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PH EFFECT ON ANTAGONISTIC ACTIVITY TOWARDS BACTERIA OF YEASTS ISOLATED FROM HUCUL DAIRY PRODUCTS AND GASTROINTESTINAL TRACT OF HUMAN

The aim of this work was to study the influence of pH of medium on antagonistic activity of isolated from authentic Hucul dairy products and gastrointestinal tract (GIT) of Hucul long-livers yeasts towards potentially harmful for humans and animals bacteria. Among 52 tested yeast isolates 14 % yeasts showed considerable antagonistic activity towards Gram-positive bacteria Staphylococcus aureus and only 6 % of them inhibited growth of Gram negative bacteria belonging to genera Escherichia and Citrobacter. Most of yeasts with antagonistic activity (over 70 %) were isolated from long-livers GIT. There were identified two optimal for antagonism areas of pH values of nutrient medium for tested yeasts being around 5.5 and 6.0 for Gram-positive bacteria and around 6.0 and 6.5 for Gram negative bacteria. It appeared that isolated from Hucul yogurt Saccharomyces pasterianus yeasts manifested their antagonistic activity in more acidic conditions compared to isolates from GIT.

K e y w o r d s: yeast, antagonistic activity, pH, dairy products, GIT.

Being unicellular microeukaryotes belonging to phylogenetically different groups of fungi yeasts are widely distributed in the environment and an essential compound in many complex ecosystems [2].

Yeasts are commonly found in milk and dairy products. Yeasts appear in milk just in a few hours after the milking process reaching at least 13 % of the total number of microorganisms. The yeasts found in milk belonged predominantly to genera *Candida*, *Pichia*, *Rhodotorula* and *Saccharomyces* [4].

Yeasts are also an important part of the normal microbiota of human and animals. It was reported that in the human body they were found mostly in saliva, skin and mucous membranes, gastrointestinal tract (GIT) and belonged to over 170 species and 25 genera [6].

Like other microscopic fungi yeasts can take an active part in various interactions with other microorganisms and macroorganisms including symbiosis, mutualism, parasitism and competition [2].

As a large and heterogeneous group of microorganisms of the great practical importance yeasts have been always of particular interest. Numerous versatile properties make them promising candidates for a wide range of applications not limited just to the food sector of industry [1, 12, 13]. One of the growing areas of interest is currently the application of certain yeasts in healthcare.

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Yeasts can manifest pronounced antagonistic effect towards various groups of microorganisms including bacteria and other yeasts and filamentous fungi [8, 13]. It provides yeasts competitive advantages by creating unfavorable conditions for other microorganisms growth and development. This antagonistic activity of yeasts can be contributed by biosynthesis of specific substances like mycocins and bacteriocins as well as by nonspecific metabolites altering environmental conditions [11].

The particular importance of yeasts in treating and prevention of the gastrointestinal tract diseases is fully recognized nowadays. There are increasing global trends for using yeasts as food supplements, probiotic product etc.

One of the requirements for the microbial probiotics is their ability to survive the passage through the gastrointestinal tract of humans and animals. It is known that, when taken orally, the best commercially established preparation of yeast probiotic *Saccharomyces boulardii* showed the 1–3 % rate of cells survival in the distal part of large intestine. It was also shown that *S. boulardii* can survive in the range of pH from 2 to 6.3 [7].

Currently there is a lack of data on the influence of pH on yeast survival under conditions of the gastrointestinal tract and only single studies on the pH effect on the yeasts antagonism towards bacteria. For example, for *Saccharomyces cerevisiae* isolates from human GIT the highest values of antagonistic activity towards pathogenic and opportunistic pathogenic bacteria were observed at range pH 5.5–6.5 [3].

The aim of this study was to investigate the effect of pH on the antagonistic activity against opportunistic pathogenic bacteria manifested by yeasts isolated from authentic Hucul dairy products and GIT of Hucul long-livers.

Materials and methods. *Microorganisms.* Yeasts were 52 cultures isolated from the authentic Hucul homemade dairy products and representing the distal part of large intestine GIT samples taken from Hucul long-livers of the highland region of the Eastern Carpathians, Ukraine. The dairy products were sour cream, Hucul yogurt Guslinka and home-made cheese (Table 1). The conventional dilution method and the malt agar medium were used for yeasts isolation.

The reference strains of bacterial cultures for testing antagonistic activity of yeasts were *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 from Ukrainian collections of microorganisms. There were also tested 3 strains of bacteria *Escherichia coli* 1621*, Citrobacter freundii* 1603*, Citrobacter intermedius* 1555 isolated from healthy air transport workers at their routine examination performed by the Central Sanitary and Epidemiological Station of

Table 1

The sources of yeasts isolates

Sources	Number of yeasts strains	Total number
Guslinka	F2, F21, F23, F25, FF1,FF2, FF5, FF6, FF7, FF8, FF9, FF10, FF11, FF12, FF13, FF18	16
Sour cream	FF4, FF14, FF15, FF16, FF18	5
Homemade cheese	F15, F27, F28, F29, F31, F35, F36	7
Long-livers GIT	S1, S2, S3, S4, S5, S7, S10, S11, S12, S13, S14, S15, S16, S17, S19,S21, S22, S23, S24, S25, S28, S30, S31, S32	24

Air Transport in Ukraine, Kiev. The preliminary identification of 7 yeasts isolates with highest antagonistic activity was performed using a standard scheme of phenotypic identification by Kurtsman [10].

Yeast and bacterial strains were maintained on malt agar and meat-peptone agar (MPA), correspondently.

Antagonistic activity evaluation. The conventional agar plug method was used to evaluate the antagonistic activity of yeasts isolates towards bacteria. Prior to the tests the microorganisms were grown for 24 hours at 28 °C for yeast and at 37 °C for bacteria. To prepare the agar plugs with yeasts biomass for tests were initially prepared by plating 1 ml of yeasts cell suspension (6×10^8 cells mL^{-1}) on the malt agar medium (20 ml per plate) with different pH values: 5.0, 5.5, 6.0, 6.5 and 7.0. The pH adjustment was performed using sterile citrate-phosphate buffer before pouring the plates. The yeasts cultures were incubated at 28 °C for 72 h and then a sterile 8-mm-diameter cork borer was used to cut the agar plugs.

The cell suspensions of 24 hours-old bacterial cultures (1 mL, 1.5×10^8 cells mL^{-1}) were pour-plated on MPA medium (20 ml per plate). The plugs with yeast biomass were placed onto MPA containing a lawn of certain bacterial strains. After an incubation period of 24 h at 37 °C, the diameter of clear zones of the inhibition of the bacterial growth surrounding the plugs with yeasts biomass was measured. As at present there are no fully accepted standard methods for evaluation of microbial antagonism, the values of antagonistic activity in our studies were the widths of the bands of the inhibition zones surrounding the plugs. This will make possible to carry out the comparative analysis of our data with the results coming from the alternative methods [3, 4].

All experiments were carried out in triplicates and the results were statistically analysed using Excel and Statistica 12.

Results and Discussion. We studied the antagonistic activity of yeasts isolated from Hucul homemade dairy foods (28 strains) and GIT samples of long-livers (24 strains) of the Carpathian highlands in Ukraine (Table 1). Out of 52 tested yeasts strains totally we found that 7 strains (13.46 %) manifested antagonistic activity towards bacteria. We evaluated the antagonistic activity as low at band widths 1–9 mm, medium -at 10–19 mm and high at values ≥ 20 mm. Our experiments showed that, in general, 42.85 % of tested yeasts (3 strains) demonstrated medium antagonistic activity and 57.14 % of yeasts (4 strains) were of low antagonistic activity.

All 7 selected yeast-antagonists were active against Gram-positive bacteria *Staphylococcus aureus*, and only 3 of them were active toward Gram-negative bacteria *Escherichia coli, Citrobacter freundii, Citrobacter intermedius*.

The high values of the bacterial growth inhibition, in general, were observed towards the reference strain *Staphylococcus aureus* ATCC 25922. However the yeasts cultures *Rhodotorula sp*. S1, *Debaryomyces hansenii* S3, *Debaryomyces hansenii* S5, *Candida sp.* S11, *Saccharomyces pasporianus* FF7 showed the maximum values of antagonistic activity towards the clinical isolate *Staphylococcus aureus* 38.

In present study the antagonistic activity of the tested yeasts depended on the source of their isolation clearly demonstrating the predominance of yeast-antagonists among the isolates from GIT of long-livers (5 strains) compared to those isolated from dairy food – Hucul yogurt guslinka (2 strains).

Only three strains out of 7 yeasts manifested antagonistic activity to Gram-negative bacteria. The widths of bacterial growth inhibition zones for *Saccharomyces pasterianus* FF7 were different for clinical and reference strains of *Escherichia coli* (Fig. 1C). This yeast strain showed the highest zones of antagonistic activity against the reference *E. coli* strain under acidic conditions with pH 5.0–5.5. At pH 6.0 and 7.0 the values of inhibition zones did not differ for the reference and clinical *E. coli* strains (Fig. 1C). Whereas at pH 6.5 *Saccharomyces pasterianus* FF7 demonstrated significantly higher antagonistic activity towards the clinical *E. coli* isolate. It was found that *Saccharomyces pasterianus* FF7 did not show any antagonism towards *Citrobacter* bacteria (Fig. 1C).

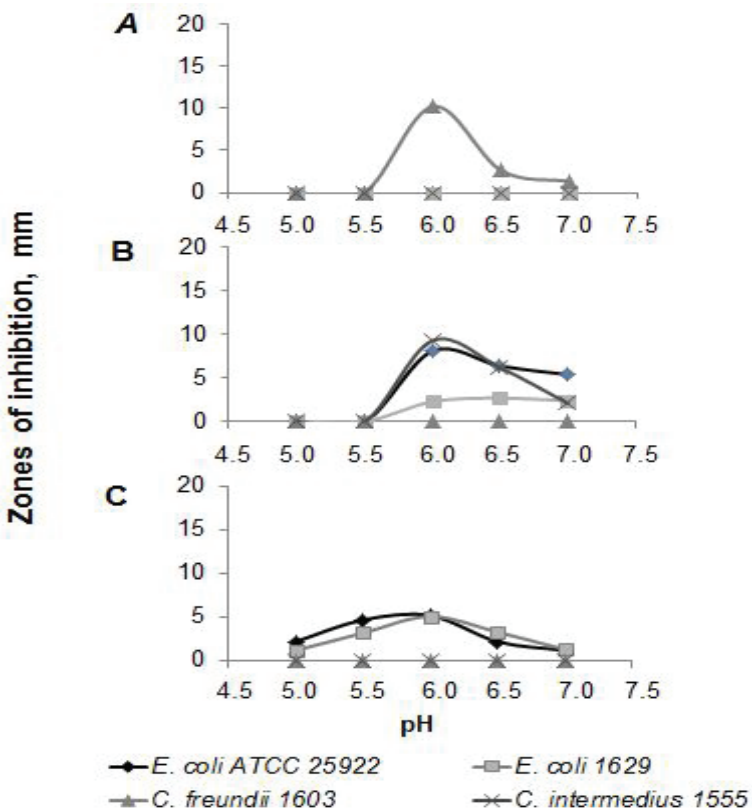

Fig. 1. Antagonistic activity of yeast strains isolated from dairy products and human GIT towards Gram-negative bacteria (genera *Escherichia* and *Citrobacter*) at different pH values: A – *Debaryomyces hansenii* S2; B – *Debaryomyces hansenii* S3; C – *Saccharomyces pasterianus* FF7. Standard errors of mean did not exceed 6 %

The difference in the width values of inhibition zones can be caused by the effect of pH on the antagonistic activity of yeasts metabolites including bacteriocines because the activation of enzymes and the rate of enzymatic reaction are primarily dependent on temperature and pH [7, 8].

Another two antagonistic yeasts against Gram-negative bacteria were GIT strains *Debaryomyces hansenii* S2 and S3 (Fig. 1 A and B). They antagonistic activity was low (S2 – 10,2 mm and S3 – 8,1 mm; 2,3 mm; 9,3mm). *D. hansenii* S3 was able to inhibit the growth of 3 bacteria (*Escherichia coli* ATCC 25922, *Escherichia coli* 1621, *Citrobacter intermedius* 1555) whereas *D. han-*

senii S2 possessed antagonistic activity to only one bacterial strain (*Citrobacter freundii* 1603).

Both guslinka isolates *Saccharomyces pasterianus* FF7 and FF18 possessed antagonistic activity towards tested Staphylococci (Fig. 2). Despite not being able to inhibit the growth of Gram-negative bacteria *S. pasterianus* FF18 demonstrated the highest observed in this study values of antagonistic activity towards Gram-positive bacteria (Fig. 2B). Thus, its maximum value of the width of the growth inhibition zone of the reference strain *Staphylococcus aureus* ATCC25923 reached 18.5 mm at pH=5.5.

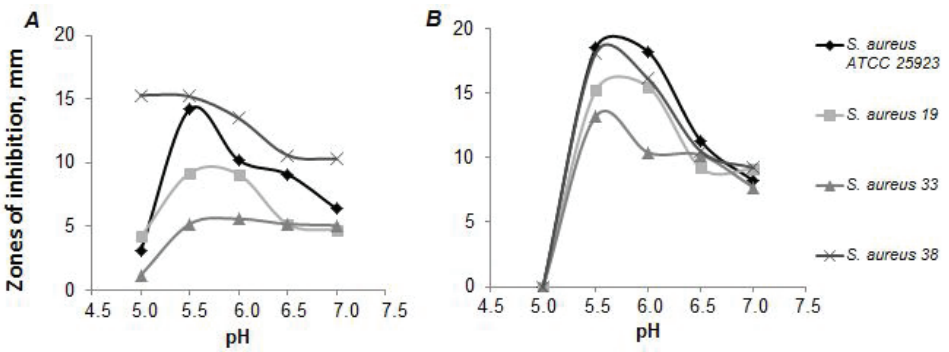

Fig. 2. Antagonistic activity of yeast strains isolated from dairy products towards Gram-positive bacteria (*Staphylococcus aureus*) at different pH values: A – *Saccharomyces pasterianus* FF7; B – *Saccharomyces pasterianus* FF18. Standard errors of mean did not exceed 6 %

Among the GIT yeasts isolates the antagonistic activity of *Debaryomyces hansenii* S2, S3 and S5 towards *Staphylococci*, if any, was low or medium (Fig. 3).

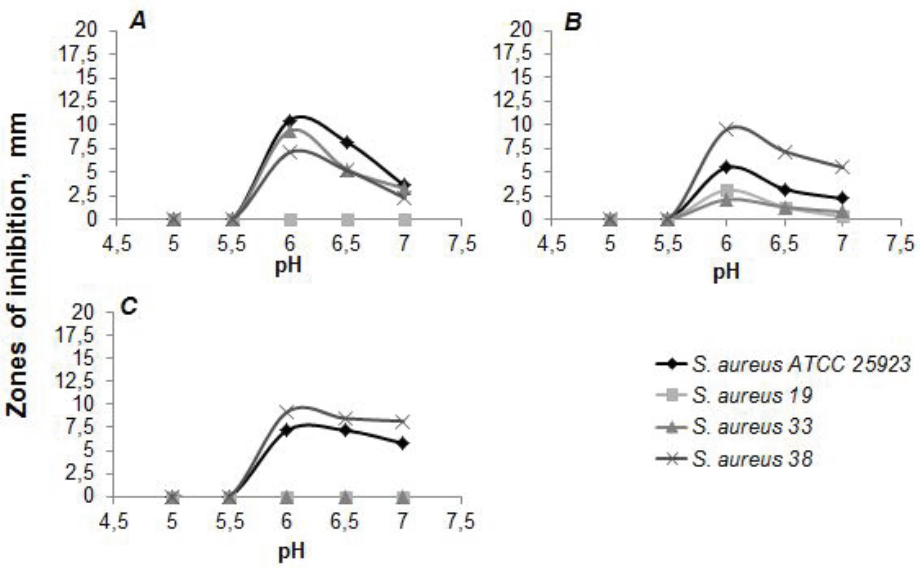

Fig. 3. Antagonistic activity of yeast strains isolated from human GIT towards Gram-positive bacteria (*Staphylococcus aureus*) at different pH values: A – *Debaryomyces hansenii* S2; B – *Debaryomyces hansenii* S3; C – *Debaryomyces hansenii* S5. Standard errors of mean did not exceed 10 %

All *D. hansenii* strains inhibited growth of the reference strain *Staphylococcus aureus* ATCC25923 however the pattern of their anti-*Staphylococci* activity differed. *D. hansenii* strains S2, S3 and S5 manifested antagonistic activity towards 3, 4 and 2 tested *S. aureus* strains, respectively. *D. hansenii* S2 demonstrated the highest antagonistic activity towards to the reference strain *Staphylococcus aureus* ATCC25923 (Fig. 3A), whereas *D. hansenii* S3 and S5 inhibited the reference bacterial strain less than the clinical isolate *S. aureus* 38 (Fig. 3B and 3C).

In general, the anti-*Staphylococci* activity of *Rhodotorula* spp. S1 was significantly higher compared to *Candida* sp. S11. Similarly to *D. hansenii* S3 and S5, *Candida* sp. S11 demonstrated considerably higher antagonistic ability towards the clinical isolate *Staphylococcus aureus* 38 (Fig. 4).

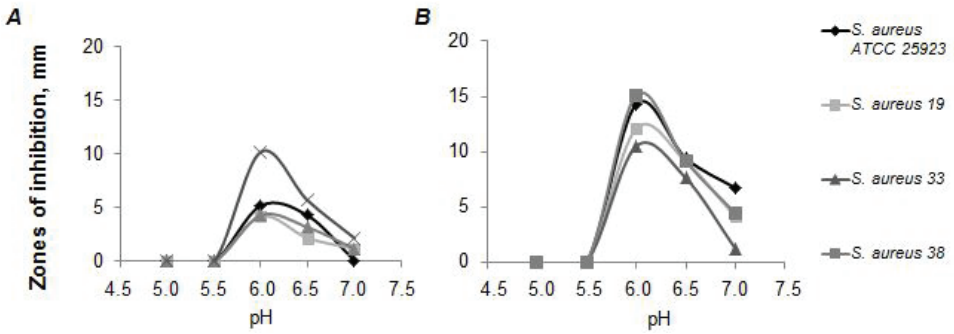

Fig. 4. Antagonistic activity of yeast strains isolated from human GIT towards Gram-positive bacteria (*Staphylococcus aureus*) at different pH values: A – *Candida* sp. S11; B – *Rhodotorula* spp. S1. Standard errors of mean did not exceed 10 %

Our results showed the significant differences between the optimal values of pH for maximum inhibition of the growth of Gram-positive and negative bacteria by yeasts originated from guslinka and GIT. To visualize such differences we plotted the relative data on antagonistic activity of yeasts expressed as a percent of maximum manifested antagonism towards bacteria by each yeast strain (Fig. 5). There were two distinct optimal ranges of pH values around 5.5 for

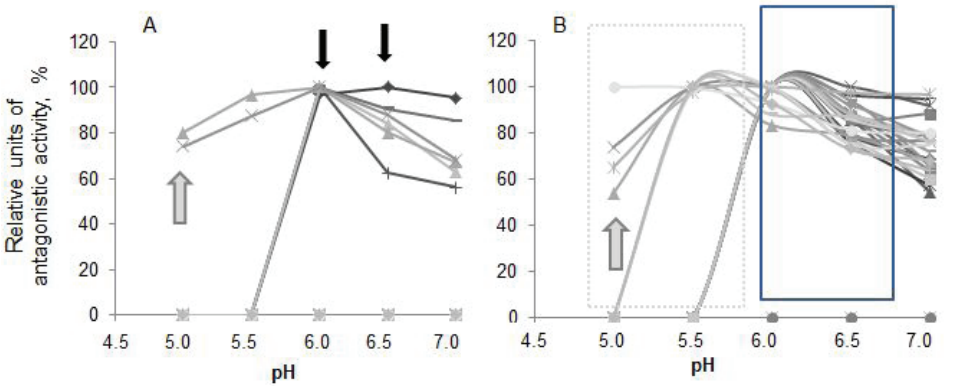

Fig. 5. Relationship between the relative units of yeasts antagonistic activity towards Gram-negative (A) and Gram-positive (B) bacteria and the values of pH. Black arrows (A) and rectangles (B) indicate the presence of two optimal ranges of pH values for antagonism manifestation, grey arrows (A, B) indicate the start of the antagonistic action of yeast isolates from guslinka under more acidic conditions. Standard errors of mean did not exceed 10%

guslinka strains and 6.5 for GIT strains in the case of antagonism towards Gram-positive bacteria. In the case of antagonism towards Gram-negative bacteria the difference between the peaks of optimal pH values was less pronounced being around 6.0 and 6.5 for guslinka and GIT isolates, respectively (Fig. 5).

We found the different effect of pH values on antagonistic activity towards bacteria for yeasts of different origin (Fig. 5). Antagonistic activity of the guslinka strains belonging to the species *Saccharomyces pasterianus* appeared at pH 5.0 whereas the isolated from GIT strains identified as *Rhodotorula* spp., *Candida* sp. and *Debariomyces hansenii* started to manifest the antagonistic activity only at pH 6.0.

This phenomenon of the antagonism manifestation at more acidic pH values for guslinka yeast isolates can be associated with the difference in the acidity of the environments where these two groups of yeasts originated from: yogurt and GIT. The pH of the yogurts is usually around 4.5-4.6 due to the lactic acid production by lactic acid bacteria [15]. Whereas the pH values of healthy human bowel's were reported to vary being in average 6.4 for proximal small bowel, 7.4 for distal small bowel, 6.3 for caecum/right colon and 6.9 for left colon/rectal [14]. It might be suggested that the pH effect on bactericidal activity manifested by two different groups of yeast-antagonists is related to the different nature of antagonistic reaction, for example the activation of bacteriocines occurring at different values of pH.

It is known that mechanisms of antagonistic activity of yeasts against Gram-positive and negative bacteria are different. Antagonistic activity toward Gram-positive bacteria was attributed to the action of bacteriocin-like substances inhibiting cellular metabolism of bacteria [5].

The inhibition of growth of Gram-negative bacteria by yeasts is thought to be connected mainly with the action of primary metabolites [5]. Such inhibiting activity is usually low. The substances like phenols, cresols and detergents can manifest antagonistic action towards Gram-negative bacteria affecting both outer membrane and the selective permeability of the plasma membrane [5].

The bacteriostatic and bactericidal effects of yeasts on bacteria can be also contributed by indirect mechanisms, for example, through the production of substances like acids and alcohols changing physico-chemical environment (e.g. pH) and in the course of competition for nutrient resources [9].

Thus, as a result of this research out of 52 yeast isolates from homemade dairy products and the Hucul long-livers GIT from the Carpathian highland in Ukraine, we found 7 yeasts with antagonistic activity (13.46 %), of which 2 strains were food isolates and 5 strains were GIT isolates.

All found yeast-antagonists inhibited Gram-positive bacteria (*Staphylococcus aureus*) growth and only three isolates showed antibacterial activity towards Gram-negative bacteria (genera *Escherichia* and *Citrobacter*).

Optimal pH ranges for the development of antagonism by different yeasts differed for isolates from different sources. It was demonstrated a general trend that antagonistic activity of yeast isolates from dairy products starts to manifest at more acidic pH values (pH 5.0 and 5.5), unlike yeasts isolated from the gastrointestinal tract (pH 6.0 and 6.5).

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ВПЛИВ РН НА АНТАГОНІСТИЧНУ АКТИВНІСТЬ ДРІЖДЖІВ, ІЗОЛЬОВАНИХ З КИСЛОМОЛОЧНИХ ПРОДУКТІВ ТА ШЛУНКОВО-КИШКОВОГО ТРАКТУ ЛЮДИНИ

Резюме

Метою цієї роботи було вивчити вплив pH поживного середовища на антагоністичну активність ізолятів дріжджів із автентичних гуцульських кисломолочних продуктів та шлунково-кишкового тракту гуцулів-довгожителів до умовно-патогенних бактерій для людини і тварин. Серед 52 досліджених дріжджових ізолятів 14 % показали значну антагоністичну активність проти грампозитивних бактерій *Staphylococcus aureus*; з них тільки 6 % інгібували ріст грамнегативних бактерій, що належать до родів *Escherichia* та *Citrobacter.* Більшість дріжджів з антагоністичною активністю (більше 70 %) було виділено з шлунково-кишкового тракту довгожителів. Також було визначено два оптимальні діапазони значень pH поживного середовища для виявлення антагоністичної активності досліджуваних дріжджів, що складало біля 5,5 і 6,0 для грампозитивних бактерій, та біля 6,0 і 6,5 – для грамнегативних бактерій. Показано, що дріжджі *Saccharomyces pasterianus*, ізольовані з гуцульського йогурту, проявляють антагоністичну активність за більш кислих умов, ніж ізоляти з шлунково-кишкового тракту людини.

Ключові слова: дріжджі, антагоністична активність, pH, кисломолочні продукти, шлунково-кишковий тракт.

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ВЛИЯНИЕ РН НА АНТАГОНИСТИЧЕСКУЮ АКТИВНОСТЬ ДРОЖЖЕЙ, ИЗОЛИРОВАННЫХ ИЗ КИСЛОМОЛОЧНИХ ПРОДУКТОВ И ЖЕЛУДОЧНО-КИШЕЧНОГО ТРАКТА ЧЕЛОВЕКА

Резюме

Целью этой работы было изучить влияние pH питательной среды на антагонистическую активность изолятов дрожжей из аутентичных гуцульских кисломолочных продуктов и желудочно-кишечного тракта гуцулов-долгожителей к условно-патогенным бактериям человека и животных. Среди 52 исследованных дрожжевых изолятов 14 % показали значительную антагонистическую активность против грампозитивных бактерий *Staphylococcus aureus*; из них только 6 % ингибировали рост грамнегативных бактерий, принадлежащих к родам *Escherichia* и *Citrobacter*. Большинство дрожжей с антагонистической активностью (более 70 %) было выделено из желудочно-кишечного тракта долгожителей. Также нами были

найдены оптимальные диапазоны значений pH питательной среды для выявления антагонистической активности исследованных дрожжей, что составило около 5,5 и 6,0 для грампозитивных бактерий, и около 6,0 и 6,5 – для грамнегативных бактерий. Показано, что дрожжи *Saccharomyces pasterianus,* изолированные из гуцульского йогурта, проявляют антагонистическую активность при более кислых условиях, нежели изоляты из ЖКТ человека.

К л ю ч е в ы е с л о в а: дрожжи, антагонистическая активность, pH, кисломолочные продукты, желудочно-кишечный тракт.

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