10.2478/v10067-010-0002-0

ANNALES
UNIVERSITATIS MARIAE CURIE-SKŁODOWSKA
LUBLIN – POLONIA

VOL. LXIV,1 SECTIO C 2009

MAGDALENA POLAK-BERECKA, ADAM WAŚKO, DANIEL KOSTON

Department of Biotechnology, Human Nutrition and Food Commodities,
University of Life Sciences in Lublin, ul. Skromna 8, 20-704 Lublin, Poland

Comparison of different methods for detection of antimicrobial activity of probiotic strains of *Lactobacillus rhamnosus* against some food spoilage microorganisms

Porównanie różnych metod wykrywania antagonistycznej aktywności probiotyków *Lactobacillus rhamnosus* wobec bakterii gnilnych i chorobotwórczych

SUMMARY

Different methods were used to detect antibacterial activity of *Lactobacillus rhamnosus* against some food spoilage microorganisms. This study demonstrates that in comparison with culture supernatant and sonificated cells of *L. rhamnosus* the strongest antimicrobial activity was observed when live test culture was used. It can be suggested that the best results were seen with two methods: the dual culture overlay assay and the agar slab method. From comparison between methodologies it can be concluded that the agar slab technique gives the most reliable and reproducible results.

STRESZCZENIE

Jednym z najważniejszych wymagań stawianych probiotykom wykorzystywanym w przetwórstwie żywności, oprócz bezpieczeństwa zdrowotnego, jest posiadanie aktywności antagonistycznej wobec bakterii gnilnych i chorobotwórczych. Z przeprowadzonych badań wynika, że bakterie z gatunku *Lactobacillus rhamnosus* hamują wzrost *Micrococcus* sp., *Pseudomonas fluorescens, Staphylococcus aureus* i *Escherichia coli*. Istnieje wiele metod służących do szybkiego wykrywania antagonistycznych właściwości bakterii mlekowych. Są one oparte na mechanizmie dyfuzji w żelu substancji hamujących wzrost drobnoustrojów, jak np. kwas mlekowy, bakteriocyny oraz związki niskocząsteczkowe, jak nadtlenek wodoru, reuteryna, diacetyl. Antagonistyczne właściwości bakterii *L. rhamnosus* oznaczono, stosując następujące metody: metodę kropelkową, metodę studzienkową z zastosowaniem (a) płynu pohodowlanego, (b) żywych oraz (c) martwych komórek *L. rhamnosus*, metodę słupkową oraz metodę krążkową. Spośród testowanych metod metoda słupkowa okazała się najefektywniejsza, dawała najbardziej spójne, powtarzalne oraz przejrzyste wyniki.

K e y w o r d s: antibacterial activity, *Lactobacillus*, food spoilage microorgan-isms

INTRODUCTION

In recent years much attention has received the application of biopreservation. Biopreservation refers to extended shelf life and enhanced safety of foods obtained by using the natural or added microflora and their antimicrobial products (Schnürer and Magnusson, 2005). Lactic acid bacteria (LAB) have been traditionally used for preserving food products for many years, and a variety of strains have been reported to be antagonistic to pathogens associated with those products (De Vuyst and Vandamme, 1994). LAB are found in many nutrient rich environments and occur naturally in various food products such as dairy and meat products, and vegetables (Carr et al., 2002). They are known to produce different antimicrobial compounds. The primary antimicrobial effect exerted by LAB is the production of lactic and acetic acids, the pH-reducing fermentation products (Daeschel, 1989). In addition, LAB produce various antimicrobial compounds, which can be classified as low-molecular-mass (LMM) compounds such as hydrogen peroxide, carbon dioxide, diacetyl (2,3-butanedione), reuterin, and high-molecular-mass (HMM) compounds like bacteriocins and enzymes (Chen and Hoover, 2003; Holzapfel et al., 1995; Klewicka et al., 1999). All of these substances can antagonize the growth of some spoilage and pathogenic bacteria in food. The precise mechanism of antimicrobial action is difficult to elucidate due to complex and commonly synergistic interactions between different compounds (Corsetti et al., 1998; Niku-Paavola et al., 1999). Lactic acid bacteria are considered to be harmless or even to improve human and animal health acting as probiotics. They have a GRAS status (generally recognized as safe) and could be promising alternatives to chemical preservatives. Consumer demands for minimally processed foods and reduced use of chemical preservatives stimulate research on antibacterial and antifungal lactic acid bacteria as biopreservatives. In the meat industry an important role of the lactic acid bacteria is to inhibit the competing natural flora, which includes spoilage bacteria, and pathogens such as *Staphylococcus aureus* and *Listeria monocytogenes*. Studies on the inhibition of *Bacillus cereus* by LAB have been reported in nonfat milk medium (Wong and Chen, 1988) and in cheese (Rukure and Bester, 2001). Stiles et al. (2002) showed antifungal activity of *Lactobacillus rhamnosus* against species of *Penicilium* spp., *Aspergillus* spp., *Fusarium* spp., *Alternaria* spp.

Over the past decade, levels of bacterial resistance to antibiotics have risen dramatically. In the context of bacterial antibiotic resistance, the non-bacteriocin, antibiotic-like molecules produced by selected lactobacilli and bifidobacteria strains are of interest in terms of innovative antimicrobial therapy.

There are several methods used to detect antimicrobial activity (Tagg and McGiven, 1971; Strus, 1998; Klewicka et al., 1999; Ammor et al., 2006). Generally, tests for antagonism are performed on solid media and involve the detection of inhibition of growth of an indicator strain caused by the test culture. The two basic methods that are commonly used are referred to as the simultaneous (or direct) and the deferred antagonism procedures. The simplest direct test is the "spot-on-lawn" antagonism. Here, the test and indicator cultures are grown simultaneously and the demonstration of antagonism is dependent upon the release of a diffusible inhibitor early in the growth of the test culture (Tagg et al., 1976). Variations of this procedure include the use of overlapping drops and also of wells cut into freshly seeded pour plate cultures and filled with agar containing the test organism. In deferred antagonism, the test organism is grown on agar for a period of time, the bacteria are then killed, and an overlay of the indicator strain in melted agar is placed on the surface. Deferred antagonism procedures often prove to be more sensitive than simultaneous antagonism and permit the independent variation of the time and conditions of incubation of the test and indicator cultures (Tagg et al., 1976).

In the present study three strains of *L. rhamnosus* were examined for antagonistic activities against some Gram-positive and Gram-negative spoilage microorganisms and pathogenic bacteria associated with food. The aim of this work was to compare methodologies used to detect antibacterial activity of *L. rhamnosus*, and to determine sensitivity of some pathogenic bacteria.

EXPERIMENTAL

Materials and methods

Bacterial strains and growth conditions

In this study three probiotic strains of *Lactobacillus rhamnosus*: OXY, PEN, E/N (Biomed, Lublin) were used as test culture. *L. rhamnosus* was grown overnight in MRS broth (BTL, Poland) at 37°C.

Target strains were chosen to represent a range of spoilage and pathogenic organisms of concern to the food industry. Indicator microorganisms used in the experiment came from our own collection (Department of Biotechnology, Human Nutrition and Food Commodities, University of Life Science in Lublin) the following cultures were used: Gram-positive bacteria: *Staphylococcus aureus, Micrococcus sp., Enterococcus faecalis, Bacillus cereus*; Gram-negative bacteria: *Pseudomonas fluorescens, Escherichia coli, Serratia marcescens* and yeast *Rhodotorula rubra* R45. All target strains were stored at -80°C in 20% glycerol. Both the Gram-negative and Gram-positive bacteria were grown overnight in nutrient broth at 37°C (only *P. fluorescens* at 25°C), yeast was grown in wort broth at 28°C.

Detection of antagonistic activity

In order to choose the best methodology for detection of antibacterial activity of *L. rhamnosus* against food spoilage and pathogenic microorganisms the following methods were examined.

The agar spot test (the dual culture overlay assay)

Overnight cultures of *L. rhamnosus* were spotted onto the surface of agar plates (MRS) and incubated for 24 h at 37°C to allow colonies to develop. The indicator strains were inoculated into 10 ml of soft MRS agar (wort agar for yeast) and poured over the plate on which *L. rhamnosus* was grown (Schillinger and Lücke, 1989). After incubation for 24 h at 37°C (25°C for *P. fluorescens* and 28°C for *R. rubra* R45) the plates were checked for inhibition zones (Klewicka et al., 1999).

The well diffusion assay

(A). MRS agar and wort agar plates were inoculated with 0.5 ml of an overnight culture of the indicator strain. Wells of 10 mm in diameter were cut into these agar plates, and 100 µl of the test culture supernatant was placed into each well. Supernatant was prepared in three methods: (a) a cell-free solution was obtained by centrifuging the overnight culture of *L. rhamnosus*, followed by filtration of the supernatant through 0.45 µm pore-size cellulose acetate filter (Milipore), (b) supernatant prepared as described above was concentrated 5-fold with vacuum evaporator (BUCHI) at conditions of 1 Atm, 35°C. The concentrated supernatant was filtered through 0.45 µm pore-size cellulose acetate filter, (c) 5-fold concentrated supernatant was adjusted to pH 7.0 with 30% NaOH and 5% HCl in order to neutralize organic acids. Inhibitory activity of hydrogen peroxide was eliminated by addition of catalase (100 U/ml) (Schillinger and Lücke, 1989).

(B). In modification of this method each well was filled with of 100 µl of culture of *L. rhamnosus* culture and then each well was poured with 20 µl of 1% agar.

(C). In another modification the wells were filled with sonificated cells of *L. rhamnosus* (SONIC Vibra cell VC 750), and poured with 20 µl of 1% agar.

The plates were incubated for 24 h at 37°C (25°C for *P. fluorescens* and 28°C for R. rubra R45) and examined for zones of inhibition.

The paper discs test

Sterile Whatman paper discs of 5 mm in diameter were placed on the plates with indicator strain lawn. Paper discs have been soaked in 10 µl of the supernatant of overnight culture of *L. rhamnosus* filtered as previously described and 5-fold concentrated supernatant, pH 7.0 (Klewicka et al., 1999). After incubation the plates were examined for zones of inhibition in the target strains cell lawn.

The agar slab method

Plates with MRS agar were inoculated with 1 ml of *L. rhamnosus* and incubated at 37°C for 24 h. Then slabs of 10 mm and 7 mm in diameter were cut and placed on agar inoculated with 0.5 ml of target strain culture (Strus, 1998). After for 24 h of incubation at 37°C (25°C for *P. fluorescens* and 28°C for R. rubra R45) the plates were checked for inhibition zones.

Statistical analysis

Antibacterial activity of *L. rhamnosus* against pathogenic bacteria was measured as the diameter of the inhibition zone (in mm). Statistical methods included calculation of the mean value, standard deviation and analysis of variance. Each experiment was performed in three repeats.

RESULTS

The agar spot test

In this test clear and big zones of growth inhibition of target microorganisms were observed. There were no significant differences between three tested strains from *L. rhamnosus*: OXY, PEN, and E/N. The strongest antimicrobial activity of *L. rhamnosus* was observed against *P. fluorescens, Micrococcus* sp., and *S. aureus*, the inhibition zones were accordingly of 43.8 mm, 30.3 mm and 28.5 mm in diameter. Small inhibitory effect was noticed on *R. rubra* R45 (8.5 mm).

The well diffusion assay

(A) High levels of antimicrobial activity against indicator strains by 5-fold concentrated supernatant of *L. rhamnosus* was observed. When unconcentrated supernatant or supernatant neutralized to pH 7.0 were used with added catalase the zones of growth inhibition were considerably smaller. *P. fluorescens* and *Micrococcus* sp. were the most sensitive strains, whose zones of growth inhibition measured 21.0 mm and 20.0 mm for 5-fold concentrated supernatant, and 10.8 mm for unconcentrated supernatant. Both supernatants did not inhibit the growth of *R. rubra* R45. When neutralized supernatant + catalase were used only

P. fluorescens, Micrococcus sp., and *S. aureus* (5.8 mm, 4.3 mm, and 4.0 mm accordingly) were inhibited.

(B) This modification showed high antimicrobial activity of *L. rhamnosus* against *Micrococcus* sp. (15.0 mm), *P. fluorescens* (13.5 mm) and *E. coli* (11.3 mm) and *R. rubra* R45 was resistant.

(C) In the case of this test similar results were observed as in previous modification. However, when sonificated cells of *L. rhamnosus* were used, the zones of growth inhibition were smaller: 4.5 mm, 5.0 mm, and 4.5 mm for *Micrococcus* sp., *P. fluorescens*, and *E. coli*, respectively.

The paper discs test

In comparison with unconcentrated supernatant significant differences in size of zones of inhibition were noticed when 5-fold concentrated supernatant was used. Difference in dimension was from 1 mm to 4 mm depending on the strain. The most sensitive strain was *P. fluorescens* (7.8 mm for 5-fold concentrated and 5.0 mm for unconcentrated supernatant) whereas *S. marcescens* was only slightly inhibited (2.3 mm for 5-fold concentrated, and 0.8 mm for unconcentrated supernatant), and *R. rubra* was not inhibited.

The agar slab method

L. rhamnosus showed high level of antimicrobial activity against all indicator strains tested. We observed clear and large zones of growth inhibition of target microorganisms. The strongest antimicrobial activity was observed against *P. fluorescens* (17.8 mm) *Micrococcus* sp. (14.0 mm), and *S. aureus* (13.8 mm), small inhibitory effect was noticed against *S. marcescens* (6.8 mm) and *R. rubra* (3.5 mm). Any significant differences in the size of the zones of inhibition were seen between the slabs of 10 mm and 7 mm.

The results of this comparative studies are summarized in Table 1 and on Figures 1 and 2. Figure 1 shows the results of standard deviation and the mean size of the zones of inhibition detected by all methods. Figure 2 illustrates the results of analysis of variance using *P. fluorescens* as an example.

DISCUSSION

Methods used in this study to demonstrate antagonism are referred to generally the simultaneous (or direct) antagonism procedures based on diffusion of inhibitory substance in the agar medium (Tagg et al., 1976). The results indicated that *L. rhamnosus* exerted strong inhibitory effect on *Micrococcus* sp., *P. fluorescens, S. aureus*, and *E. coli*. Any inhibitory effect on *S. marcescens* and *R. rubra* was

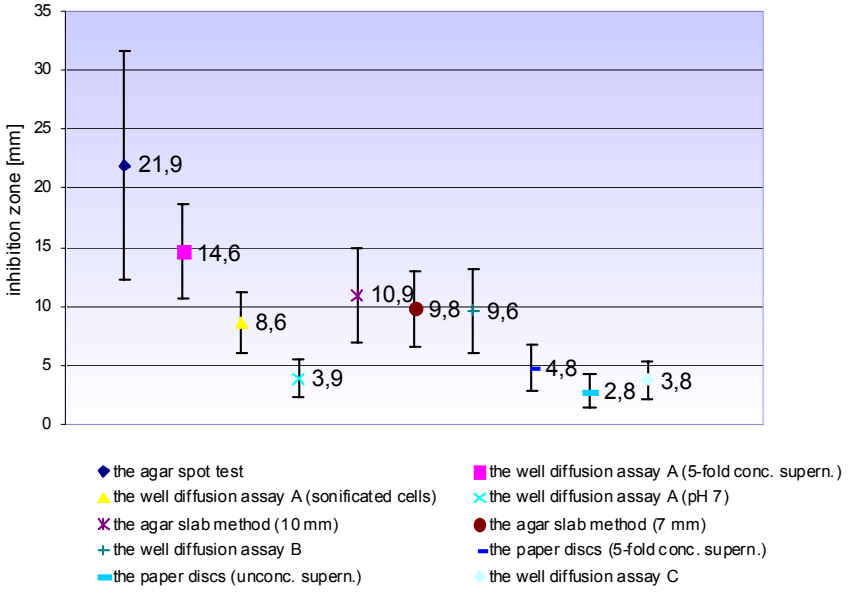

Figure 1. Standard deviation and mean size value of inhibition zone diameters obtained in tested methods for estimation of antimicrobial activity of *L. rhamnosus* against some food pathogens

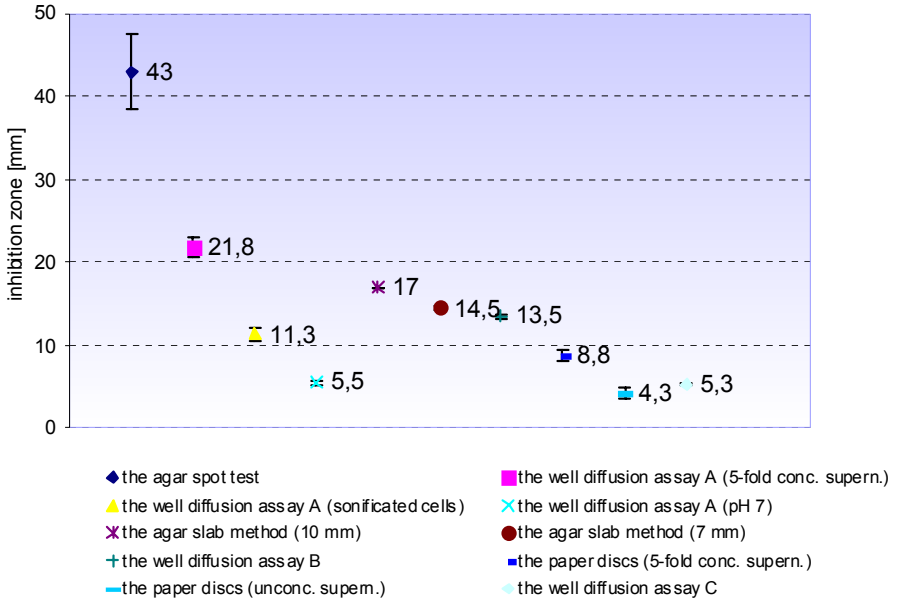

Figure 2. Analysis of variance as exemplified by the inhibition of *P. fluorescens* by *L. rhamnosus*

detected. The composition of the medium and culture conditions may indirectly affect the sensitivity of the indicator strain (Tagg et al., 1976). It may be stated that the size of inhibition zones depends not only on sensitivity of the target strain on antimicrobial compounds produced by *L. rhamnosus* but also on the method used for detection. The most reliable results of antimicrobial activity of *L. rhamnosus* were observed in case of three methods: the agar spot test (the dual culture overlay assay), the well diffusion assay (A), and the agar slab method. The highest mean value of inhibition zones (22 mm) and the larger dispersion (between12 and 32 mm) were in case of the agar spot test (Fig. 1). Taking into account mean value of inhibition zones good results were observed in case of the well diffusion assay (A) and the agar slab method. Results of variance analysis indicate that the most reliable and reproducible results were observed with the agar slab method (Fig. 2). From our study it may be concluded that when indicator strain was grown in agar medium the larger and most clear inhibition zones were observed with the agar spot test and the agar slab method. Antimicrobial property of *L. rhamnosus* against various food pathogens is probably connected with production of the extracellular, diffusable inhibitory substances. Inhibitory effect was caused mainly by lactic acid produced by test strain of LAB (Annuk et al., 2003). Raczyńska-Cabaj and co-workers (2005) confirm antagonistic role of lactic acid produced by *Lactobacillus*.

In comparison with supernatant, the strongest antimicrobial activity was observed when the live cells of *L. rhamnosus* were used. It is confirmed by Fernandez, Boris and Barbes (2003). Good results were also obtained with the well diffusion assay with 5-fold concentrated supernatant but the drawback of this method is labour-consuming. Klewicka et al. (1999) also suggest that the most reliable results were obtained with two methods: the dual culture overlay assay and the agar slab method. Moreover, it was found in one study (Sip, 1999) that *Lactobacillus* grown on agar medium is able to synthetize other inhibitory substances – bacteriocins in significantly greater amount than that in liquid culture. Production and release of antimicrobial molecules by *Lactobacillus* is known to be variable with factors such as cell density and population kinetics (Delgado et al., 2007; Stoyanova and Levina, 2006). Such differences could account for the failure to detect inhibition zones in well-diffusion tests. Growth of pathogens was less inhibited by cell-free extracts from *L. rhamnosus* than by live culture of *L. rhamnosus*. Calculating mean values and standard deviations for diameters of inhibitory zones and making variance analysis showed that in comparison to other methods with the agar slab technique the most consistent and reproducible results were obtained. These results are in agreement with another study in which three methods were compared: paper disc, double layer and agar slab test (Strus, 1999).

REFERENCES

1. Ammor S., Tauveron G., Dufour E., Chevallier I., 2006. Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small-scale facility. 1 – Screening and characterization of the antibacterial compounds. Food Control 17, 454–461.
2. Annuk H., Schopetova J., Kullisaar T., Songisepp E., Zilmer M., Milelsaar M., 2003. Characterization of intestinal lactobacilli as putative probiotic candidates. J. Appl. Microbiol. 94, 403–412.
3. Carr F. J., Chill D., Maida N., 2002. The lactic acid bacteria: a literature survey. Crit. Rev. Microbiol. 28, 281–370.
4. Chen H., Hoover D.G., 2003. Bacteriocins and their food applications. Compr. Rev. Food Sci. Food Saf. 2, 82–100.
5. Corsetti A., Gobbetti M., Rossi J., Damiani P., 1998. Antimould activity of sourdough lactic acid bacteria: identification of a mixture of organic acids produced by *Lactobacillus sanfrancisco* CB1. Appl. Microbiol. Biotechnol. 50, 253–256.
6. Daeschel M. A., 1989. Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technol. 43, 164–166.
7. De Vuyst L., Vandamme E. J., 1994. Lactic acid bacteria and bacteriocins: their practical importance. In: De Vuyst, L., Vandamme, E. J. (Eds.), Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications, Blackie Academic and Professional, Glasgow, 1–12.
8. Delgado A., Arroyo López F.N., Bito D., Peres C., Fevereiro P., Garrido-Fernández A., 2007. Optimum bacteriocin production by *Lactobacillus plantarum* 17.2b requires absence of NaCl and apparently follows a mixed metabolite kinetics. J. Biotechnol. 130, 193–201.
9. Fernández M. F., Boris S., Barbés C., 2003. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. J. Appl. Microbiol. 94, 449–455.
10. Holzapfel W. H., Geisen R., Schillinger U., 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. Int. J. Food Microbiol. 24, 343–362.
11. Klewicka E., Libudzisz Z., Czajka D., Kuc K., 1999. Antagonistic activity of lactic acid bacteria *Lactobacillus acidophilus*. [Antagonistyczna aktywność bakterii fermentacji mlekowej *Lactobacillus acidophilus*.] Żywność 4 (21) 168–175.
12. Niku-Paavola M. L., Laitila A., Mattila-Sandholm T., Haikara A., 1999. New types of antimicrobial compounds produced by *Lactobacillus plantarum*. J. Appl. Microbiol. 86, 29–35.
13. Raczyńska-Cabaj A., Kraszewska J., Wzorek W., Sztando E., 2005. Antagonistic activity of lactic acid bacteria from strain *Lactobacillus plantarum*. Aktywność antagonistyczna bakterii fermentacji mlekowej z gatunku *Lactobacillus plantarum*. Acta Scie. Pol., Tech. Alimentaria 4 (1), 39–51.
14. Rukure G., Bester B. H., 2001. Survival and growth of *Bacillus cereus* during Gouda cheese manufacturing. Food Control 12, 31–36.
15. Schillinger U., Lücke F. K., 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. and Environ. Microbiol. 55, 1901–1906.
16. Schnürer J., Magnusson J., 2005. Antifungal lactic acid bacteria as biopreservatives. Trends Food Sci. Technol. 16, 70–78.
17. Sip A., 1999. Bacteriocin production by lactic acid bacteria. Produkcja bakteriocyny przez bakterie mlekowe. Biotechnologia 2, 145–165.
18. Stiles J., Penkar S., Plockova N., Chmuchalova J., Bullerman L.B., 2002. Antifungal activity of sodium acetate and *Lactobacillus rhamnosus*. J. Food Protect. 65, 1188–1191.

19. Stoyanova L. G., Levina N. A., 2006. Components of fermentation medium regulate bacteriocin synthesis by the recombinant strain *Lactococcus lactis* subsp. Lactis F-116. Microbiol. 75, 286–291.
20. Strus M., 1998. A new method for evaluation of the antagonistic action of bacterial lactic acid (LAB) on selected pathogenic indicator bacteria. Nowa metoda oceny antagonistycznego działania bakterii kwasu mlekowego (LAB) na wybrane, chorobotwórcze bakterie wskaźnikowe. Med. Dośw. Mikrobiol. 50, 123–130.
21. Tagg J. R., Dajani A. S., Wannamaker L. W., 1976. Bacteriocins of Gram-positive bacteria. Bacteriol. Rev. 40, 722–756.
22. Tagg J. R., McGiven A. R., 1971. Assay system for bacteriocins. Appl. Microbiol. 21, 943.
23. Wong H. C., Chen Y. L., 1988. Effects of lactic acid bacteria and organic acids on growth and germination of *Bacillus cereus*. Appl. Environ. Microbiol. 54, 2179–2184.

Table 1. Antimicrobial inhibition spectra of *L. rhamnosus* OXY measured as inhibition zone diameters [mm] of indicator strains

Indicator strain	The agar spot test	The well diffusion assay					The agar slab method		The paper disc method	
		A			B	C	Slabs of 10 mm in diam.	Slabs of 7 mm in diam.	5-fold conc.	Unconc. supern.
		Unconc. supern.	5-fold conc.	pH 7.0 + catalase						
P. fluorescens	43.8 (±1.64)	10.8 (±0.43)	21.0 (±0.71)	5.8 (±0.43)	13.5 (±0.50)	4.5 (±0.50)	17.8 (±0.43)	15.5 (±0.50)	7.8 (±0.83)	5.0 (±0.71)
Micrococcus sp.	30.3 (±0.83)	10.8 (±0.83)	20.0 (±0.71)	4.3 (±0.43)	15.0 (±0.71)	5.0 (±0.71)	14.0 (±0.00)	13.0 (±0.00)	6.3 (±0.83)	4.0 (±0.71)
E. coli	20.5 (±0.50)	10.3 (±1.09)	16.0 (±1.00)	4.0 (±0.00)	11.3 (±0.83)	4.5 (±0.50)	13.0 (±0.00)	11.8 (±0.43)	6.3 (±0.83)	3.0 (±0.71)
S. anatum	19.0 (±0.71)	10.0 (±0.71)	15.3 (±0.83)	0.0	10.5 (±1.12)	3.8 (±0.43)	11.8 (±0.43)	10.0 (±0.00)	5.3 (±0.83)	3.0 (±0.71)
E. fecalis	16.5 (±0.50)	9.3 (±0.43)	14.3 (±0.83)	0.0	8.5 (±0.50)	3.5 (±0.50)	9.8 (±0.43)	8.0 (±0.00)	4.8 (±0.83)	2.8 (±0.83)
S. aureus	28.5 (±0.50)	9.0 (±0.71)	14.0 (±0.71)	0.0	9.3 (±0.83)	2.8 (±0.43)	13.8 (±0.43)	11.5 (±0.50)	4.0 (±0.71)	2.5 (±0.50)
B. cereus	20.0 (±0.71)	7.8 (±0.43)	10.8 (±0.83)	0.0	8.8 (±0.83)	2.0 (±0.71)	12.0 (±0.00)	10.8 (±0.83)	2.8 (±0.43)	1.5 (±0.50)
S. marcescens	14.8 (±0.43)	2.8 (±0.83)	9.0 (±0.71)	0.0	5.3 (±0.83)	1.0 (±0.00)	6.8 (±0.43)	5.8 (±0.43)	2.3 (±0.43)	0.8 (±0.43)
R. rubra R45	8.5 (±0.50)	0.0	0.0	0.0	0.5 (±0.50)	0.0	3.5 (±0.50)	2.0 (±0.71)	0.0	0.0

A – cell free supernatant
B – cultured cells
C – sonicated cells