

CHANGES OF THE SELECTED PROPERTIES OF *LACTOBACILLUS PLANTARUM* ATCC 4080 DURING STORAGE OF MALT BEVERAGE

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Abstract. It is possible to obtain malt beverage, which includes high number of viable lactic acid bacteria and has a good sensor quality for eight weeks of storage at the temperature of 22°C. *L. plantarum* ATCC 4080 strain after four weeks of storage did not reveal antagonistic activity against spoilage and pathogenic bacteria. This strain after two, six and eight weeks of storage had antagonistic properties. The tested strain after two and four weeks of storage did not survive during incubation at pH 2.5 and next in malt beverage with 3 mmol/dm³ deoxycholate sodium, while survived in these conditions after six and eight weeks. In case of incubation at pH 2.5 and next in aqueous solution of deoxycholate sodium tested strain after four and six weeks of storage had survival ability. The survival ability in these conditions of the tested strain after two and eight weeks of storage were not investigated.

Key words: lactic acid bacteria, malt beverage, probiotics, antagonism activity, low pH, deoxycholate sodium

INTRODUCTION

Probiotics are defined as live microorganisms which when are administered in adequate amounts confer a health benefit on the host [FAO/WHO 2002]. Probiotic foods and specimens include usually strains of lactic acid bacteria of genera *Lactobacillus* and *Bifidobacterium*. In human organism not all lactic acid bacteria cause the same effect improvement of health, because not all have probiotic properties. Criteria which are used for selection of probiotic strains are updated permanently. In 2002 a Working Group was convened by FAO/WHO to generate guidelines and recommended criteria for probiotic strain. This strain should be characterized by, among other things, antagonistic activity against potentially pathogenic bacteria and resistance to gastric acidity and bile acid resistance.

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What is more some people suffer from lactose intolerance and allergy to milk protein [Bielecka 1998, Pełczyńska 1997]. People who suffer from this affection should eat vegetable products or fermented dairy products with viable lactic acid bacteria. These bacteria release β -galactosidase during transit through alimentary tract. β -galactosidase is responsible for the hydrolyze of lactose. People who suffer from allergy to milk protein must eliminate from diet dairy products. Kvass is popular as beverage which slakes one's thirst in Russia and in the countries of the former Soviet Union. Kvass is a product of alcoholic and lactic acid fermentation of aqueous extracts from cereal products (bread, rye and barley malt, rye flour) [Pietschmann et al. 2000, Šatnjuk and Spiričev 2002]. This beverage has a refreshing sweet-sour taste, dark brown colour and rye bread flavour. Qualities of used raw materials and compounds which form during alcoholic and lactic acid fermentation (melanoidins, lactic acid, acetic acid, carbon dioxide, volatile compounds for example esters, aldehydes) form organoleptic qualities of kvass. Kvass includes also amino acids, vitamin of group B (B_1 , B_2), vitamin PP and others [Kretov and Antipov 1997].

The aim of the investigation was to determine the influence of time of storage of malt beverage on antagonistic activity strain that was used for production this beverage. We tried to determine also the influence of time of storage of malt beverage on survival ability of this strain at pH 2.5 and next in deoxycholate sodium solution.

MATERIALS AND METHODS

Strain *Lactobacillus plantarum* ATCC 4080 (American Type Culture Collection) was used for preparation starter culture and malt beverage.

In the research we used indicator microorganisms:

- *Proteus mirabilis* 180 (Wojewódzka Stacja Sanitarno-Epidemiologiczna, Warszawa),
- *Citrobacter freundii* 488 (Wojewódzka Stacja Sanitarno-Epidemiologiczna, Warszawa),
- *Staphylococcus epidermidis* ATCC 12228 (American Type Culture Collection),
- *Bacillus megaterium* (Państwowy Zakład Higieny, Warszawa),
- *Enterococcus faecalis* ATCC 29212 (American Type Culture Collection).

For preparation of malt beverage, starter culture and 3 mmol/dm³ solution of deoxycholate sodium in malt used malt wort. Malt wort obtained by dissolution (in ratio used in initial tests) of the following components: industrial malt concentrate about 70.0% of extract (produced by Wytwórnia Ekstraktu Słodowego Wolsztyn), consumption sugar, caramel and water.

Caramel obtained by slow heating of consumption sugar with constant mixing till moment of start boiling. Next water was added in the quantity identical as the quantity of the used sugar. Caramel was cooled and stored at 4°C.

Experiment course

Initially malt wort was soured by citric acid to pH 4.15 to limit possibilities of spore-forming bacteria growth then poured into dark bottles (4/5 of capacity), closed with cotton wool closure and pasteurized at temperature 80°C for 15 minutes. Bottles were cooled and inoculated with starter culture of *Lactobacillus plantarum* ATCC 4080 in

amount 5 cm³. Starter culture was prepared for incubation (48 hours at 28°C) of this strain in sterile (20 min at 117°C) malt wort. Bottles were closed with crown closure and stored at 22 ± 1°C for period of 12 weeks. Four series of experiments, each in two repetitions, were conducted.

During storage of beverage was determined antagonistic activity of lactic acid bacteria and their survival ability during complex incubation that is at low pH and next in solution of deoxycholate sodium. Also a number of lactic acid bacteria, total acidity and pH were determined. The sensory analysis of the product was also carried out.

Determination of antagonistic activity lactic acid bacteria isolated from malt beverage. Samples of beverage were taken after two, four, six and eight weeks of storage and antagonistic activity of lactic acid bacteria against selected indicator microorganisms was determined. Well diffusion assay was used in this study [Kraszewska et al. 2005 b], but 0.1 cm³ of malt beverage was poured into the well. Three series of experiments, each in two repetitions were conducted.

Determination of the influence of time of storage of malt beverage on survival ability of lactic acid bacteria at pH 2.5 and next in 3 mmol/dm³ solution of deoxycholate sodium. The aim of this experiment was to determine the influence of time of storage of malt beverage on ability transit barrier of stomach and small intestine for bacteria. Survival ability of lactic acid bacteria was determined in malt wort (pH 2.5) and next in aqueous 3 mmol/dm³ solution of deoxycholate sodium. In parallel this survival was determined also in wort (pH 2.5) and next in malt wort with 3 mmol/dm³ deoxycholate sodium. Aqueous solution of deoxycholate sodium and malt wort with deoxycholate sodium was neutralized to pH 8.0 and was sterilized at temperature 117°C for 20 minutes.

In the case of bacteria incubated at pH 2.5 and in malt wort with deoxycholate sodium, samples for studies were taken in week "0" and after two, four, six and eight weeks of storage of beverage. In the case of incubation of bacteria at pH 2.5 and in aqueous solution of deoxycholate sodium the samples were taken in week "0" and after four and six weeks of storage. From the stored beverage was taken 45 cm³, acidified with 1 mol/dm³ HCl to pH 2.5, determined number of lactic acid bacteria (time "0") and next sample was incubated for four hours at the temperature of 37°C. After this time the number of lactic acid bacteria was determined. Later samples were neutralized with 1 mol/dm³ NaOH to pH 8.0, transmitted 1 cm³ of neutralized sample to 9 cm³ aqueous solution of deoxycholate sodium or to 9 cm³ solution of malt wort with deoxycholate sodium. The number of lactic acid bacteria was determined, and next samples were incubated at the temperature of 37°C for 24 hours. After 3 and 24 hours of incubation in solution of deoxycholate sodium the number of lactic acid bacteria was determined. Three series of experiments, each in two repetitions were conducted.

Analytic methods

The number of lactic acid bacteria was determined by applying a plate method and MRS medium with 1.5% agar. Plates were carried out for 48 hours at the temperature of 28°C in an atmosphere containing 5% CO₂ (v/v) [Drago et al. 1997, Kraszewska et al. 2005 a]. The result was given in cfu/cm³.

Value pH was determined in the glass electrode a calomel electrode system [AOAC 1995].

Total acidity was determined by potentiometric titration of sample by using 0.1 mol/dm³ NaOH to pH 8.0 after previous degassing [AOAC 1995]. The result was given in grams calculated as lactic acid in 100 cm³ of the beverage.

The sensory analysis was carried out by a panel of five people in accordance with the principle of sensory analysis by applying five-scores scale of nine quality levels. The colour, smell and taste were estimated. Overall quality was calculated as weighed average factor coefficient (coefficient ponderability): colour – 1, smell – 2, taste – 6 [Baryłko-Pikielna 1975].

Statistic analysis

Majority results were elaborated statistically by means of Statgraphics Plus Ver. 4.1 program with application of Multifactor Anova ($\alpha = 0.05$) and NIR calculation according to Tuckey's (as HSD – Honestly Significant Differences).

RESULTS AND DISCUSSION

The important technological characteristic of the probiotic product is a minimal number of bacteria cells, which a particular product should contain. Because in the available literature there is a divergence of opinions concerning this number (10^5 - 10^9 in cm³) the value 10^6 cells/cm³ was accepted that is selected by most often.

The average number of lactic acid bacteria directly after inoculation of bacteria amounted to about $3.24 \cdot 10^6$ cfu/cm³ (Fig. 1). After one week of storage a significant growth in the number of bacteria cells ($2.39 \cdot 10^8$ cfu/cm³) was observed in relation to the number directly after inoculation of bacteria (week "0"). In the following weeks of storage gradual drop in the number of cells was noted and distinctions between four and seven weeks were contained within the limit of error. During eight weeks of storage malt beverages contained the number of lactic acid bacteria at a higher level than minimum which is required for probiotic products. After 12 weeks this number was a little lower and averaged $7.15 \cdot 10^5$ cfu/cm³, but between eight and twelve weeks of storage the number of bacteria cells was not determined.

Sensory quality was determined also during the period of storage of beverages (Fig. 1). After 1 week of storage significant improvement of sensory quality of beverages (3.9 pkt) was observed in comparison with products directly after inoculation of bacteria (3.4 pkt). In the following weeks (until week 6) a further gradual growth of sensory quality of beverages (overall quality – 4.1 pkt) was noted and next a gradual decrease of quality was observed. Distinction between seven and eight weeks was contained within the limit of error. After the storage period overall quality of beverages was 3.7 pkt.

It was stated that malt beverages containing strain *L. plantarum* ATCC 4080 for 8 weeks of storage at room temperature (22°C) were characterized by a good sensor quality and high number of lactic acid bacteria ($> 10^6$ cfu/cm³).

Lactic acid bacteria produce a lot of metabolites among other things lactic acid and acetic acid that decrease pH environment. The influence of time of storage on pH value of malt beverage was shown on Figure 2. Initial pH value of malt beverage amounted to 4.15 and after one week of storage dropped to 3.18. In the following weeks of storage

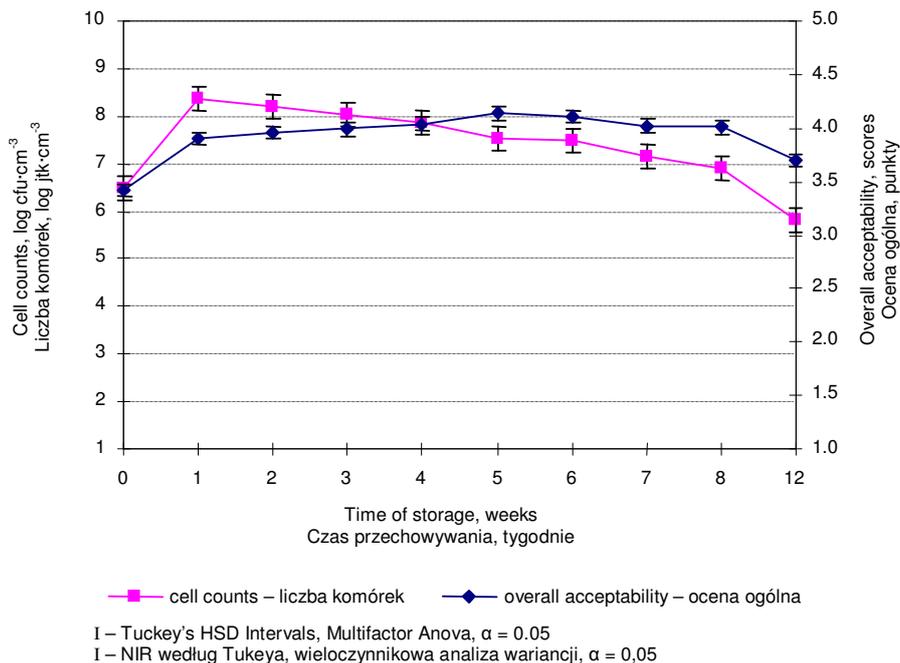


Fig. 1. The influence of time of storage of malt beverage on the survival ability of lactic acid bacteria and sensory analysis of product (mean from four series)

Rys. 1. Wpływ czasu przechowywania napoju słodowego na przeżywalność bakterii mlekowych oraz ocenę sensoryczną produktu (średnia z czterech serii)

gradual and slow decrease in pH value was observed. This further slow drop in pH value could be caused by the inhibition of bacterial cells metabolic activity. After 12 weeks of storage the average pH value amounted to 2.91.

During storage the pH value of malt beverages decreased to pH below 3.0 but despite this low pH products were estimated sufficient high. According to members of panel high acidity of beverages gave the products refreshing properties.

Changes of total acidity of beverages during period of storage were shown on the Figure 2.

Directly after inoculation of lactic acid bacteria total acidity of beverage amounted to average 0.06 g/100 cm³. After one week of storage an increase of total acidity to 0.32 g/100 cm³ was observed. During the following period of storage this increase was slow and distinctions between four and twelve weeks were contained within the limit of error. After the storage period was over total acidity was 0.56 g/100 cm³.

It should be pointed out that in the case of the biggest increase of total acidity simultaneously the biggest decreased was stated of pH value of beverage and also biggest increase of the number of lactic acid bacteria.

Wojtatowicz and Chrzanowska [1998] communicated, that bacteria of genus *Lactococcus* can decrease pH of dairy products to pH 4.5, while bacteria of genus *Lactobacillus* which participate in souring vegetables sour the environment to value pH 2.8-3.0.

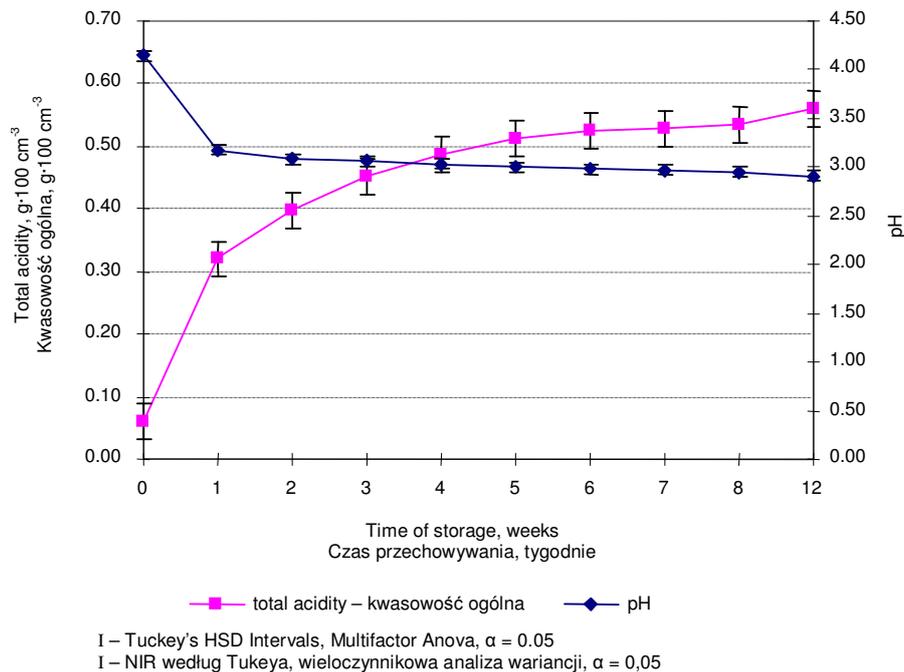


Fig. 2. Value pH and total acidity of malt beverage during period of storage (mean from four series)

Rys. 2. Wartość pH oraz kwasowość ogólna napoju słodowego podczas jego przechowywania (średnia z czterech serii)

Also Hutkins and Nannen [1993] think that growth of strains belonging to genus *Lactobacillus* in medium rich in nutrient components can cause decrease of pH to value 3.0.

Pietschmann et al. [2000] determined value pH of bread beverage ("Brottrunk") and beverages which were fermented on the basis wheat and barley malt ("Weiznmalzgetränk"). The authors stated that pH value of beverage obtained on the basis of malts was 3.1 and in the case of bread beverage – 3.0. It was observed that while the time of incubation goes value of pH of kvass ("Kwas Getränkepulver") decreased; after 1 hour averaged 4.3, after 1 day – 3.6 and after 2 days – 3.2.

Hutkins and Nannen [1993] stated that growth of lactic acid bacteria continues as long as carbohydrates, amino acids and other nutrients are available; toxic compounds (such as hydrogen peroxide) are removed, and the hydrogen ion concentration is maintained above the level that a specific strain can tolerate. The authors write that low pH connected with production of lactic acid is frequently growth limiting for lactic acid bacteria that are grown in milk or in weakly buffered bacteriological media.

In the earliest studies [Kraszewska et al. 2005 b] antagonistic activity was determined among other things strain *L. plantarum* ATCC 4080 against selected spoilage and pathogenic bacteria but then antagonistic properties were not examined of lactic acid bacteria in dependence on time of storage beverage. This experiment aimed at checking if during the period of storage of product the changes of this activity took place (Fig. 3).

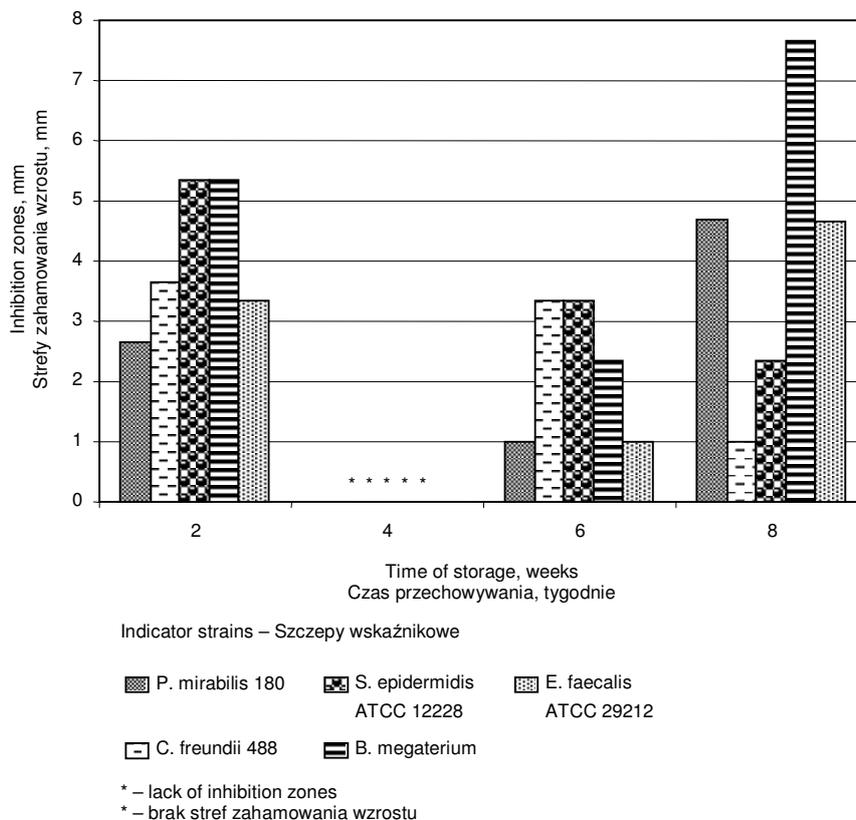


Fig. 3. The influence of time of storage of malt beverage on the antagonistic activity of *L. plantarum* ATCC 4080 (mean from three series)

Rys. 3. Wpływ czasu przechowywania napoju słodowego na aktywność antagonistyczną *L. plantarum* ATCC 4080 (średnia z trzech serii)

In beverages stored for four weeks lack of antagonistic activity of used strain was noted while in residual date of determination (two, six and eight weeks) these bacteria had this activity.

The explanation of this phenomenon requires carrying out a further research. During the period of storage of product, the observed size of inhibition zones for particular indicator bacteria underwent changes. The biggest inhibition zones of strain *P. mirabilis* (4.7 mm), *B. megaterium* (7.7 mm) and *E. faecalis* (4.7 mm) were observed for antagonistic bacteria taken after eight weeks of storage of beverages. For strain taken after two weeks the biggest inhibition zones were noted in the case of *S. epidermidis* ATCC 12228 (5.3 mm) and *B. megaterium* (5.3 mm).

It was stated that application of strain *L. plantarum* ATCC 4080 for production of malt beverage is advisable although antagonistic properties of this strain change during the storage of the product. According to the literature data [Klewicka and Libudzisz 1998, Klewicka et al. 1999], the ability of lactic acid bacteria for production of com-

pounds with antagonistic activity depends on external factors such as composition of medium, pH of environment, temperature or time of incubation.

Earlier it was noticed [Kraszewska et al. 2005 a] that all from six tested strains which belong to the species *L. plantarum* survived both in environment with low pH and in the presence of used bile salt. This fact suggest possibility for using them in production of malt beverage.

Also the influence of time of storage of malt beverage on survival of lactic acid bacteria at low pH and next in solution of deoxycholate sodium was determined.

Aqueous solution of deoxycholate sodium was used in the studies to which bacterial culture incubated in malt wort was added. It caused dilution of wort in ratio 1:9. This way of procedure was applied because the variety of food which is consumed is big and their concentrations in alimentary tract gradually decrease. In the small intestine, food is digested to simple compounds which are absorbed in this part of tract into blood and lymph. Besides, glands which are in mucous membrane of small intestine produce digestive juice (3-6 dm³ within a day). This juice causes dilution of contents of the alimentary tract. Malt wort with deoxycholate sodium was used parallelly in order to compare the survival ability of lactic acid bacteria in the environment of bile salts containing bigger amount of nutrient components towards aqueous solution.

In work 4 hours incubation of lactic acid bacteria in the environment with pH 2.5 was applied because food remains in the stomach usually 2-4 hours (depending what kind of meal) [Huang and Adams 2004]. Owing to the fact that food after transit through the small intestine (about 1-4 hours) gets to the large intestine where it usually stays for 8-12 hours, the number of lactic acid bacteria was determined after 3 and 24 hours of incubation in solution of deoxycholate sodium. Łaniewska-Moroz et al. [1996] and Vinderola and Reinheimer [2003] determined survival ability of lactic acid bacteria in presence of bile salts also after 24 hours of incubation.

At the beginning of incubation at pH 2.5 (week "0", time "0") the number of bacteria cells of *L. plantarum* ATCC 4080 amounted to $3.11 \cdot 10^6$ cfu/cm³ (Fig. 4 and 5). During the period of storage of beverages, changes of survival ability of lactic acid bacteria at complex incubation (at pH 2.5 and next in malt wort with deoxycholate sodium) were observed (Fig. 4). After 4 hours of incubation at pH 2.5 decrease of the number of bacteria cells (10^3 - 10^5 cfu/cm³) was stated in relation to time "0". After two and four weeks of storage and 3 and 24 hours of incubation in bile salt did not determine viable lactic acid bacteria. This phenomenon repeated in the following series and it is difficult to explain unequivocally. Bacteria taken after six and eight weeks of storage beverage survived (10^2 - 10^4 cfu/cm³) during 24 hours of incubation in wort with deoxycholate sodium.

According to Begley et al. [2005] pre-exposure of bacteria to one stress may also confer protection of this bacteria against other stresses. The author informed that adaptation strain belonging to species *E. faecalis* to heat (50°C), high concentration of sodium chloride (6.5%) or alkaline pH (10.5) confer an increase survival ability of the tested strain during incubation in presence of high concentrations of bile salts. Incubation of strain *L. monocytogenes* LO28 at low pH (5.5), heat (42°C) or sodium chloride (5%) cause increase of bile salts tolerance [Begley et al. 2005]. Adaptation of strain belonging to species *Vibrio parahaemolyticus* to acidic or alkaline environment induced increase of deoxycholic acid tolerance [Koga et al. 2002].

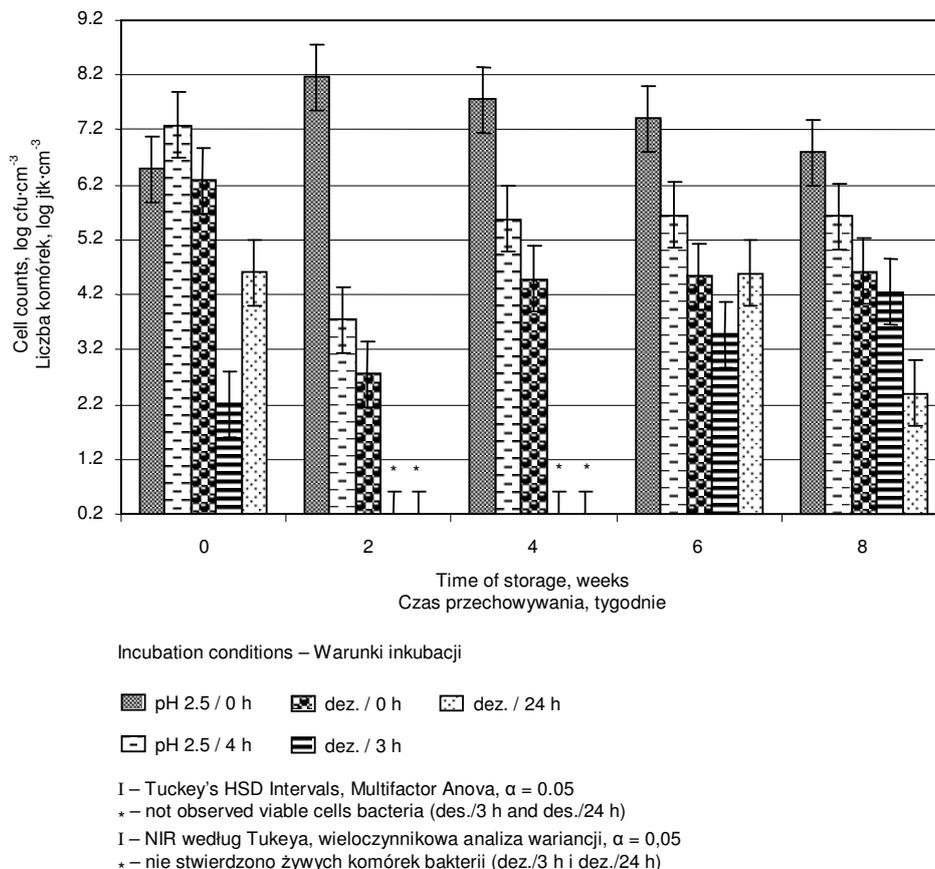


Fig. 4. The influence of time of storage of malt beverage on the survival ability *L. plantarum* ATCC 4080 at pH 2.5 and next in malt wort with deoxycholate sodium (mean from three series)
 Rys. 4. Wpływ czasu przechowywania napoju słodowego na przeżywalność *L. plantarum* ATCC 4080 kolejno w pH 2,5 oraz w brzeczce słodowej zawierającej dezoksycholalan sodu (średnia z trzech serii)

It should be pointed out that decrease in the value of pH and increase of total acidity of product was observed during the period of storage of beverages. It is possible that this fact influenced the adaptation of the tested strain to acidic environment and in consequence during complex incubation, better survival ability of bacteria taken after six and eight weeks of storage of beverage was observed in relation to microorganisms which were taken after two and four weeks. Besides in the case of samples of beverages taken after six weeks of storage a significant growth in the number of bacteria cells ($5.05 \cdot 10^4$ cfu/cm³) was noted after 24 hours of incubation (in the presence of deoxycholate sodium) in relation to incubation that lasted 3 hours ($1.57 \cdot 10^2$ cfu/cm³). We can presume that these bacteria adapted for tested conditions and thanks to this bacteria can still multiply.

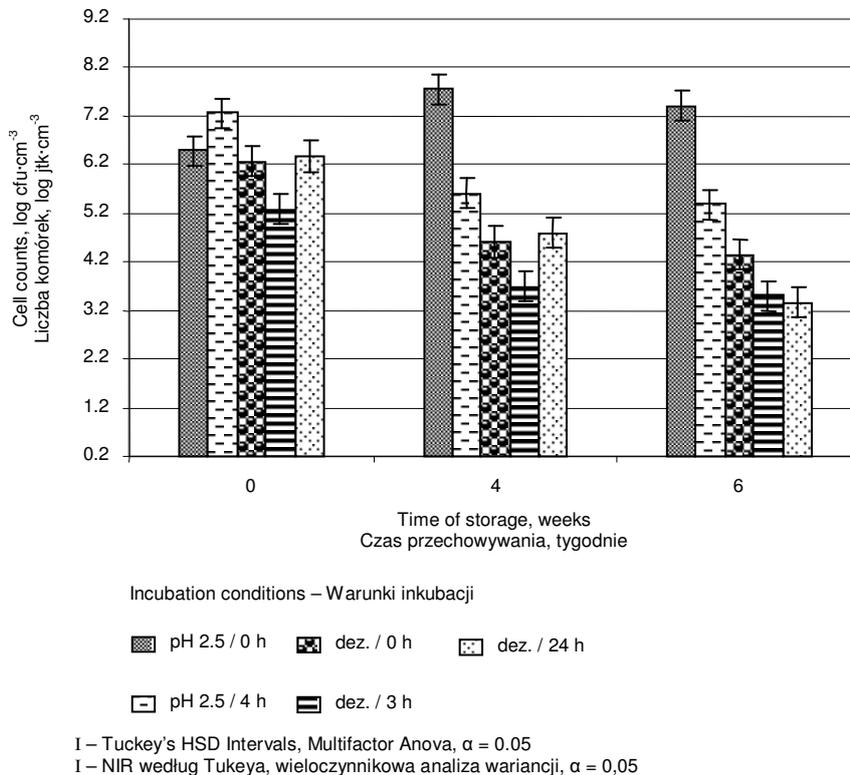


Fig. 5. The influence of time of storage of malt beverage on the survival ability *L. plantarum* ATCC 4080 at pH 2.5 and next in water solution deoxycholate sodium (mean from three series)

Rys. 5. Wpływ czasu przechowywania napoju słodowego na przeżywalność *L. plantarum* ATCC 4080 kolejno w pH 2,5 oraz w wodnym roztworze dezoksycholalanu sodu (średnia z trzech serii)

In opinion of Guchte et al. [2002] genetic and biochemical analyses indicate that lactic acid bacteria acid responses are intricate processes which require the synthesis of a variety of proteins and contribution of several mechanisms. Knowledge about these mechanisms is not yet complete. Also mechanisms which are used by bacteria to respond to bile salts are currently unknown but are likely to be similar to those used for other stress responses [Begley et al. 2005].

In the case of application of aqueous solution of deoxycholate sodium (Fig. 5) no considerable differences were found in survival ability of lactic acid bacteria during the period of product storage.

After 24 hours of incubation in solution of deoxycholate sodium samples of beverages stored four weeks was noted $6.38 \cdot 10^4$ cfu/cm³, and in the case of samples taken after six weeks – $2.60 \cdot 10^3$ cfu/cm³. We should underline that in the case of beverages stored for four weeks and used malt wort with deoxycholate sodium after complex incubation did not state viable lactic acid bacteria. In connection with this fact high

a number of bacteria cells in aqueous solution of deoxycholate sodium can result from the fact that the tested strain in the environment poor in nutrient components adapts faster to adverse conditions of the environment.

Deficiencies of nutrients create conditions the so-called stress or shock. Kunicki-Goldfinger [1998] write that lack of nutrients for bacteria lead to activation of numerous genes and next to production regulator proteins which allow to survive in these conditions.

According to Begley et al. [2005] the stresses encountered in food-processing could influence an increase of bacterial bile salts tolerance. This influence can be the result of changes of expression of particular genes or indirectly changes of membrane characteristics for example permeability, fluidity or charge.

Tetteh and Beuchat [2003] think that bacteria respond to different kinds of stress by starting synthesis specific proteins that allow them surviving in conditions of this stress. Identification of proteins in which the changes took place is carried out by using the two-dimensional electrophoresis (2DE) [Guchte et al. 2002]. Further analysis of proteins consists in isolation of proteins from gel electrophoresis and identification by using mass spectrometry (MALDI-TOF).

Mechanism of adaptation of lactic acid bacteria to adverse conditions of the environments requires further studies. These studies should contain determination, among other things, of changes of proteins profiles, changes of gene expression or group of genes which are responsible for adaptation process and changes of composition of fatty acids in the cell wall of bacteria.

CONCLUSIONS

1. Changes of antagonistic properties lactic acid bacteria were observed during 8-week beverage storage. The strain *L. plantarum* ATCC 4080 which was taken after four weeks of storage of beverage did not have antagonistic properties against indicator bacteria, while on another occasion of storage the product (after two, six and eight weeks) had the properties.

2. Changes of survival ability of the tested strain at pH 2.5 and next in presence of deoxycholate sodium were observed during the period of storage of malt beverage. Lactic acid bacteria which were taken after two and four weeks of storage did not survive during complex incubation (pH 2.5 and deoxycholate sodium in wort), while survived in these conditions after six and eight weeks. Lactic acid bacteria which were taken after four and six weeks of storage survived in aqueous solution of deoxycholate sodium.

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ZMIANY WYBRANYCH CECH *LACTOBACILLUS PLANTARUM* ATCC 4080 PODCZAS PRZECHOWYWANIA NAPOJU SŁODOWEGO

Streszczenie. Stwierdzono możliwość otrzymywania smacznego napoju słodowego, w którym przez osiem tygodni przechowywania w temperaturze 22°C liczba żywych komórek bakterii mlekowych przekracza 10^6 jtk/cm³. Podczas przechowywania napoju badano zmiany przeżywalności *L. plantarum* ATCC 4080 w pH = 2,5, a następnie w 3 mmol/dm³ dezoksychoalanu sodu oraz zmiany aktywności antagonistycznej tych bakterii. Szczep *L. plantarum* ATCC 4080, pobrany z napoju słodowego przechowywanego cztery tygodnie, nie wykazywał aktywności antagonistycznej w stosunku do bakterii gnilnych i chorobotwórczych, natomiast wykazywał tę cechę w pozostałych tygodniach przechowywania. W inkubacji kombinowanej (w pH = 2,5, a następnie w brzezce słodowej zawierającej 3 mmol/dm³ dezoksychoalanu sodu) szczep pobrany z napoju po dwóch i czterech tygodniach nie wykazywał zdolności przeżywania w tych warunkach, natomiast charakteryzował się wspomnianą zdolnością po sześciu i ośmiu tygodniach. W wypadku inkubacji w wodnym roztworze dezoksychoalanu sodu o stężeniu 3 mmol/dm³, zamiast w brzezce z dezoksychoalanem sodu, badany szczep po czterech i sześciu tygodniach przechowywania wykazywał zdolność przeżycia. Nie badano zdolności przeżywania w tych warunkach bakterii mlekowych pobranych po dwóch i ośmiu tygodniach przechowywania napoju.

Słowa kluczowe: bakterie fermentacji mlekowej, napój słodowy, probiotyki, aktywność antagonistyczna, niskie pH, dezoksychoalan sodu

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