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ANTAGONISTIC ACTIVITY OF *LACTOBACILLUS ACIDOPHILUS* BACTERIA TOWARDS SELECTED FOOD-CONTAMINATING BACTERIA

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Key words: *Lactobacillus acidophilus*, antagonistic activity, products of metabolism

The aim of this study was to determine the antibacterial properties of 20 *Lactobacillus acidophilus* strains against spoilage or pathogenic bacteria. The relationship between contents of various metabolites and the inhibitory activity of *Lb. acidophilus* against *Escherichia coli*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and vegetative cells and spores of *Bacillus mycoides* and *Bacillus subtilis* was studied. The antibacterial activity was determined by measuring zones of growth inhibition on agar media. The antibacterial activity was markedly diversified. Some *Lb. acidophilus* strains also inhibited the germination of *Bacillus* spp. spores. Lactic and acetic acids as well as hydrogen peroxide were the most potent inhibitory agents produced by the *Lb. acidophilus* studied. However, some strains of *Lb. acidophilus* producing relatively small amounts of lactic acid in milk had a higher inhibitory activity than those producing a large quantity of lactic acid.

INTRODUCTION

Lactic acid bacteria (LAB) are widely used in the food industry, primarily due to their ability to metabolise saccharides present in foods and beverages to organic acids, mainly to lactic acid and, to a smaller extent, to acetic acid, which depends on the organism and the type and source of carbon. In fermented food, lactic acid bacteria determine the sensory features of food, such as consistency, taste and flavour. They are also responsible for increasing its nutritive value, prolonging its shelf life and improving its microbiological safety, as well as for probiotic effects, especially for maintaining natural intestinal microflora, alleviating symptoms of lactose intolerance and inhibiting the synthesis of enzymes which transform procarcinogenic compounds into carcinogenic ones, thus reducing the risk of cancer.

The role of lactic acid bacteria in the preservation and improvement of microbiological safety of food is of great importance. The antagonistic activity of lactic acid bacteria towards pathogenic bacteria provides an opportunity to discontinue using foreign substances such as salt, sugar and other food preservatives [Klewicka & Libudzisz, 1998]. The metabolites of lactic acid bacteria are capable of inhibiting the growth of many microbial species and their activity is usually based on the synergistic interaction of many factors. In addition to the basic acidic products of saccharide metabolism, lactic acid bacteria produce cell metabolism by-products of antagonistic activity, namely acetaldehyde, diacetyl, ethanol, hydrogen peroxide and bacteriocines [Bredholt *et al.*, 1999; Gomes & Malcata, 1999].

Of particular interest is the species *Lactobacillus acidophilus*, whose antagonistic metabolites control both the development of a food-contaminating microflora and the growth of pathogenic and toxinogenic bacteria in the alimentary tract of humans and animals [Kaur *et al.*, 2002].

The type and quantity of metabolic products with antagonistic activity depend not only on the species and strain, but also on external factors such as the chemical composition of the medium, temperature, microorganism growth phase and pH of the growth medium.

The aim of the work was to characterise the antibacterial properties of *Lactobacillus acidophilus* strains and to correlate them with the quantity of metabolic products with the antagonistic activity formed by these strains.

MATERIALS AND METHODS

Cultures of 20 single strains of *Lb. acidophilus* have been studied. Strains denoted as Ros, 172, H-1, Ch-2, In3, Cz-1, 343, 336, Ind1, 20T1, were obtained from Rhodia Food Biolacta in Olsztyn; the cultures: Bauer, Ch-5, A92, Diat, Nestle from Prague Technical University, Dept. Milk Fat Technology; strains NCAINB 1075, NCAINB 1152 came from the National Collection of Agricultural and Industrial Microorganisms in Budapest, whereas cultures 1, 0.3, B from the collection ŁOCK 105 of the Institute of Fermentation Technology and Microbiology, Technical University of Łódź.

The indicatory microorganisms tested for inhibition by *Lb. acidophilus* were: *E. coli* ATCC 25922, *E. coli* ŁOCK 0836, *P. aeruginosa* ATCC 27853, *P. fluorescens* ŁOCK 0887, *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633 and *B. mycioides* ŁOCK 0811. These organisms are psychrotrophic food spoilers or pathogens. The latter two strains of *Bacillus* were inoculated in a growth medium as a suspension of vegetative cells or as a suspension of spores.

The activity of inter-strain antagonism was investigated on an MRS medium containing 2% of lactose using the

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agar slab method [Strus, 1998]. The method was based on the observation of parallel growth of the strains under study (the indicator and the antagonistic ones). Agar slabs of 10-mm in diameter were aseptically cut off the MRS agar overgrown with a lawn of *Lb. acidophilus* incubated for 24 h at 37°C, and placed on plates with the agar media (Nutrient Agar, Merck) inoculated with the indicator strain (10^5–10^6 CFU/mL). After 18 h of incubation, the diameters of growth inhibition zones around the agar slabs were measured. The results are given in mm, minus the agar slab diameter.

At the same time, the concentration of metabolites formed during 24 h of growth in milk of *Lb. acidophilus* strains was determined. The samples were incubated at 37°C in the presence of 5% CO_2.

Lactic acid, acetic acid, acetaldehyde and ethanol were determined by enzymatic methods, using commercial kits provided by Boehringer Mannheim (Germany).

The amount of hydrogen peroxide was determined in deproteinised (10% TCA) samples by titration with 0.001 N potassium permanganate to a light pink colour, which did not disappear for 60 sec.

To compare the antagonistic activity of the strains of lactic acid bacteria against the indicator cultures, the index of total antagonistic activity (IAA) was determined. The index was expressed as a sum of scores determined by the diameters of growth inhibition of all 6 species of the indicator bacteria. The growth inhibition diameter above 14 mm – 3 points, 9–14 mm – 2 points, 1–8.9 mm – 1 point. For the IAA, the coefficient K definite was determined as quotient IAA and total acidity or the concentration of hydrogen peroxide depending on metabolism products ($K=IAA/C_{ko}$, C_{ko} – total acidity in °SH, or $K=IAA/C_{nw}$ where C_{nw} – concentration of hydrogen peroxide in μg/mL).

RESULTS AND DISCUSSION

A broad antagonistic spectrum is one of the features which should be characteristic of probiotic strains of lactic acid bacteria [Dunne *et al.*, 1999]. Their antagonistic activity range should in particular cover the strains pathogenic for humans, such as *Yersinia enterolitica*, *Bacillus* sp., *Staphylococcus aureus*, *Salmonella* sp. and enteropathogenic strains *Escherichia coli* [Drago *et al.*, 1997]. Recently, the pathogens *Helicobacter pylori* [Aiba *et al.*, 1998] and *Listeria monocytogenes* [Bredholt *et al.*, 1999] have been found to be sensitive to the action of probiotic cultures.

All strains of *L. acidophilus* studied have revealed the ability to inhibit the growth of the strains of pathogens or spoilers under investigation (Table 1). However, the level of this activity was different. Of the twenty cultures of *L. acidophilus* investigated, twelve (Ch-2, 172, A92, B, 1, 0.3, B, Ch-5, Ros, In-3, 1152, Nestle) also inhibited the growth of vegetative cells of bacteria *B.subtilis*. Some of the strains of *L. acidophilus* studied also inhibited spore germination (Table 1).

The highest IAA value (18 points) was found for the strain *L. acidophilus* NCAINB 1152, whereas the lowest

TABLE 1. Antagonistic activity of *Lb. acidophilus* bacteria.

	1	2	3	4	5	6	7	8	9	IAA
L. acidophilus 336	+	++	+	++	++	++	-	-	-	10
L. acidophilus Cz-1	+	++	+	++	++	++	-	-	-	10
L. acidophilus Ros	+	++	+	+	++	+	+	-	+	10
L. acidophilus Nestle	+	-	++	++	++	-	++	-	+	10
L. acidophilus I nd 1	+	++	+	++	++	++	-	+	-	11
L. acidophilus H-1	+	+++	+	++	++	++	-	-	-	11
L. acidophilus Diat	++	++	+	++	++	++	-	-	-	11
L. acidophilus Ch-5	+	++	+	+	++	++	+	+	+	12
L. acidophilus In-3	++	++	+	+	++	+	+	+	+	12
L. acidophilus Ch-2	+	++	+	++	++	++	++	-	+	13
L. acidophilus 1	+	++	+	++	++	++	++	+	+	14
L. acidophilus 343	+	++	+++	+++	++	++	-	+	-	14
L. acidophilus A92	++	++	++	++	++	++	++	-	+	15
L. acidophilus 0.3	++	++	++	++	++	++	++	-	+	15
L. acidophilus Bauer	+	++	+++	+++	++	++	++	-	-	15
L. acidophilus 20T1	+++	++	+++	+++	++	++	-	-	-	15
L. acidophilus NCAINB 1075	++	++	+++	+++	++	++	-	+	-	15
L. acidophilus B	+	++	+++	+++	++	++	++	-	+	16
L. acidophilus 172	++	+++	++	++	++	++	++	-	+	17
L. acidophilus NCAINB 1152	++	++	+++	+++	++	++	++	+	+	18

+++ growth inhibition zone above 14 mm, ++ growth inhibition zone 9–14 mm, + growth inhibition zone 1–8.9 mm, – no antagonistic activity was found. 1 – *E. coli* ATCC 25922, 2 – *E. coli* ŁOCK 0836, 3 – *S. aureus* ATCC 25923, 4 – *P. aeruginosa* ATCC 27853, 5 – *P. fluorescens* ŁOCK 0887, 6 – *B. mycoides* ŁOCK 0811 (mixture of vegetative cells and spores), 7 – *B. subtilis* ATCC 6633 (mixture of vegetative cells and spores), 8 – *B. mycoides* ŁOCK 0811 (spores), 9 – *B. subtilis* ATCC 6633 (spores).

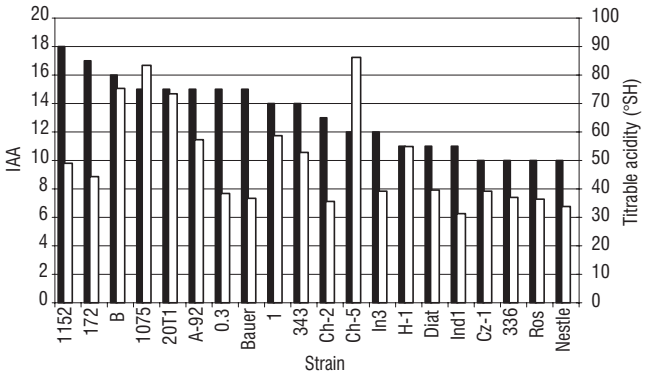

FIGURE 1. Antagonistic and acidifying activity of lactic acid bacteria *Lactobacillus acidophilus*.
■ – IAA, □ – Titrable acidity. °SH – degree of Soxhlet-Henkl: The amount of 0.25 N NaOH (mL) used for titration of 100 mL of milk in the presence of fenolophtaleine. One mL of 0.1 N NaOH corresponds to 0.009 g of lactic acid.

IAA (10 points) was reported for 4 cultures of lactic acid bacteria denoted by the symbols 336, Cz-1, Ros and Nestle (Table 1). The comparison of the IAAs and the total acidity (Figure 1), showed that strains: NCAINB 1152, 172, 0.3, Bauer and Ch-2, despite their low acidifying activity had high inhibitory activity IAA values of 15–18 points. Hence, it can be assumed that these bacteria are capable of synthesising other inhibitors, *e.g.* bacteriocins.

In bacterial cultures that were capable of synthesising lactic acid in amounts smaller than 10.0 g/L, acetic acid could play an important role.

The bacterial cultures studied grown both in milk and in synthetic media [Klewicka & Libudzisz, 2001] for 24 h at 37°C, decrease the pH value to about 4.0 or lower (Table 2). *L. acidophilus* Ros had the lowest ability to ferment lactose, lowering pH to 4.7 and producing 8.12 g/L of lactic acid and 0.06 g/L of acetic acid (Table 2).

It is known that the antibacterial effect of organic acids is the result of a rapid decrease in the medium pH below the range of the optimal value for microorganism growth as well as by the inhibition of their biochemical activity caused by undissociated acid molecules present in the medium [Freese *et al.*, 1976; Eklund, 1989; Adams & Hall, 1998]. Due to its pKa dissociate, acetic acid is a better inhibitor than lactic acid.

A high level of acetic acid synthesis, reaching 13–22% of the total acidity in 8 cultures (Table 2) (strains: 336, Ch-2, 0.3, Bauer, Cz-1, Ch-5, In-3, Nestle) was observed. Homo-fermentative bacteria, including *Lactobacillus acidophilus* produce lactic acid from the saccharides present in the medium, with a yield of 1.8 mole per one mole of glucose, the rest, *i.e.* 0.2 mole, being composed of the mixture of trace quantities of other metabolic products (acetic acid, acetaldehyde, ethanol) [Gomes & Malcata, 1999]. An increased synthesis of acetic acid by some of the strains tested occurs, although the EMP path, including a pentose phosphoketolase path, is active in these bacteria. It has been shown that the following pentoses: L-arabinose, rybose and D-xylose can be fermented by the strains (336, Cz-1, Ch-2, Ch-5, Bauer) of the *Lb. acidophilus* used in this study [Libudzisz *et al.,* 2001].

Acetaldehyde can be a product of transformation of saccharides (Table 2), proteins or nucleic acids [Lees & Jago, 1978]. Lactic acid bacteria with threonine aldolase activity (*Lactococcus lactis* spp. *cremoris*, *Lactococcus lactis* spp. *lactis*, *Streptococcus thermophilus*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*) form acetaldehyde also as

TABLE 2. Metabolic products of *Lb. acidophilus* after 24 h growth in 10% powder milk.

Strain	pH	Lactic acid (g/L)	Acetic acid (g/L)	Ethanol (g/L)	Acetaldehyde (mg/L)	Hydrogen peroxide (μg/mL)
L. acidophilus I nd 1	3.8	7.04	0.57	0.05	1.36	18.7
L. acidophilus 336	4.3	6.65	1.67	0.67	0.66	44.9
L. acidophilus H-1	4.0	11.75	0.61	0.27	0.34	18.72
L. acidophilus Ch-2	3.8	6.91	1.10	0.19	0.48	33.7
L. acidophilus 172	3.8	9.03	0.93	0.25	0.42	33.7
L. acidophilus A92	3.9	12.02	0.87	0.00	0.61	31.8
L. acidophilus 1	4.0	12.80	0.40	0.36	1.00	5.7
L. acidophilus 0.3	4.2	7.48	1.16	0.26	0.710	44.9
L. acidophilus Bauer	4.1	7.07	1.19	0.29	0.44	15.1
L. acidophilus 343	3.8	11.15	0.72	0.05	3.43	48.6
L. acidophilus 20T1	3.8	15.78	0.74	0.04	0.56	15.0
L. acidophilus Cz-1	3.9	7.55	1.28	0.85	0.61	33.7
L. acidophilus Diat	4.0	7.81	1.10	0.18	0.35	16.8
L. acidophilus B	4.1	16.23	0.71	4.80	0.68	20.6
L. acidophilus Ch-5	4.0	14.93	4.47	0.75	0.61	5.7
L. acidophilus Ros	4.7	8.12	0.06	0.12	2.93	24.3
L. acidophilus In–3	3.8	7.46	1.37	0.20	6.61	7.6
L. acidophilus NCAINB 1075	4.0	18.23	0.54	0.15	1.47	13.1
L. acidophilus NCAINB 1152	3.8	10.98	0.05	0.04	0.26	52.4
L. acidophilus Nestle	3.9	6.02	1.58	0.10	0.58	20.6

a result of degradation of threonine released by proteolysis. The amount of synthesised acetaldehyde is a strain characteristic feature, thus these quantities can differ significantly within one species. *Streptococcus thermophilus* bacteria produce from 1.0 to 8.3 mg/L, while in the case of a culture *Lactobacillus delbrueckii* ssp. *bulgaricus* from 2.0 to 41 mg/L of this compound. Acetaldehyde present in the medium in the amount of 44 mg/L effectively inhibits the cell division of *E. coli* [Egyad, 1967]. Within the cultures tested, *Lb. acidophilus* In-3 was characterised by the highest acetaldehyde productivity, reaching (6.61 mg/L). An acetaldehyde concentration slightly lower than that mentioned above was reached by the strains: 343 – 3.43 mg/L, Ros – 2.93 mg/L, NCAINB 1075 – 1.47 mg/L, Ind 1 – 1.36 mg/L, 1 – 1.00 mg/L. The other strains of *Lb. acidophilus* under study synthesised acetaldehyde in small quantities (0.26 to 0.71 mg/L). On the basis of the results obtained, it can be assumed that aldehyde was only a factor contributing to the enhancement of inter-strain antagonism.

The bacteriocidal action of ethanol is observed only at high concentrations, *e.g.* 10–15% (wt/wt) or higher. The bacteria *Lb. acidophilus* studied could synthesise ethanol at very low concentrations, ranging from 0.04 to 4.8 g/L. Therefore, this compound is probably significant in contributing to the total antagonistic activity.

Hydrogen peroxide is another metabolic product of lactic acid bacteria which demonstrates antibacterial activity. *Lactobacillus* species have been identified as the most efficient producer of hydrogen peroxide among LAB [Vanderbergh, 1993]. The most active are *Lb. acidophilus*, *Lb. plantarum* and *Lb. delbrueckii* spp. *bulgaricus*. The latter ones produce H_2O_2 at concentrations of 11.0 to 15.5 μg/mL, whereas *Lb. acidophilus* produces 56.0 to 72.0 μg/mL of this compound in milk. It is known that the concentration of about 10.0 μg/mL hydrogen peroxide in the medium can efficiently inhibit the growth of *S. aureus*, while higher concentrations, *e.g.* 50.0 μg/mL and more, can prolong the lag phase of *Pseudomonas* species [Gudkow, 1986; Vandervoode *et al.*, 1992].

Due to incubation conditions – the presence of 5% (v/v) of CO_2 – the formation of hydrogen peroxide by the cultures investigated was remarkably reduced; the maximum concentration of 52.4 μg/mL H_2O_2 was obtained in the culture of *Lb. acidophilus* NCAINB 1152 (Table 2). A high accumulation of hydrogen peroxide was also characteristic of the cultures of *Lb. acidophilus* 20T1, 336, 0.3, Ch-2, 172, Cz-1, A92 that produced from 32 to 49 μg/mL. Some strains produced <10 μg of H_2O_2/mL.

On the basis of these results, it may be assumed that besides organic acids, hydrogen peroxide is one of the most important agents that have a direct effect on the antibacterial properties of *L. acidophilus*.

The analysis of the level of non-specific substances which are responsible for the inhibitory activity of the LAB studied showed that for most strains the major antibacterial agent was the lactic acid, especially when occurring even with a small quantity of acetic acid. A more efficient accumulation of acetic acid and hydrogen peroxide was observed in cultures which produced less than 10.0 g/L of lactic acid. An exception was *Lb. acidophilus* In-3 which, despite the low concentrations of lactic acid and hydrogen peroxide produced, inhibited the growth of all the indicator cultures used. However, this strain produced large amounts of acetic acid (1.37 g/L) and the highest concentrations of acetaldehyde (6.61 mg/L) in all the cultures investigated.

A non-linear regression analysis of the relation that represents the general antagonistic activity expressed as IAA and the total acidity for particular lactic acid bacteria strains as well as the level of the hydrogen peroxide produced, led to determination of the coefficient K for both metabolite groups, defined as the ratio of the antagonistic activity index (IAA), and the total acidity (Figure 2) or the concentration of hydrogen peroxide (Figure 3). Figures 2 and 3 show the value of the coefficient K for each strain, as well as the total acidity and the hydrogen peroxide concentration corresponding to it in an increasing order, respectively.

It can be stated that the coefficient K is inversely proportional to the total acidity and the hydrogen peroxide concentration for the strains of lactic acid bacteria. Moreover, non- linear regression curves of a high degree of correlation can be determined (Figure 2). The R^2 and K values for non-linear regression coefficient for hydrogen peroxide concentration are also high (Figure 3).

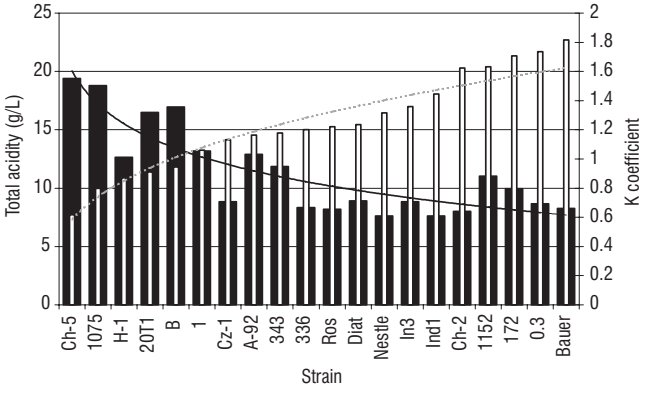

FIGURE 2. Total acidity *vs*. coefficient K.
■ – total acidity; □ – K coefficient; — Log. (total acidity) R^2 = 0.7578; ····· power (K-coefficient) R^2=0.9464.

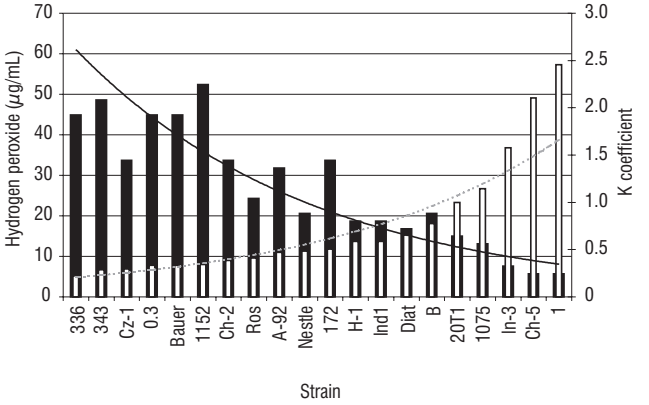

FIGURE 3. Hydrogen peroxide concentration *vs*. coefficient K.
■ – hydrogen peroxide; □ – K coefficient; — exponent (hydrogen peroxide) R^2 = 0.8463; ····· exponent (K coefficient) R^2 = 0.9200.

On the basis of the results obtained in this study and the mathematical analysis, it was found that a key role in the antagonistic action of lactic acid bacteria is played by organic acids which are the main product of the metabolic transformations of hydrocarbons; hydrogen peroxide was a supportive inhibition agent *Lb. acidophilus*. The other products of this metabolism may only slightly contribute to

the total antagonistic activity against spoilage and pathogenic bacteria studied.

CONCLUSIONS

1. During the fermentation of saccharides contained in the medium, all the cultures of *Lb. acidophilus* under study produce – in addition to the main product, *i.e*. lactic acid – other non-specific products of cell metabolism with an antibacterial effect.

2. The main substances with antagonistic activity produced by *L. acidophilus* are lactic and acetic acids and hydrogen peroxide.

3. Due to a low level of accumulation, acetaldehyde and ethanol can play only a small supporting antibacterial role.

4. The cultures of *Lb. acidophilus* investigated reveal a variety of antibacterial activities, expressed by the antagonistic activity towards Gram-negative bacteria: *P. aeruginosa*, *P. fluorescens*, *E. coli*, and Gram-positive bacteria: *S. aureus*, *B. subtilis* and *B. mycoides*.

5. Within 20 strains of *Lb. acidophilus,* 12 strains were capable of inhibiting the germination of bacterial spores of *Bacillus* spp.

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ANTAGONISTYCZNA AKTYWNOŚĆ BAKTERII *LACTOBACILLUS ACIDOPHILUS* W STOSUNKU DO WYBRANYCH BAKTERII ZANIECZYSZCZAJĄCYCH ŻYWNOŚĆ

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Celem pracy było określenie właściwości przeciwbakteryjnych bakterii *Lactobacillus acidophilus* oraz skorelowanie jej z ilością tworzonych produktów metabolizmu o aktywności antagonistycznej. Badane kultury bakterii mlekowych charakteryzowały się zdolnością hamowania wzrostu *E. coli*, *P. fluorescens*, *S. aureus*, *B. mycoides*, *B. subtilis*. Poziom tej aktywności był wyraźnie zróżnicowany. Niektóre z badanych szczepów *Lb. acidophilus* hamowały również kiełkowanie przetrwalników bakterii z rodzaju *Bacillus* (tab. 1). Głównymi czynnikami o działaniu antagonistycznym wytwarzanymi przez badane bakterie mlekowe są kwasy organiczne (mlekowy i octowy) oraz nadtlenek wodoru (tab. 2).