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PHYSICAL AND IMMUNOBIOLOGICAL STUDIES OF TEICHOIC ACIDS OF PROBIOTIC LACTOBACILLI STRAINS

Aim. The aim of this study was to establish the chemical structure of teichoic acids of probiotic lactobacilli strains and to investigate their influence on murine macrophages phagocytosis and proliferation of the murine macrophages cell line J 774. **Methods.** Physical (infrared spectroscopy methodology, ¹H, and ¹³C NMR experiments), immunobiological and statistical methods were used. **Results.** It was shown that cell walls of *Lactobacillus plantarum* 11/16 and *Lactobacillus plantarum* 195D contained glycerol teichoic acids with unusually high number of sugar elements. The teichoic acid of the strain *L. plantarum* 11/16 had 9 different pyranoses and furanoses sugars in alfa-configuration and *L. plantarum* 195D had 4 hexosopyranoses. The teichoic acids of *L. plantarum* 195D and 11/16 increased the percent phagocytosis by 15.00±5.72% and 13.75±4.65% respectively. Both teichoic acids in the doses of 32 and 64 µg/ml stimulated cell proliferation of murine macrophage cell line by 35–53%. It was not observed any considerable effect of studied teichoic acids on phagocytic index, spontaneous and stimulated NBT-test of peritoneal macrophages of mice.

Key words: teichoic acids, NMR spectroscopy, infrared spectroscopy, phagocytosis, probiotics.

The role of teichoic acids (TA) of grampositive bacteria in many functions related to mechanical stability, adhesive properties, biofilm formation ability, cation balance and immune activity is widely discussed. These biopolymers are of interest as patogenicity factor of causative agents of infectional diseases and as bioactive molecules. At the same time TA of normal human microbiota representatives and its impact to bacteria biological activity have not been enough studied yet. Nowadays a large part of modern investigations in the area of health keeping is devoted to probiotics and functional foods. Traditionally probiotics are represented by lactic acid bacteria, particularly, belonging to genus *Lactobacillus*. *Lactobacillus plantarum* is a lactic acid bacteria that is widely isolated from the environment and home-made foods and used in industrial productions of various fermented foods [17]. Probiotic

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effects are contributed by antagonistic effects against pathogenic microorganisms and immune system stimulation. However, very little has been known about the molecular mechanisms by which health-promoting probiotic bacteria act as the host cell modulators [7, 10]. As bacterial envelope components are the first to establish bacterial-host cell interactions are very little-studied TA may play an essential role.

The aim of this study was to establish the chemical structure of TA of probiotic lactobacilli strains and to investigate their biological effects.

Materials and methods

Strains. The objects of the study were probiotic strains of lactic acid bacteria *Lactobacillus plantarum* 11/16 and *L. plantarum* 195D. These strains were obtained from Ukrainian collection of microorganisms of D.K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine. The studied strains attract the scientific interest due to the source of isolation and the high biological activity as well [3].

Teichoic acids were obtained from native cells by trichloroacetic acid extraction and purified using ion exchange chromatography and dialysis as it was previously described [9].

To perform NMR analysis lyophilized material (20 mg) was dissolved in 0.5 ml deuterium oxide. NMR spectra were recorded using a Bruker Avance DRX500 spectrometer at ambient temperature 20 °C with dioxan (^1H (3,75) and ^{13}C (67,19) as the internal standard and frequencies 500 MHz and 125 MHz. Spectra processing was performed with standard software Bruker Top Spin, Version 2.1.

Infrared spectra were obtained with a IR Fourier spectrometer FSM-1202 in the wavenumber range of 4000-600 cm^{-1} . To perform measurements the lyophilized samples were mixed with KBr (1:300) mg and pressed into transparent tablets (d = 13 mm) [14].

The effect of TA on the phagocytic and cytotoxic activities (nitro-blue tetrazolium (NBT-test)) of peritoneal macrophages was studied on albino mice aged 18-20 weeks. Due to the fact that the value of functional activity of macrophages is specific for every macroorganism only the degree of its change in the experiments was assessed. Also in order to implement the most general and the most appropriate assessment of influence of substances on studied parameters nonlinear animals (males and females equally) were used. All mouse experiments were conducted following bioethics guidelines and were in full accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

To obtain macrophage the mice were euthanized by cervical dislocation and the peritoneal fluid was collected in 5 ml of modified 199 medium (containing 10% bovine serum) after gentle massage of the abdomens of the animals. Portion of the peritoneal cell suspension containing 10^6 cells per ml was used for *in vitro* phagocytosis assays.

In order to investigate the influence of TA on phagocytosis peritoneal macrophages TA were added to the cells before the incubation (0.1, 1, 2 mkg/ml). To measure phagocytosis activity aliquots of peritoneal macrophages (10^6 cells per ml)



were incubated for 30 min at 37 °C with the same volume of latex suspension (latex particles; 10^7 particles per ml). The percentage of macrophages that had ingested latex particles (percent phagocytosis) and the average number of ingested latex particles (phagocytosis index) were estimated with the Zeiss microscope immersion method by counting 100 cells.

To estimate cytotoxic activity (nitro-blue tetrazolium (NBT-test)) the macrophages were incubated in 0.2% NBT PBS solution at 37 °C for 30 min. The percent of cells containing diformazan granules was calculated [8].

In order to investigate the influence of teichoic acids on cell proliferation the murine macrophage cell line J 774 was used. The cells were cultivated in DMEM medium in the presence of 10% newborn calf serum (PAA, Austria) at the temperature of 37 °C in the presence of 5% CO₂ in the humid atmosphere. After the monolayer had been formed the cells were passaged every 3–4 days using trypsin-versen solution (Sigma, USA) at a ratio of 1:10. Suspension of cells was incubated with TA (500 mkg/ml) for 48 hours at the same conditions.

The estimation of the results was performed by colorimetric test with vital stain MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) use. The percentage of living cells was measured by determining of optical density on spectrophotometer MCC-340 (Labsystem, Finland) at 540 nm.

Statistical Techniques. Data for phagocytosis and cell proliferation are represented as the mean \pm standard deviation of means. All data were tested for significance by analysis of variance with the Statistica Program, release 5.0. The level of significance tested in each instance was $P < 0.05$.

Results

In order to be able to clarify the possible mechanisms of biological activity and, in particular, probiotic action of TA their chemical structure was studied. When both teichoic acid preparations were examined by infrared spectroscopy, specific absorbencies were observed at the bands of 839, 870, 964, 1022, 1146, 1148, 1216, 1336, 1509, 1640, 1750 cm⁻¹. The absorption bands $\nu \sim 3500$ cm⁻¹ and $\nu \sim 1640$ cm⁻¹ are specific for stretching and deformation vibrations of OH bonds (Fig. 1 a, c).

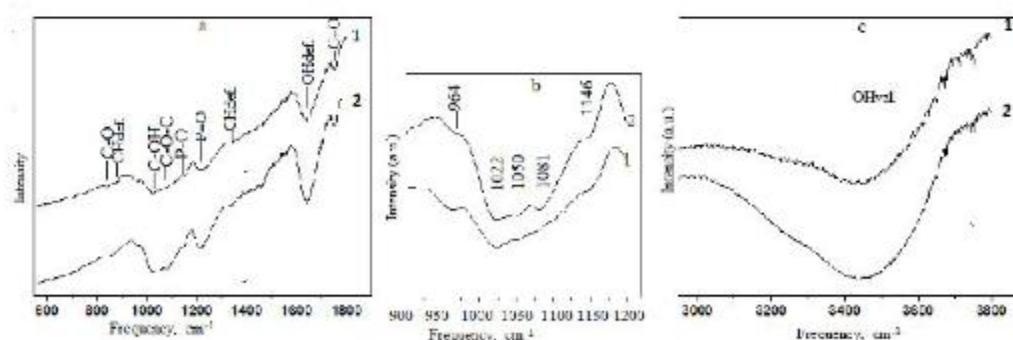


Fig. 1. Infrared spectra of teichoic acids from *Lactobacillus plantarum* 11/16 (1) and *Lactobacillus plantarum* 195D (2)



The signals $\nu\sim 2870$, 1336 and 964 cm^{-1} belong to stretching and deformation vibrations respectively of CH bonds. Absorption band in the $\nu\sim 1750\text{ cm}^{-1}$ corresponds to the C=O bonds. There were intense bands ($1250\text{--}1050\text{ cm}^{-1}$) which can correspond either to stretching vibrations C-O-C of ester group, or to C-O vibrations of COOH. The interpretation difficulties of this area occurred due to the proximity and overlay of these groups signals. The absorption bands at $\nu\sim 870$, 1022 cm^{-1} , corresponding C-O, C-OH, respectively, were also observed (Fig. 1) [1, 12, 13].

The significant disparity in the spectra was the different intensity at 3500 cm^{-1} (-OH group), indicating their different amounts in the samples – TA of the strain *L. plantarum* 195D contained more OH-groups than TA of the strain *L. plantarum* 11/16. In addition, the range $1200\text{--}900$, corresponding to the C-O-C and C-O bonds was also different, which may indicate the differences in the carbohydrate components of the investigated substances. Thus, all functional groups, that are specific for the TA, were identified. These data formed the basis for further studies using NMR analysis.

^{13}C spectra revealed chemical shifts specific for glycerol and α -hexose containing compounds. The signals were partly expanded or split into duplets or triplets due to interaction with phosphorus. Glycerol residues were identified by signals in the characteristic areas (chemical shifts corresponding to 1,3polyglycerol phosphate links – 61.15 and 66.84 ppm – for *L. plantarum* 11/16; and 61.11 , 66.81 ppm – for *L. plantarum* 195D – CH_2 -atoms according to APT-spectra; 71.87 ppm – for *L. plantarum* 11/16 and 71.82 in case *L. plantarum* 195D – CH-atoms, according to APT-spectra (Fig. 2 A, B).

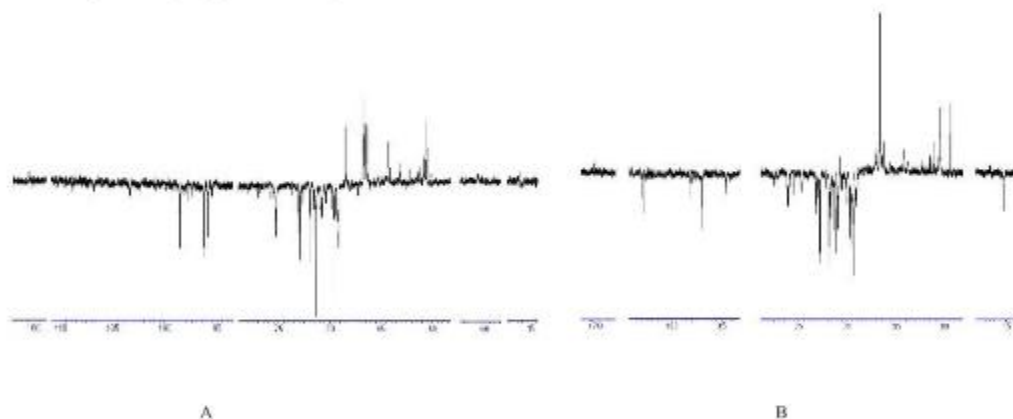


Fig. 2. Carbon NMR-spectra of teichoic acids from *Lactobacillus plantarum* 11/16 (A) and *Lactobacillus plantarum* 195D (B)

^1H -spectroscopy also showed the presence of the signals characteristic for polyglycerol chain – 3.79 ppm , 3.97 ppm and 3.92 ppm for the spectra of the strain *L. plantarum* 11/16, and 3.79 ppm , 3.97 ppm and 3.89 ppm – for *L. plantarum* 195D with specific splits because of interaction with phosphorus (Fig. 3).

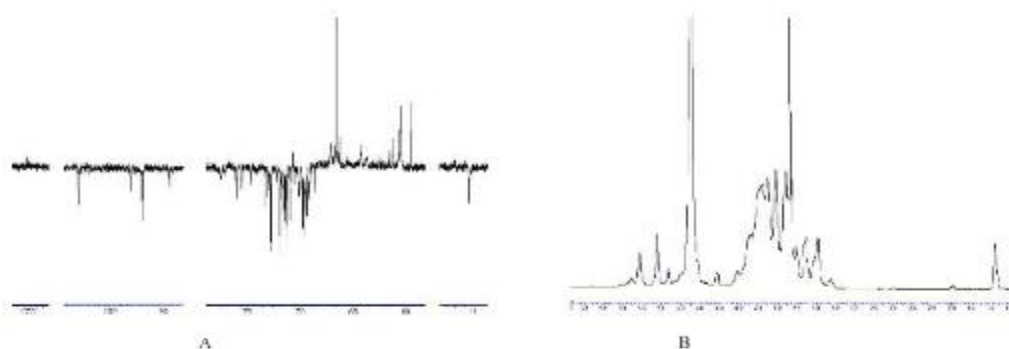


Fig. 3. Proton NMR-spectra of teichoic acids from *Lactobacillus plantarum* 11/16 (A) and *Lactobacillus plantarum* 195D (B)

Sugar components identification was complicated due to signal overload of the spectra. Nonetheless we can definitely make a conclusion about the presence, quantity and configuration of the sugar components. The ^1H and ^{13}C spectra contained anomeric signals corresponding to α -atoms of the sugar residues. The spectra of TA of the strain *L. plantarum* 11/16 revealed the presence of 9 sugar units the two of which were represented by furanose configuration (signals 107.26 and 109.33 ppm) and seven – pyranoses (95.97, 96.31, 96.66, 97.91, 98.35, 99.07, 103.88 ppm). The difference of signal intensity indicated the different amount of these substituents. The domination of three α -pyranoses with chemical shifts (96.31, 96.66, 99.07 ppm) was observed. This also has been reflected in ^1H spectrum, where some of peaks could not be observed.

The ^{13}C and ^1H spectra of the TA obtained from *L. plantarum* 195D strain showed 4 anomeric signals indicating the presence of 4 α -hexose substitution in the polymer (chemical shifts are 95.14, 97.51, 98.60 and 103.50 ppm and 4.50, 5.00, 5.12, 5.30 ppm, respectively).

When the ability of the teichoic acids to activate macrophages *in vitro* was tested, only the concentration of 2 mkg/ml was able to cause statistically significant influence on phagocytic activity.

TA of *L. plantarum* 195D and 11/16 increased the percent phagocytosis by $15,00 \pm 5,72\%$ and $13,75 \pm 4,65\%$, respectively (Fig. 4). Other doses of teichoic acids had no statistically significant effect on the value of percent phagocytosis.

None of the studied TA concentrations had significant impact on phagocytic index of mice peritoneal macrophages. There was no detected effect of the TA on the spontaneous and stimulated NBT-test.

The study of the lactobacilli TA effect on the proliferation of murine macrophage cell line J774 has demonstrated a dose-dependent effect of both investigated substances on the cells. TA of *L. plantarum* 11/16 and 195D in doses 32 and 64 $\mu\text{g/ml}$ stimulated cell proliferation by $35,25 \pm 23,64\%$, $53,54 \pm 23,26\%$ and $53,03 \pm 17,13\%$, $44,97 \pm 18,16\%$, respectively (Fig. 5). The rest of the studied doses of teichoic acids has not significantly effected the level of macrophage proliferation.

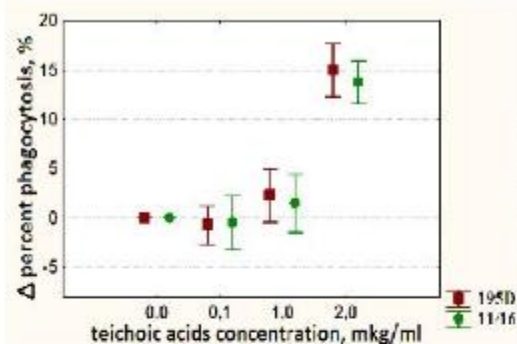


Fig. 4. The effect of teichoic acids from *Lactobacillus plantarum* 11/16 and 195D on the mice peritoneal macrophages percent phagocytosis change

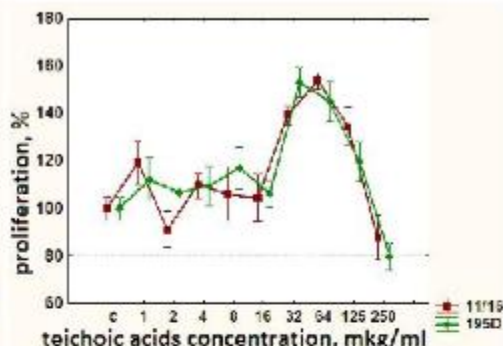


Fig. 5. The effect of teichoic acids from *Lactobacillus plantarum* 11/16 and 195D on the cell proliferation of cell line J774 mice macrophages.

Discussion

The use of infrared spectroscopy in this study was aimed to determine the functional groups in the TA molecules of tested strains and compare them with each other. Infrared spectroscopy has rather limited use in studies of most organic compounds due to their high molecular weight. Nevertheless we have found that all absorption bands characteristic for TA functional groups were present in the obtained spectra.

The biological role of teichoic acids has drawn great attention since it is known to be a key molecule that triggers a range of diseases by pathogens and immunity in case of “beneficial” bacteria as well. Among lactobacilli, wall teichoic acids (WTA) ultrastructures of *L. plantarum* are the most studied. It is known that *L. plantarum* is the only lactobacilli species which can contain either polyribitolphosphate or polyglycerophosphate in its WTA [15, 17]. Our data showed the presence of polyglycerophosphate in WTA of the tested strains.

The glycosyl residues of teichoic acids are commonly represented by multiple glucose residues in a range of configurations [15], but usually no more 1 or 2 kinds per molecule [5, 6, 11]. However our study revealed the presence of unusually high number of sugar units (up to 9). So it is not clear yet whether our finding may contribute to investigation of biological activity of lactobacilli. It was suggested that such high structural diversity is important for their lifestyle [16]. It was also discussed that the sugar components could be responsible for biological characteristics of these molecules [4]. There is further necessity in detailed and comprehensive study of molecular structure of TA and its connection with biological activity of probiotic bacteria.

It is known that TA of gram-positive bacteria can demonstrate biological activity and influence upon host immune system parameters. Teichoic acids of the pathogenic bacteria can act as pathogenicity factors in the infectious process whereas the role of these polymers in probiotic properties of lactic acid bacteria is poorly understood.

It is known that oral and parenteral administration of TA of lactobacilli to laboratory animals can cause increase of the functional activity of macrophages including

results of NBT-test [2]. In our work we have investigated the effect of teichoic acids of *Lactobacillus plantarum* 195D and *L. plantarum* 11/16 on the functional activity of murine macrophages in *in vitro* system.

The obtained results have indicated that the TA of the studied lactobacilli strains indeed showed some biological effects in particular increased the percent phagocytosis of murine macrophages and stimulated proliferation of macrophage cell line. Such effects were dose-dependent. The teichoic acids stimulating effect on proliferation of macrophage cell line J 774 is of special interest, because such data have not been found in the available sources. Moderate increasing of percent phagocytosis can be considered as one of the mechanisms of probiotic properties of investigated lactobacilli.

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ДОСЛІДЖЕННЯ ТЕЙХОЄВИХ КИСЛОТ ПРОБІОТИЧНИХ ШТАМІВ ЛАКТОБАЦИЛ ІЗ ЗАСТОСУВАННЯМ ФІЗИЧНИХ ТА ІМУНОБІОЛОГІЧНИХ МЕТОДІВ

Реферат

Мета. Метою роботи було встановити хімічну будову тейхоєвих кислот пробіотичних штамів лактобацил та дослідити їх вплив на фагоцитоз макрофагів мишеї та проліферацію макрофагів клітинної лінії J 774. **Методи.** Було використано фізичні (інфрачервона спектроскопія та спектроскопія ЯМР ¹H, ¹³C), імунобіологічні та статистичні методи. **Результати.** Показано, що клітинні стінки *Lactobacillus plantarum* 11/16 і *Lactobacillus plantarum* 195D містять гліцепротейхоєві кислоти із нехарактерно великою кількістю глікозильних залишків. Тейхоєві кислоти штаму *L. plantarum* 11/16 містили 9 різних піраноз та фураноз в альфа-конфігурації, *L. plantarum* 195D – 4 гексозопіранози. Тейхоєві кислоти штаму *L. plantarum* 195D та 11/16 підвищували показник фагоцитозу на 15,00±5,72% та 13,75±4,65%, відповідно. Обидва препарати тейхоєвих кислот у дозах 32 та 64 мкг/мл стимулювали проліферацію макрофагів мишеї клітинної лінії на 35–53%. Не спостерігалось впливу тейхоєвих кислот лактобацил на фагоцитарне число та показники функціональної активності макрофагів, отримані за допомогою індукованого та не індукованого НСТ-тесту.

Ключові слова: тейхоєві кислоти, ЯМР спектроскопія, інфрачервона спектроскопія, фагоцитоз, пробіотики.



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ШТАММОВ ЛАКТОБАЦИЛЛ С ПРИМЕНЕНИЕМ ФИЗИЧЕСКИХ
И ИММУНОБИОЛОГИЧЕСКИХ МЕТОДОВ****Реферат**

Цель. Целью работы было установить химический состав тейхоевых кислот пробиотических штаммов лактобацилл и исследовать их влияние на фагоцитоз макрофагов мышей и пролиферацию макрофагов клеточной линии J 774. **Методы.** Было использовано физические (инфракрасная спектроскопия и спектроскопия ЯМР ¹H, ¹³C), иммунобиологические и статистические методы. **Результаты.** Показано, что клеточные стенки *Lactobacillus plantarum* 11/16 и *Lactobacillus plantarum* 195D содержат глицеротейхоевые кислоты с нехарактерно большим количеством гликозильных остатков. Тейхоевые кислоты штамма *L. plantarum* 11/16 содержали 9 различных пираноз и фураноз в альфа-конфигурации, *L. plantarum* 195D – 4 гексозопиранозы. Тейхоевые кислоты штамма *L. plantarum* 195D и 11/16 повышали показатель фагоцитоза на $15,00 \pm 5,72\%$ и $13,75 \pm 4,65\%$, соответственно. Оба препарата тейхоевых кислот в дозах 32 и 64 мкг/мл стимулировали пролиферацию макрофагов мышей клеточной линии на 35–53%. Не наблюдалось влияния тейхоевых кислот лактобацилл на фагоцитарное число и показатели функциональной активности макрофагов, полученные с помощью индуцированного и не индуцированного НСТ-теста.

Ключевые слова: тейхоевые кислоты, ЯМР спектроскопия, инфракрасная спектроскопия, фагоцитоз, пробиотики.

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