQ, the concentration level was as high as 25  $\mu\text{g/ml}$ ; although such levels are unrealistic for consumers, they are not toxic for bacteria during a 120h period of incubation. In many reports, much lower and much higher concentrations (1, 10, 100 and 1000 mg/l) of HCA were investigated [23]. The objective of the study was to study the phenomenon *in vitro*. Our probiotic strains are resistant to even high concentrations of IQ, MeIQx, and PhIP. As for human gastrointestinal tracts, most substances spend, on average, 24 to 72 hours therein; 18 - 64 h of this period, they are in the large bowel; that is why the transit time is mainly a colonic event [6]. When considering the colonic transit time, the incubation of bacteria in the phosphate buffer continued for 120h. Colon transit time varies greatly from person to person and it depends on such factors as diet, stress, hormones, colonic anatomy, microbiota, age (old persons), sex (females more often), physical activity, and colon disturbances (*e.g.* irritable bowel syndrome). All the named factors can lead to constipations and a slow transit time; all this is associated with the prevalence of large bowel disorders, particularly with colon cancer [6, 21].

Probiotic lactobacilli can reduce the colonic transit time when carcinogens within the colon are in contact with the intestinal mucous, thereby they can suppress carcinogenesis. It was reported that *Bifidobacterium animalis* DN 173010 reduce the colonic transit time in healthy women [13]. Probiotics bind with HCAs and, in this way, remove carcinogens from the human body; it is a very important characteristic of probiotics. As soon as the bacterial cells absorb carcinogens, they are excreted in faeces and the colon epithelial cells are no longer exposed to the impact of those compounds.

In the research study, all the lactobacilli appeared to be mostly resistant to high concentration levels (25 µg/m) of PhIP, i.e. to the amine most commonly occurring in diets; its daily intake by humans is the highest among all HCAs [7]. Of all HCAs, this amine appears to be the most mutagenic and genotoxic for colonic epithelial cells [14]. Lactobacilli revealed their definite sensitivity to IQ, MeIQx, and PhIP. *Lb. casei* 0900 was the most sensitive to IQ, *Lb. casei* 0908, *Lb. paracasei* 0919 to MeIQx and PhIP, and *Lb. plantarum* 0945 was resistant to all the amines analyzed. Generally, the number of living cells of bacteria decreased from the initial  $10^9$  to  $10^7$  CFU/ml during 120h; this decrease was reported even in the case of the control sample. IQ, MeIQx, and PhIP slightly affected the viability of the probiotic lactic acid bacteria tested ( $p < 0.01$ ). The impact of those compounds depended on the dosage and the compound itself.

## Conclusion

The conclusion is that none of the IQ, MeIQx, and PhIP amines (5 and 25 µg/ml) impacted the growth of probiotic lactobacilli in MRS broth. Additionally, the probiotic strains had a high viability level in their presence during a period up to 120 h (normal colon transit time). Thus, probably, the probiotic bacteria can efficiently interact with HCAs in the human body (so that they bind with them and, then, are removed in faeces).

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## WZROST I PRZEŻYWALNOŚĆ BAKTERII PROBIOTYCZNYCH W OBECNOŚCI HETEROCYKLIKOWYCH AMIN AROMATYCZNYCH

### Streszczenie

Heterocykliczne aminy aromatyczne (HCA) spożywane wraz z termicznie przetworzonym mięsem mogą przyczyniać się do rozwoju raka jelita grubego. W pracy badano wpływ amin IQ, MeIQx oraz PhIP (każda w stężeniu 5 i 25 µg/ml) na wzrost (24-godzinna hodowla w pożywce MRS) i przeżywalność (do 120 h inkubacji w buforze fosforanowym) czterech szczepów probiotycznych z rodzaju *Lactobacillus*. Zaobserwowano, że żadne z badanych stężeń HCA nie wpływało na wzrost bakterii. Wyższe stężenie IQ (25 µg/ml) obniżało żywotność szczepu *Lb. casei* 0900 od 24 h inkubacji. Aminy MeIQx i PhIP obniżały żywotność *Lb. paracasei* 0919 również od 24 h inkubacji. Generalnie liczba żywych komórek obniżyła się z  $10^9$  jtk/ml do  $10^8$  jtk/ml. Trzy szczepy były całkowicie odporne na obecność PhIP podczas 120 h inkubacji. Uzyskane rezultaty wskazują, że badane szczepy probiotyczne zachowują wysoką żywotność w obecności testowanych amin i prawdopodobnie mogą wiązać się z nimi w jelicie grubym, po czym te zagregowane cząstki usuwane są z organizmu.

**Słowa kluczowe:** probiotyki, *Lactobacillus*, heterocykliczne aminy aromatyczne ☒