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Article

The degradation of acrylamide by microorganisms present in food products

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Abstract: Acrylamide (AA) present in food is considered a harmful compound for humans, but it exerts impact on microorganisms too. The aim of the study was to evaluate the impact of AA (at conc. 0-10 μg/mL) on the growth of bacteria (*Leuconostoc mesenteroides, Lactobacillus acidophilus* LA-5) and yeasts (*Saccharomyces cerevisiae, Kluyveromyces lactis* var. *lactis*), which naturally occur in food products. Moreover, we decided to verify whether these microorganisms could decompose acrylamide. Our results proved that AA can stimulated the growth of *L. acidophilus* and *K. lactis*. We have also reported, to the best of our knowledge for the first time, that probiotic strain of bacteria *L. acidophilus* LA-5 is able to degrade AA by amidase production and hence can utilize AA as a source of carbon and nitrogen if they lack in the environment. The conducted Response Surface Methodology indicated that pH as well as incubation time and temperature significantly influenced the amount of ammonia released from acrylamide by the bacteria. Concluding, our studies suggest that some strains of bacteria present in milk fermented products can exert additional beneficial impact by acrylamide degradation and preventing against its harmful impact on human body and other members of intestinal microbiota.

Keywords: lactic acid bacteria; probiotic; yeast; acrylamide; amidase; degradation; Response Surface Methodology

1. Introduction

Changes in the standard of living, eating habits and the increased awareness of the impact of nutrition on the human health resulted in more and more consumers that pay attention not only to the nutritional value of food products. The consumers are increasingly convinced that food affects their health, enables the prolongation of life and well-being, and may also prevent non-transmissible chronic diseases [1]. Hence, each year we can find more types of the so-called functional food on the market. It is estimated that 60 % – 80 % of functional products available on the market are probiotics [2]. Dairy products contain a variety of lactic acid bacteria (LAB) and yeasts, which are responsible for the organoleptic and health-promoting properties of these products. Among the products containing many microorganisms, first of all distinguish we can natural yoghurts, kefirs, ripening cheeses, fermented and acidophilic milk and koumiss. The microorganisms present in them are bacteria strains belonging mainly to the species *Lactobacillus bulgaricus, Lactobacillus acidophilus, Bifidobacterium lactis,* and *Streptococcus thermophilus*, and less often to other species e.g., *Bifidobacterium longum, B. bifidum, B. breve, B. infantis, Lactobacillus casei, L. rhamnosus, L. acidophilus, L. lactis, L. gasseri, L. brevis, L. salivarius, L. paracasei, L. plantarum,* and *Propionibacterium freudenreichii*. Fermented milk drinks also contain yeasts of various types and species, e.g., *Kluyveromyces marxianus (Candida kefyr), K. lacits, Saccharomyces cerevisiae, S. exiguus, S. omnisporus* [1].

It is known that the human gastrointestinal tract can be colonized by even 1200 various species of microorganisms, most residing in the colon, where they are detected most frequently and reach 10^{12} bacteria per gram of intestine content [3]. The intestinal

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microbiota (IM) uses mainly carbohydrate and protein products from our diet as sources of carbon and nitrogen, but is also able to degrade other dietary compounds, such as polysaccharide fibers, that are not digested by human enzymes. In biochemical processes, IM can synthesize from them, among others biotin, vitamin K or folic acid, which are absorbed by the human body. Metabolites formed in the processes of bacterial fermentation of carbohydrate substrates may provide additional benefits to the host organism, whereas during the decomposition of protein the products of often harmful or even toxic effect are formed [4]. By focusing on the positive aspects of the activity of microorganisms in the human body, according to experts from the International Scientific Association for Probiotics and Prebiotics, consumption of dairy fermented products containing live microbes brings many benefits, e.g. nutritional benefits, production of important digestive enzymes (e.g., β-galactosidase), prevention of intestinal infection, stimulation of the host immune system and modulation of immune response, prevention of colorectal cancer and diabetic type 2, good effect on the brain central system, as well as reduction of cholesterol level and obesity [5]. All above causes that functional foods are often LAB-containing products. Recent studies indicate their likely new function – protecting the human body against the toxic effect of acrylamide present in food [6].

Acrylamide (AA) is formed in food mainly by the reaction of free asparagine with reducing sugars (especially fructose and glucose) during the Maillard reaction, however, it can also be produced by other pathways (e.g. the acrolein pathway) [7]. It means that high temperatures (above 120 °C) used during the thermal processing of food, in addition to impart flavor and aroma, may lead to the formation of harmful substances in the product, such as acrylamide. On the basis of the laboratory tests in animals, the International Agency for Research on Cancer [8] assigned acrylamide to the group of compounds "probably carcinogenic to humans", while according to the USEPA [9], acrylamide at a concentration of 0.8 μg/L causes a cancer risk of 1: 10,000. Acrylamide has been shown to exert neurotoxic, genotoxic, cytotoxic, and carcinogenic impact on the animal and human organisms, but it does not exert a mutagenic effect in bacterial cells. It was concluded that carcinogenic activity of AA is related to glycidamide (GA) – a metabolite of acrylamide formed in mammalian cells [7].

Some studies have shown that selected strains of *Lactobacillus* spp. can alleviate the toxicity of AA against rats [10, 11]. However, when the studies have looked at the detailed mechanism of this action they have shown that it consists in removing acrylamide from the solution by its physical binding to the bacterial cell wall [12]. The significant role of peptidoglycan structure, especially the structure of teichoic acids and the contents of four amino acids (alanine, aspartic acid, glutamic acid, and lysine) was also studied in details by Serrano-Niño et al. [13] and Zhang et al. [14].

It is known that some microbial enzymes can catalyze the acrylamide degradation, and the most important among them are amidases [7]. Amidases are enzymes (EC. 3.5.1.4) that occur ubiquitously in nature. They are characterized by a broad spectrum of catalyzed reactions and can use various chemicals as a substrate. They play an important role in the bacteria proliferation of cells and separating young cells (i.g., amidases AmiA, AmiB and AmiC synthetized by *E. coli*), however, the substrate for those enzymes is peptidoglycan [15]. There have been no reports describing whether bacteria containing amidases, which degrade amide bonds in peptidoglycan, are able to degrade acrylamide as well, however, this cannot be excluded. There are also other enzymes that operate similarly. One of them is N-acetylmuramidase (AcmA, EC 3.5.1.28) synthesized by various strains of *Lactococcus lactis* demonstrating autolysin activity, that has been already applied in cheese maturation. The result of cell autolysis is the release of bacterial peptidases leading to the formation of peptides and amino acids providing desired aroma and flavor of cheese [16, 17].

The participation of intestine microbiota in chemical changes of AA has not been investigated yet; however, it has been shown that various bacteria, including those that naturally occur in human gut or those that are delivered with food, might degrade that compound. The ability of synthesizing amidases by bacteria present in digestive tract,

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such as *Escherichia coli, Enterococcus faecalis, Bacillus clausii, Helicobacter pylori* [6] indicates the possible existence of an undiscovered mechanism of acrylamide degradation in human gut.

In our previous work we demonstrated that acrylamide could influence the viability of beneficial intestinal bacteria from *Lactobacillus* genus [6]. First of all we demonstrated that the tested lactic acid bacteria strains were tolerant to acrylamide even at high concentrations (up to 1 g/mL) and the growth of *Lactobacillus plantarum, L. brevis,* and *Lactococcus lactis* subsp. *lactis,* as well as probiotic strain *Lactobacillus acidophilus* LA-5, was more intense in the presence of AA at high concentration than in medium with limited accessibility of carbon and nitrogen compounds. It was concluded that acrylamide had no toxic impact on LAB, and that some strains probably could utilize acrylamide as a source of carbon and nitrogen if they lacked in the environment/medium. Moreover, the viability of bacteria was modulated by AA, the viability of probiotic strain *L. acidophilus* LA-5 increased, that of *L. plantarum* decreased, while *L. brevis* was the less sensitive.

Basing on the above, the aim of the present study was to check whether the microorganisms naturally occurring in milk products (lactic acid bacteria and yeasts), which growth was stimulated by acrylamide, are able to amidase production and acrylamide degradation.

2. Materials and Methods

2.1. Microorganisms and Materials

Pure cultures of naturally occurring in food products yeasts (*Saccharomyces cerevisiae* DSM 70478, *Kluyveromyces lactis* var. *lactis* DSM 70799) and lactic acid bacteria (*Streptococcus salivarius* subsp. *thermophilus* DSM 20617, *Leuconostoc mesenteroides* subsp. *cremoris* DSM 20346) were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany), while probiotic strain *Lactobacillus acidophilus* LA-5 was provided by Chr. Hansen (Hørsholm, Denmark). All microbiological media and components of growth media were purchased from Bio-Maxima (Lublin, Poland) unless otherwise stated. Maximum Recovery Diluent (MRD) was composed of 0.45% (w/v) of bacteriological peptone (as the only source of carbon and nitrogen in this medium) and 0.45% NaCl (w/v) (POCh, Gliwice, Poland). YM agar was prepared by dissolving yeast extract (3.0 g/L), malt extract (3.0 g/L), peptone from soybeans (5.0 g/L), glucose (10.0 g/L), agar (15.0 g/L) and chloramphenicol (0.1 g added after sterilization) in distilled water. All media were sterilized using a Microjet Microwave Autoclave (process parameters: 135 °C, 80 s, 3.6 bar; Enbio Technology Sp. z o.o., Gdynia, Poland). Acrylamide (AA, purum, ≥98%, GC) was provided by Sigma-Aldrich (Sp. z o.o, Poznan, Poland).

2.2. Bacteria and Yeasts Preparation

All microorganisms were cultured 24 h before experiments. Yeasts were grown at 28 °C in Sabouraud broth supplemented with chloramphenicol (100 mg/L) in 100 mL flasks on the rotary shaker (Orbit 1000, Labnet International Inc., Edison, NJ, USA) at 120 rpm. Bacterial strains were grown in MRS broth at 37 °C (*L. acidophilus* LA-5, *S. salivarius* subsp. *thermophilus*) and 30 °C (*L. mesenteroides* subsp. *cremoris*). Just before experiments the cultures were centrifuged for 15 min at 194 × g (MPW-35JR centrifuge, MPW MED Instruments, Warsaw, Poland) and washed twice with sterile water to remove traces of growth media. The resulting pellets were resuspended in MRD so as to obtain the bacterial/fungal suspensions containing 10^7 cfu/mL. For that purpose, the optical density of microorganisms solution was measured by Den-1B densitometer (Biosan, Latvia) and expressed in McFarland units. The bacteria and yeast concentrations were calculated from the relationship established as described below.

2.3 Measurement of Optical Density of Bacterial Suspension: Calibration

To tubes containing 5 mL of sterile MRS medium, a volume of 0.1 mL of 24-h liquid bacterial culture was added, the contents were mixed, and the tubes were incubated for

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24 h at the optimum temperature for the tested strain. After incubation, bacterial cultures were centrifuged at 194 × g for 15 min (MPW-35JR centrifuge, MPW MED Instruments, Warsaw, Poland), and the supernatant was discarded. The pellets were rinsed by mixing with 5 mL of sterile distilled water followed by centrifugation (parameters as above). The resulting pellets were resuspended in sterile water so as to obtain an optical density of the bacterial suspensions equal to McFarland standard 1.0 (using a Den-1B densitometer, Biosan, Latvia). Then, serial 10-fold dilutions were made in sterile water, and 1 mL of subsequent dilution was spread over the surface of the MRS medium (in triplicate). After 72 h of incubation at an optimal temperature, bacterial colonies were counted, and the mean bacterial cell density (expressed in cfu/mL) from 3 replicates was calculated for each tested strain. The relationship between the optical density of McFarland = 1 and bacterial cell density was determined. Exactly the same experiments were done for tested yeasts strains, except that Sabouraud broth and Sabouraud agar were used instead of MRS, and the incubation temperature was 28 °C.

The relationships obtained for individual strains were as follows (the optical density value that corresponds to the cell concentration of 10^7 cfu/mL): *L. acidophilus* LA-5 (0.22), *L. mesenteroides* (0.03), *S. thermophilus* (0.03), *K. lactis* (1.23) and *S. cerevisiae* (7.89). In the case of *S. thermophilus* and *L. mesenteroides* optical density was firstly adjusted to 0.3 and then diluted 10-fold.

2.4. Preparation of Acrylamide "Stock" Solution

Concentrated (100 mg/L) aqueous solution of acrylamide was sterilized by membrane filtration (pore φ = 0.22 μm; PES Millex-GP, Bionovo, Poland) and diluted (if needed) with sterile distilled water to obtain "stock" solutions of acrylamide (concentrations: 25.0, 50.0, and 75.0 mg/L).

2.5. The impact of AA concentration on microbial growth

The impact of acrylamide on tested microorganisms was determined in liquid cultures. Control samples were prepared by mixing 8 mL of MRD, 1 mL of microbial suspension (10^7 cfu/mL) and either 1 mL of sterile water (negative control, C_{neg}) or 1 mL of MRD (positive control, C_{pos}). Test samples contained 8 mL of MRD, 1 mL of microbial suspension (10^7 cfu/mL) and 1 mL of adequate AA "stock" solution so that the final concentration of acrylamide was 2.5, 5.0, 7.5 and 10.0 μg/mL. Therefore, it was possible to track how growing amount of carbon and nitrogen source influence the microbial growth (starting from the lower amount in negative control, through the samples with growing AA concentration up to positive control). Experimental variants were incubated at temperatures optimal for the growth of each tested strain, i.e. 28 °C (both yeast strains), 30 °C (*L. mesenteroides*), 37 °C (LA-5 and *S. thermophilus*). Separate sets of experimental variants were prepared for each time interval (0, 24 h and 48 h) for each microorganism. The number of microorganisms was enumerated by the spread plate method on MRS agar (for LAB) or YM agar (yeast), wherein 1 mL of 10-fold serially diluted samples were inoculated on the plates. Plates were incubated at optimal temperatures for 72 h and colonies were counted. The experiment was performed in 5 replicates.

2.6. Assessment of the Impact of pH and Temperature on Microbial Growth in the Presence of AA

Basing on the results of experiment described in section 2.5., where the presence of acrylamide seemed to stimulate the growth of *L. acidophilus* LA-5 (at 7.5 μg/mL) and *K. lactis* (at 10 μg/mL), these two microorganisms were selected for further studies. A set of tubes with MRD with appropriate AA concentration (causing the highest growth stimulation) was prepared and pH in the media was adjusted with 0.1 M HCl or 0.1 M Na_2CO_3 ranging from 2.0 to 9.0. Then 1 mL of microbial suspension (10^7 cfu/mL) prepared as described in section 2.3. was added to 9 mL of MRD with corrected pH, incubated and enumerated as above. Controls for each strain had constituted of 9 mL of MRD (without AA) adjusted to certain pH value inoculated with 1 mL of microbial suspension. They were prepared to exclude pH conditions that inhibited microbial growth. Then pH range 4-9 was selected for *K. lactis* and pH range 5-8 for *L. acidophilus* LA-5.

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After selecting the optimum pH for microbial growth, each strain was incubated at such pH and at three different temperatures: 4 °C (corresponding to the cooling conditions), 20 °C (room temperature) and 37 °C (the temperature of human body, e.g., in the human intestine). Enumeration of microorganisms was carried out as above. Each experiment was performed in 5 replicates.

2.7. Assessment of the Microorganism's Ability to Acrylamide Degradation

On the basis of the above experiments the microorganism which growth was stimulated by acrylamide had been chosen to check whether they are able to degrade acrylamide by amidase production (the ammonia released during such reaction can be detected by commercial kit). The experiments were carried out in MRD with the addition of acrylamide at concentration of 7.5 μg/mL for *L. acidophilus* LA-5 and 10.0 μg/mL for *K. lactis*. Medium was inoculated with 1 mL of microbial suspension prepared from 24-h culture (the final amount of microorganisms was 10^6 cells/mL) and incubated for 48 h at 37 °C (bacteria) or 28 °C (yeast). After 24 h and 48 h of incubation the medium was collected and the ammonia concentration was determined spectrophotometrically at 340 nm using Ammonia Assay Kit (cat. AA0100, Sigma Aldrich, St. Louis, USA). Control samples contained 9 mL of MRD without acrylamide inoculated with 1 mL of the same microbial suspension as test sample. The ammonia concentration in test samples was compared to the control. Each experiment was performed in 5 replicates.

2.8. Response Surface Methodology (RSM)

The Response Surface Methodology (RSM) can be used to evaluate the relative significance of several factors in the presence of complex interactions. In our study, RSM was carried out according to the procedure described by Lenth [18] and it was applied to evaluate the effects of 3 various parameters on acrylamide degradation rate measured as the concentration of ammonia released by amidase. The experimental design was based on Box-Behnken design matrix. Seven experimental runs were carried out and the experimental design, variables, coded and decoded levels, and responses are listed in Table 3 (section 3. Results and Discussion). We considered 3 variables that proved to be significant for the growth of *Lactobacillus acidophilus* LA-5 which were: pH, time and temperature. The variable that was measured as the outcome was the concentration of ammonia (μg/mL). Obtained model was prepared according to the equation (1), which expresses the relationship between the predicted response and independent variables in coded values:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j \quad (1)$$

where Y is the estimated response, β0 , βi , βii and βij are the regression coefficients for intercept, linearity, square and interaction, respectively, while Xi and Xj are the independent coded variables.

2.9. Basic Statistics and Principal Component Analysis (PCA)

All experiments described in the current paper were carried out in 5 replicates. The results are shown as arithmetic mean ± standard deviation (SD). The normality of distribution was assessed by Shapiro–Wilk test and one-way variance analysis (ANOVA) with Tukey's honest significant difference (HSD) posthoc test was used to compare mean values and determine the significance of differences. A p-value < 0.05 was considered statistically significant.

The Principal Component Analysis (PCA) with varimax rotation was applied to assess correlations among variables. All statistical analyses were carried out using R: A language and environment for statistical computing, version 3.5.0 (Foundation for Statistical Computing, Vienna, Austria, 2015). ANOVA was carried out using "lm" function and Tukey's test was done using "HSD.test" function in the "agricolae" package. PCA

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was carried out after each experiment for various acrylamide concentrations considering bacteria and yeast separately; pH and temperatures. We considered those variables as scores and numbers of microorganisms determined at particular time of collecting samples as loadings. The PCA was carried out in "psych" package [19]. Cortest-Bartlett test was carried out using "cortest.bartlett" function in "psych" package as well. The data demonstrated normal distribution so its transformation was not necessary. Strong correlations between loads and scores were considered when values obtained in correlation matrix exceeded 0.3 or –0.3. Based on the basic statistics and results obtained in the PCA we selected optimum conditions for continuing experiments.

3. Results and Discussion

3.1. The Impact of Various Acrylamide Concentrations on Microbial Growth

In the current paper we decided to verify whether yeast and/or bacteria, which naturally occur in food products could decompose acrylamide and therefore limits its negative impact on human body. Therefore we selected microorganisms which can naturally occur in fermented milk products (2 strains of LAB and 2 strains of yeasts) and one bacterial strain that demonstrate probiotic properties (*L. acidophilus* LA-5).

The lactic acid bacteria *S. thermophilus* did not growth in the MRD, the medium in which the carbon and nitrogen source are very limited (only 0.45% of bacteriological peptone), regardless of the absence or presence of AA. In turn, *L. mesenteroides* DSM 20343 showed weak growth in MRD, the number of cells decreased in all experimental variants (both with and without acrylamide) after 24 h and remained constant till 48 h (Table 1). Although differences between negative and positive control were not statistically significant, it can be seen that additional 1 mL of MRD (in positive control) enhanced the *L. mesenteroides* growth, suggesting that bacteria were ready to quickly utilize any additional source of carbon and nitrogen. This also means that not only MRD did not provide enough nutrients for the basal metabolism of *L. mesenteroides*, but also that AA was not used as an additional carbon/nitrogen source by these bacteria. Contrary to *L. mesenteroides*, the number of *L. acidophilus* LA-5 cells was increasing with the concentration of acrylamide, reaching the maximum at 7.5 μg of AA/mL and it was significantly higher than all other tested variants, including positive control (Table 1). Therefore, *L. acidophilus* LA-5 has been chosen for further experiments.

When *S. cerevisiae* DSM 70478 was considered, it was shown that after 24 h yeast number was equal between controls and experimental variants. This suggests that the presence of AA did not affect *S. cerevisiae* growth and indicates that the tested yeast are not able to assimilate AA. Moreover, the number of yeast cells decreased drastically after 48 h in all tested variants (to values below 3×10^4 cfu/mL), indicating that the majority of nutrients present in MRD have already been exhausted (Table 1) and cells started to die. This would also mean that the MRD was the only source of available nutrients required for tested strain. On the contrary, the growth of other tested yeast, *Kluyveromyces lactis* var. *lactis* DSM 70799, was enhanced when AA concentration in culture equaled 10 μg/mL and reached the values similar to those of positive control. This suggests that AA was utilized by *K. lactis* as additional source of carbon and nitrogen, so this strains was selected for further tests too.

Table 1. The impact of various acrylamide (AA) concentrations on the growth of selected bacteria and yeast (n=5)

Incubation time	C_{neg}	AA 2.5 μg/mL	AA 5.0 μg/mL	AA 7.5 μg/mL	AA 10 μg/mL	C_{pos}
***Leuconostoc mesenteroides* subsp. *cremoris* DSM 20346 [× 10^6 cfu/mL]**						
0 h	10.4 ± 11.4	18.0 ± 7.8	18.3 ± 8.8	8.8 ± 9.9	23.1 ± 11.0	19.7 ± 1.7

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	abc	ab	ab	abc	a	ab
24 h	1.2 ± 0.5 c	1.2 ± 0.3 c	1.5 ± 0.4 c	1.3 ± 0.6 c	2.1 ± 0.5 c	2.54 ± 0.8 c
48 h	1.4 ± 0.2 c	2.3 ± 0.7 c	1.9 ± 0.3 c	1.5 ± 0.5 c	2.3 ± 0.2 c	1.16 ± 0.6 c
***Lactobacillus acidophilus* LA-5 [× 10^6 cfu/mL]**						
0 h	2.6 ± 1.0 cd	2.4 ± 0.5 cd	2.9 ± 0.2 cd	1.62 ± 0.25 d	1.8 ± 0.7 d	2.0 ± 0.8 d
24 h	2.62 ± 1.3 cd	11.8 ± 3.2 bc	51.8 ± 5.4 ab	65.4 ± 47.0 a	7.2 ± 2.2 cd	32.6 ± 0.9 abc
48 h	6.0 ± 7.6 cd	1.0 ± 0.0 d	2.0 ± 0.7 d	46.3 ± 17.6 ab	19.6 ± 11.1 bc	2.6 ± 0.9 cd
***Saccharomyces cerevisiae* DSM 70478 [× 10^4 cfu/mL]**						
0 h	7.6 ± 1.3 ab	5.8 ± 1.0 ab	6.7 ± 1.2 ab	6.0 ± 1.3 ab	6.4 ± 1.3 ab	5.5 ± 1.3 ab
24 h	4.8 ± 4.9 ab	7.2 ± 2.4 ab	9.0 ± 2.6 a	8.4 ± 2.0 ab	7.2 ± 1.1 ab	9.8 ± 1.1 a
48 h	<3.0 ± 0.0 c	<3.0 ± 0.0 c	<3.0 ± 0.0 c	<3.0 ± 0.0 c	<3.0 ± 0.0 c	<3.0 ± 0.0 c
***Kluyveromyces lactis* var. *lactis* DSM 70799 [× 10^5 cfu/mL]**						
0 h	4.7 ± 1.4 b	3.7 ± 0.5 b	4.2 ± 0.7 b	2.8 ± 0.6 b	4.4 ± 1.3 b	4.6 ± 0.8 b
24 h	4.8 ± 0.8 b	5.0 ± 0.8 b	5.1 ± 1.4 b	4.64 ± 1.1 b	17.2 ± 8.2 a	14.2 ± 2.3 a
48 h	3.8 ± 1.3 b	6.4 ± 3.8 ab	3.8 ± 2.6 b	5.2 ± 4.2 b	9.0 ± 5.3 ab	9.6 ± 4.0 ab

The same letters below the standard deviations within the results for particular microorganism indicate the lack of statistical differences at $p < 0.05$.
C_{pos} – positive control (9 mL of Maximum Recovery Diluent inoculated with 1 mL microbial suspension), Cneg – negative control (8 mL of Maximum Recovery Diluent inoculated with 1 mL of microbial suspension and 1 mL of sterile water).
For DSM 70478 the number of yeast colonies after 48 h was <30 on the plate or they were not detected at all. So the results are presented as < 3.0 ± 0.0 (the lowest sample dilution spread on the plate with YM medium was 10^{-3}).

It was only possible to carry out PCA for the results that were obtained in the experiment investigating the responses of both tested yeast strains to various AA concentrations. In all other tested cases, the results of Cortest-Bartlett test did not allow to continue PCA. Based on PCA, in the case of *S. cerevisiae* and *K. lactis* we observed negative correlation between the number of cells of those strains determined at time "0"and after 24 h, respectively (Figure 1A, B and C).

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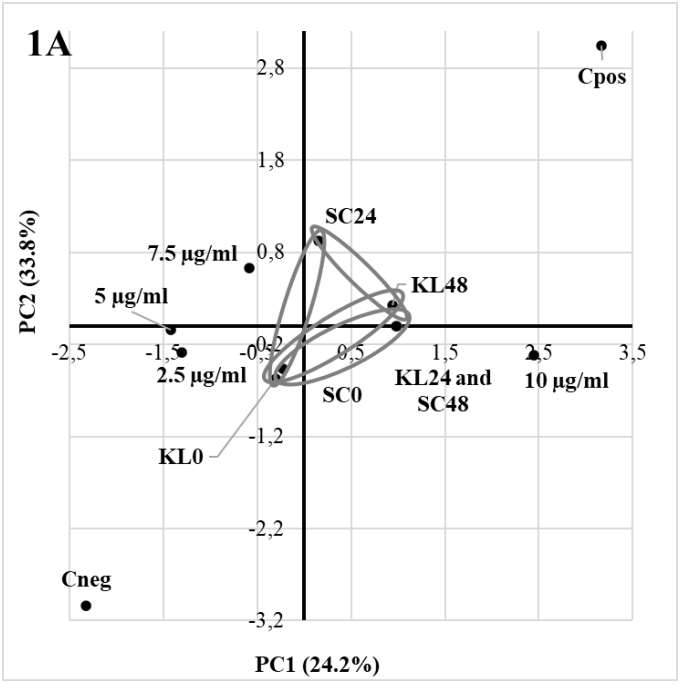

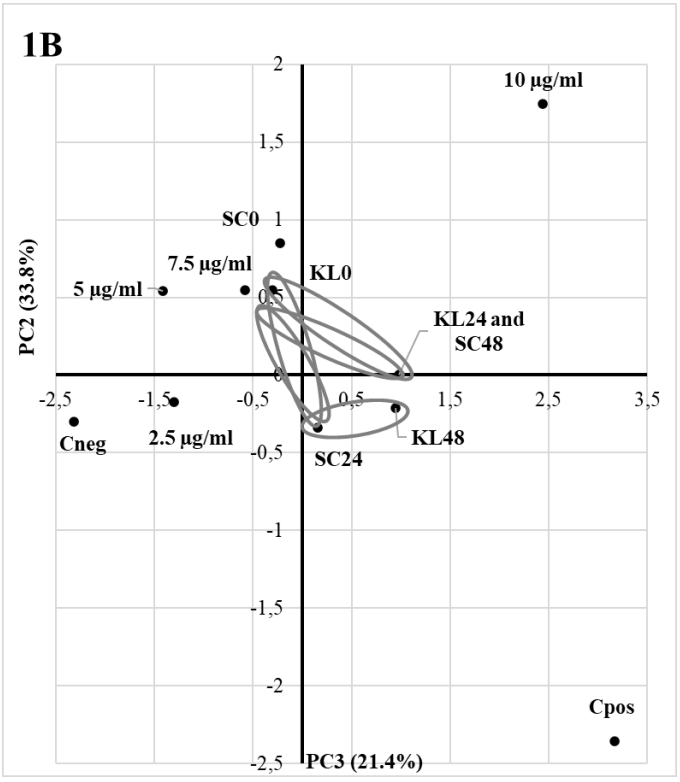

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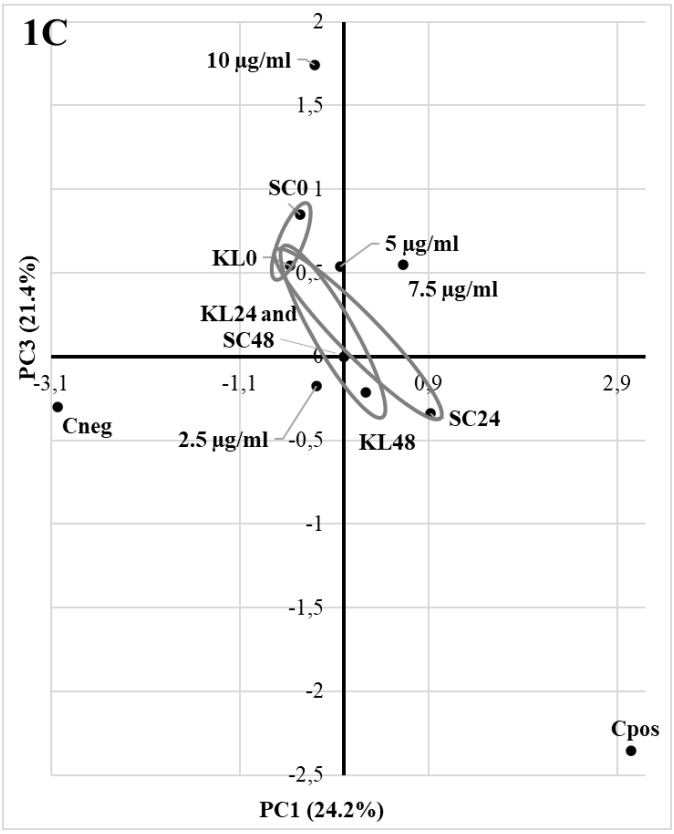

Figure 1. PCA analysis of ammonia released at different times and initial acrylamide (AA) concentrations (scores) under various yeast strains – *Saccharomyces cerevisiae* DSM 70478 and *Kluyveromyces lactis* var. *lactis* DSM 70799 (loadings) for the first three components (A, B and C); circles indicate correlated loads and scores. Cneg – negative control; Cpos – positive control; KL0 and SC0 – ammonia concentration at time 0; KL24 and SC24 – ammonia concentration at time 24 h; KL48 and SC48 – ammonia concentration at time 48 h.

It took place because the number of cells remained constant within 24 h for *S. cerevisiae*, while it increased for the latter yeast. Another negative correlation occurred for *K. lactis* after at time "0"and 48 h. It is that initially the number of fungi was the same in all variants, while it varied after 48 h depending on the AA concentration – it was greatest in positive control (C_{pos}) and when AA concentration was 10 µg of AA/mL (Table 1). We also noted positive correlations between cells concentration (cfu/mL) after 24 h and 48 h for *S. cerevisiae* – the decrease took place regardless AA dose. Another negative correlation (1C) indicated that AA concentration of 5 µg/mL was not sufficient to sustain *K. lactis* growth after 48 h.

3.2. The Impact of pH and Temperature on Microbial Growth in the Presence of Acrylamide

Basing on the results from first experiment, for further analysis describing the impact of pH and temperature on the microorganisms growth in the presence of AA only *Kluyveromyces lactis* var. *lactis* and *Lactobacillus acidophilus* LA-5 were selected.

Firstly, we chose pH range that occurs within human digestive tract (2-9) and verified how it affects microbial growth without the addition of AA. We noted that selected bacterium strain was not affected at pH from 5 to 8, while the growth of fungal strain was unaffected from pH 4 to 9 (data not shown). Due to those findings we continued experiments by adding acrylamide to the MRD adjusted to particular pH values. The acrylamide concentration that was used for this experiment was selected based on the results presented above (Table 1) as the AA concentration that most strongly stimulated the growth of the test microorganisms, i.e. 7.5 µg/mL for *L. acidophilus* and 10 µg/mL for *K. lactis*.

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We demonstrated that after 24 h at pH 4, 6 and 7 the presence of AA in medium did not affect yeast number, however, at pH 7 it was slightly higher (lack of statistical significance) in the variant that included AA than in control (Table 2). The growth of yeast at pH 8 and 9 was significantly reduced when compared to conditions closer to optimal. In turn, at pH 4 the presence of AA allowed maintaining similar number of cells after 24 and 48 h, while in control sample the yeast concentration decreased significantly after 48 h. At pH 5, the addition of AA caused slower fungal growth after 24 h, but after 48 h there were no statistically significant differences between control and MRD with AA (Table 2). When the growth of probiotic L. acidophilus was analyzed, the greatest growth at pH 6 in the presence of AA was demonstrated, especially after 48 h (Table 2). It must be highlighted that at all tested pH values the addition of acrylamide caused the increase of bacterial cells in comparison to controls.

Table 2. The impact of pH on the growth of *Kluyveromyces lactis* var. *lactis* and *Lactobacillus acidophilus* LA-5 in the presence of acrylamide (AA) (n=5)

***Lactobacillus acidophilus* LA-5 [× 10⁵ cfu/mL]** (incubation at 37°C, 7.5 μg AA/mL)							
Time	***Sample***	***pH 4***	***pH 5***	***pH 6***	***pH 7***	***pH 8***	***pH 9***
0 h	Control	NA	2.3 ± 0.5 e	2.5 ± 0.7 f	4.3 ± 0.9 e	3.7 ± 0.7 e	NA
	AA	NA	6.6 ± 1.1 d	8.0 ± 1.4 d	2.6 ± 0.2 f	3.7 ± 1.0 e	NA
24 h	Control	NA	9.1 ± 1.40 bc	6.3 ± 1.9 d	5.0 ± 0.5 e	< 0.01 i	NA
	AA	NA	9.2 ± 2.8 bc	12.1 ± 1.9 b	7.1 ± 0.9 d	1.3 ± 0.2 b	NA
48 h	Control	NA	0.6 ± 0.2 h	2.3 ± 0.4 f	1.0 ± 0.5 g	< 0.01 i	NA
	AA	NA	1.3 ± 0.2 g	>30 a	5.3 ± 1.3 d	9.5 ± 1.7 bc	NA
***Kluyveromyces lactis* var. *lactis* [× 10⁵ cfu/mL]** (incubation at 28°C, 10 μg AA/mL)							
Time	***Sample***	***pH 4***	***pH 5***	***pH 6***	***pH 7***	***pH 8***	***pH 9***
0 h	Control	6.6 ± 1.0 d	5.2 ± 0.6 d	6.4 ± 1.1 d	6.2 ± 1.1 d	5.3 ± 0.4 d	5.6 ± 1.0 d
	AA	7.3 ± 0.7 d	6.5 ± 1.0 d	8.3 ± 1.3 d	7.8 ± 1.4 d	6.5 ± 1.0 d	6.9 ± 0.7 d
24 h	Control	14.8 ± 4.6 a	12.8 ± 3.6 a	13.2 ± 2.7 a	13.6 ± 1.3 a	3.2 ± 0.9 e	0.7 ± 0.2 f
	AA	12.4 ± 2.3 a	9.4 ± 0.3 c	13.8 ± 1.9 a	18.0 ± 5.6 a	0.4 ± 0.2 f	2.9 ± 0.6 e
48 h	Control	7.5 ± 1.4 d	13.9 ± 0.3 a	11.2 ± 1.1 ab	12.1 ± 1.7 ab	5.4 ± 2.3 de	0.9 ± 0.2 f
	AA	16.3 ± 2.1 a	13.2 ± 1.8 a	12.7 ± 1.5 ab	10.9 ± 1.2 b	5.2 ± 0.7 de	0.7 ± 0.1 f

The same letters below the arithmetic means for each microorganism indicate the lack of statistical differences at $p < 0.05$. Abbreviations: AA – acrylamide present in medium at concentration of 10 μg/mL (yeast) or 7.5 μg/mL (bacteria); Control – control prepared by adding 1 mL of microbial suspension to 9 mL of MRD adjusted to adequate pH.; NA – not analyzed.

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For determining the impact of temperature on the growth of tested microorganisms we chose pH 7 for *K. lactis* var. *lactis* and pH 6 for probiotic bacteria. Three different temperatures were analyzed: 4 °C (representing the temperature of the storage of fermented milk beverages), 20 °C (the mean room temperature) and 37 °C (the temperature of human body). We demonstrated that the most optimal temperature for microbial growth was 20 °C for yeast and 37 °C for bacterium (Table 3), however the differences between culture with and without acrylamide were not statistically significant, except for test carried out at 37 °C after 48 h (Table 3). In both cases the growth was better in the AA presence.

Table 3. The impact of temperature on the growth of *Kluyveromyces lactis* var. *lactis* and *Lactobacillus acidophilus* LA-5 in the presence of acrylamide (AA) (n=5)

***Lactobacillus acidophilus* LA-5 [× 10⁵ cfu/mL]** (pH 6, 7.5 μg AA/mL)				
Time	***Sample***	***4 °C***	***20 °C***	***37 °C***
0 h	Control	4.0 ± 0.7 bc	4.8 ± 1.0 bc	3.7 ± 0.7 bc
	AA	3.9 ± 0.6 bc	4.8 ± 1.3 bc	4.5 ± 1.1 bc
24 h	Control	3.7 ± 0.6 bc	5.3 ± 0.8 b	4.8 ± 1.6 bc
	AA	3.5 ± 0.7 bc	4.9 ± 1.6 bc	6.3 ± 1.5 b
48 h	Control	3.3 ± 0.8 bc	1.9 ± 0.68 c	5.7 ± 0.6 b
	AA	2.8 ± 0.4 bc	2.6 ± 0.6 bc	17.0 ± 7.1 a
***Kluyveromyces lactis* var. *lactis* [× 10⁵ cfu/mL]** (pH 7, 10 μg AA/mL)				
Time	***Sample***	***4 °C***	***20 °C***	***37 °C***
0 h	Control	1.4 ± 0.1 c	1.6 ± 0.1 c	1.2 ± 0.2 c
	AA	1.5 ± 0.4 c	1.4 ± 0.3 c	1.4 ± 0.3 c
24 h	Control	1.8 ± 0.6 c	6.8 ± 1.4 b	0.8 ± 0.1 c
	AA	2.2 ± 0.3 c	7.1 ± 1.6 b	1.0 ± 0.1 c
48 h	Control	1.6 ± 0.3 c	21.7 ± 3.3 a	1.4 ± 0.4 c
	AA	1.9 ± 0.3 c	19.7 ± 0.9 a	8.2 ± 2.3 b

The same letters below the arithmetic means for each microorganism indicate the lack of statistical differences at $p < 0.05$. Abbreviations: AA – acrylamide present in medium at concentration of 10 μg/mL (yeast) or 7.5 μg/mL (bacteria); Control – control prepared by adding 1 mL of microbial suspension to 9 mL of MRD adjusted to adequate pH.

3.3. Acrylamide Degradation by Amidases Produced by Microorganisms

For this part of experiment we chose a probiotic strain *L. acidophilus* LA-5 and yeast *K. lactis* var. *lactis*. Our results have shown that only *L. acidophilus* LA-5 was able to degrade acrylamide and use it as a source of nitrogen and/or carbon. The ability to amidase synthesis by LA-5 was proved (Figure 2) by showing that content of ammonia (released

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from acrylamide by amidase) was slightly (but statistically significant) higher in medium inoculated with LA-5 (after 24 h) than in control medium without bacteria. In case of *K. lactis* var. *lactis* the ammonia concentration in samples with addition of 10 µg/l AA did not differ significantly from the control samples (data not shown).

There are several ways to reduce the acrylamide content in the food products. For example, the formation of acrylamide can be reduced by selecting the appropriate vegetable varieties (poor in AA precursors like asparagine and reducing sugars), by limiting the time and temperature during thermal processing, as well as by using the appropriate frying media or antioxidants [7, 20]. It is also possible to use the lactic fermentation before frying, e.g., *Lactobacillus plantarum* fermentation reduced the simple sugars content in potato rods, which allow to decrease the AA formation in French fries [21]. It was also reported that application of selected strains of LAB or the usage of enzymes that catalyze deamination of asparagine and glutamine (L-asparaginase and L-glutaminase, respectively) allowed for a significant decrease of AA formation [22–24]. Esfahani et al. [25] proved that the reduction of acrylamide content of whole-wheat breads can be obtained by combining lactobacilli and yeast in sourdough fermentation, because those microorganisms use AA precursors as the source of carbon and nitrogen. It has also been reported that acrylamide can be decomposed under the influence of microbial amidases resulting in the release of ammonia and acrylic acid, which might be then transformed in various pathways to β-hydroxypropionate, propionate, lactate or CO_2, however, the studies were focused on environmental bacteria species [26–28].

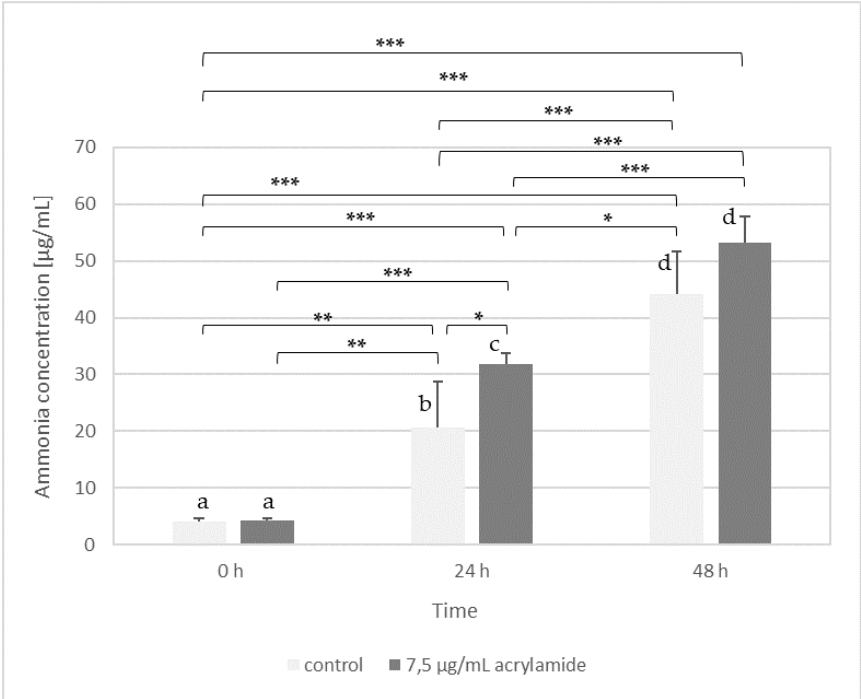

Figure 2. The ability to amidase synthesis by *L. acidophilus* LA-5 assessed by the measurement of ammonia concentration in medium without or with 7.5 µg/mL of acrylamide after 48 h of incubation with *L. acidophilus* LA-5 at 37 °C. The same letters next to means indicate the lack of statistical differences. Significance level: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

The ability to amidases synthesis has been already demonstrated in various bacteria, including LAB. For example, the synthesis of N-acetylmuramoyl-L-alanine amidase, involved in the degradation of peptidoglycan in the bacterial cell wall [29] was reported in *Lactobacillus sakei*, as well as the synthesis of N-acetylmuramidase with a key role in autolysis was demonstrated for *Lactobacillus bulgaricus* [30]. Amidases with activity of murein hydrolase are produced by staphylococci and are necessary for the generation of

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the equatorial ring on the cell surface and complete cell division and separation [31]. Similarly, the division of *Escherichia coli* depends on the activity of amidases AmiA, AmiB and AmiC [15]. However, there are no published papers demonstrating that amidases produced by lactic acid bacteria can utilize acrylamide as a substrate for deamination. Our results for the first time report that probiotic strain *Lactobacillus acidophilus* LA-5 has the ability to degrade acrylamide and then used the ammonia released by amidase as a source of nitrogen, while the rest of molecule (acrylic acid or its metabolites) as a carbon source.

Our studies revealed therefore that some LAB strains can exert beneficial impact on human organisms after intestine colonization, not only by their well-known impact on digestion and maintenance of proper gastrointestinal balance, but also by acrylamide degradation and preventing against its harmful impact on human body.

3.4. Response Surface Methodology

Since we demonstrated that only *Lactobacillus acidophilus* LA-5 was a putative amidase producer, that strain was selected for the experiments involving RSM. We considered variables that significantly influenced the growth of mentioned bacterium in the presence of acrylamide: pH (X_1), temperature (X_2) and time (X_3). The response in the model was ammonia concentration (μg/mL) measured at particular experimental time. For the first run of experiments for central point we decided to apply the following values: pH 6.0, temperature 20 °C and time 24 h (Table 4). As increments we selected the following values: 1 for pH, 17 for temperature and 24 for time. We demonstrated that only first order model explains investigated phenomenon with satisfactory reliability (R^2 = 0.9807 and adjusted R^2 = 0.9663). After the first and second run of experiments we have moved center points to: pH 6.78, temperature 33 °C, and time 48 h. This operation caused significant decrease in R^2 and adjusted R^2 (0.02244 and -0.7107, respectively for experimental run 3; 0.02441 and –0.3303, respectively for experimental run 4, Table 4), therefore we concluded that first order model could be reliable only in the range of pH 6 to 7, temperature 20 to 37 °C and time 24 to 48 h. When we tested if the data could fit to the second order model it was proved to fail, because R^2 and adjusted R^2 were too low (0.6914 and 0.4084, respectively for experimental run 5, Table 4). Those figures did not improve when we changed central points to: pH 6.67, temperature 34.7 °C, and time 75 h.

Table 4. The summary of Response Surface Methodology carried out against *Lactobacillus acidophilus* LA-5 to examine its ability to produce ammonia in the presence of acrylamide. The table present Box-Behnken design matrix and response values.

Experimental run	Coded variables			Actual variables			Ammonia conc. [μg/mL]
	X_1	X_2	X_3	pH	Temperature [°C]	Time [h]	
1	-1	1	-1	5	37	0	0
	1	-1	-1	7	3	0	0
	0.0	0	0	6	20	24	10.20
	-1	-1	1	5	3	48	4.90
	0	0	0	6	20	24	7.80
	1	1	1	7	37	48	38.60
	0	0	0	6	20	24	6.70
	0	0	0	6	20	24	8.20
2	1.50	1.57	2.02	7.57	46.64	72.53	1.00
	1.05	1.05	1.35	7.05	37.77	56.35	16.25
	0.78	0.78	1.01	6.78	33.31	48.26	11.48
	0.52	0.52	0.67	6.52	28.87	40.18	3.66
	1.30	1.31	1.69	7.31	42.20	64.44	2.16
	0.26	0.26	0.34	6.26	24.44	32.09	2.45

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3	1.61	1.68	2.01	7.78	50	72	4.05
	1.40	1.11	1.34	6.78	33	48	13.36
	1.61	0.54	0.67	7.78	16	24	4.24
	1.40	1.11	1.34	6.78	33	48	13.18
	1.20	0.54	2.01	5.78	16	72	5.98
	1.20	1.68	0.67	5.78	50	24	5.79
	1.40	1.11	1.34	6.78	33	48	13.18
	1.40	1.11	1.34	6.78	33	48	13.01
4	1.20	0.54	0.67	5.78	16	24	4.33
	1.61	1.68	0.67	7.78	50	24	3.88
	1.40	1.11	1.34	6.78	33	48	15.05
	1.61	0.54	2.01	7.78	16	72	4.65
	1.20	1.68	2.01	5.78	50	72	6.26
	1.40	1.11	1.34	6.78	33	48	15.49
	1.40	1.11	1.34	6.78	33	48	15.49
	1.40	1.11	1.34	6.78	33	48	15.94
5	1.40	1.11	2.28	6.78	33.00	81.94	29.17
	1.11	1.11	1.34	5.37	33.00	48.00	10.50
	1.70	1.11	1.34	8.19	33.00	48.00	6.82
	1.40	1.11	1.34	6.78	33.00	48.00	10.43
	1.40	1.92	1.34	6.78	57.04	48.00	9.78
	1.40	1.11	0.39	6.78	33.00	14.06	2.14
	1.40	1.11	1.34	6.78	33.00	48.00	19.61
	1.40	0.30	1.34	6.78	8.96	48.00	4.63
6	-0.137	0.04	1.708	6.643	33.68	88.992	27.1
	-0.078	0.041	0.565	6.702	33.697	61.56	26.67
	-0.126	0.043	1.422	6.654	33.731	82.128	27.65
	-0.158	0.032	2.28	6.622	33.544	102.72	26.75
	-0.148	0.036	1.994	6.632	33.612	95.856	25.11
	-0.113	0.044	1.136	6.667	33.748	75.264	29.05
	-0.097	0.044	0.85	6.683	33.748	68.4	27.5
7	1.17	0.59	1.42	5.67	17.7	51	8.56
	1.59	1.74	2.76	7.67	51.7	99	9.65
	1.17	1.74	2.76	5.67	51.7	99	7.70
	1.59	0.59	2.76	7.67	17.7	99	18.23
	1.17	0.59	2.76	5.67	17.7	99	8.51
	1.17	1.74	1.42	5.67	51.7	51	10.06
	1.59	0.59	1.42	7.67	17.7	51	14.11
	1.59	1.74	1.42	7.67	51.7	51	5.47
	1.38	1.17	2.09	6.67	34.7	75	24.56
	1.38	1.17	0.75	6.67	34.7	27	19.62
	1.38	1.17	3.43	6.67	34.7	123	27.51
	1.38	1.17	2.09	6.67	34.7	75	12.08
	1.38	0.02	2.09	6.67	0.7	75	1.95
	1.80	1.17	2.09	8.67	34.7	75	27.55
	0.97	1.17	2.09	4.67	34.7	75	11.63
	1.38	2.31	2.09	6.67	68.7	75	8.47

When we tested cube blocks of the model we obtained R^2 = 0.8224 and adjusted R^2 = 0.4672. That improvement was not sufficient to provide reliable results. Moreover, based

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on the analysis of that model, center points were: pH = 3.25, temperature = 52.98 °C, and time = 107.2 h. Those figures could lead to false conclusions because at those conditions, bacterial cells could be disintegrated which could lead to the release of significant ammonia amount. Due to this fact we did not present contour plots (response surface plots) in the current paper.

Therefore, the final version of the model includes only first order and it is:

$$Y = 9.55 + 1.204 \cdot X_1 + 0.254 \cdot X_2 + 0.227 \cdot X_3 \quad (2)$$

At the end, the validation of the model built with RSM was carried out within pH 6 – 7, temperature 20 to 37 °C and time 24 to 48 h (Table 5).

Table 5. Experimental variants used for the validation of the model.

pH	Temperature [°C]	Time [h]	Predicted values [μg/mL]	Experimental values [μg/mL]
6.1	33	48	36.17	32.27
6.7	37	48	37.91	29.87
6.5	30	24	30.44	33.90
6.5	20	24	27.90	30.29
6	28	48	34.78	36.35
7	20	24	28.51	31.76
6	30	24	29.84	32.92
6.7	28.9	40	34.04	33.90
6.3	30	48	35.65	33.68
6.1	37	24	31.74	32.62

Predicted and experimental values refer to ammonia concentrations

The obtained value of regression coefficient ($R^2 = 0.9885$) between predicted values and experimental values of ammonia concentration (Figure 3) had confirmed that equation (2) is well suited.

4. Conclusions

Acrylamide present in food product can exert both positive and negative impact on intestinal microbiota. Fortunately, some bacteria strains, including beneficial LAB, are resistant to the presence of this toxic substance, and what's more important, they can use AA as an additional source of carbon and nitrogen in a situation where the environment (medium) lacks them. To the best of our knowledge, we showed for the first time, that probiotic strain of bacteria *Lactobacillus acidophilus* LA-5, often added to yogurts by food producers, has the ability to degrade acrylamide and used the ammonia released by amidase as a source of nitrogen, while the rest of molecule (acrylic acid or its metabolites) as a carbon source. The analysis of variance and RSM indicated that pH as well as incubation time and temperature significantly influenced the amount of ammonia released from acrylamide by bacteria.

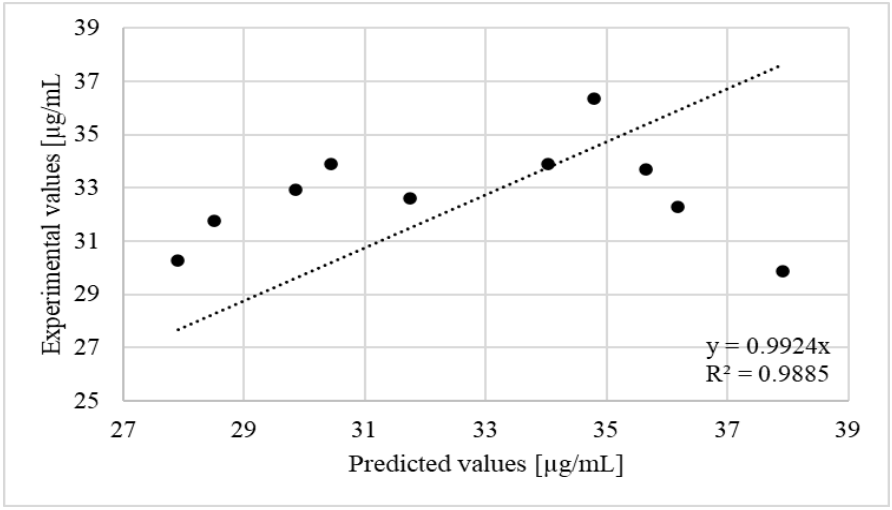

Figure 3. Validation of the model describing ammonia release built with RSM – regression analysis of experimental and predicted values.

Our studies suggest that some strains of bacteria present in milk fermented products can exert beneficial impact on human organisms after intestine colonization, not only by their well-known impact on digestion and maintenance of proper gastrointestinal balance, but also by acrylamide degradation and preventing against its harmful impact on human body and other members of intestinal microbiota.

Further studies are required to show whether the ability of acrylamide degradation is carried out by intestinal bacteria in the colon milieu, where the concentration of nutrients is high. Moreover, the impact of acrylamide on the metabolism pathways of lactic acid bacteria should be also checked.

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