

MATERIALS AND METHODS

Research object was the strain *E. coli* M-17 (a component of the Bificol and Colibacterin probiotics). Bacteria were grown on meat infusion agar for 24 h at 28–30°C. The cells were harvested by centrifugation (20 min, 5000g) and dried with acetone and ether.

LPS isolation. LPS was extracted from dried cells with 45% aqueous phenol solution at 65–68°C. Aqueous fractions were dialyzed against tap and then against distilled water for phenol removal [4].

Determination of the content of carbohydrates, nucleic acids and protein. The amount of carbohydrates was determined by the method of Dubois [4]. The results were assessed spectrophotometrically (490 nm) by color change resulting from the reaction of phenol with sulfuric acid. The content of carbohydrates was determined in accordance with the standard calibration curves plotted for glucose.

The content of nucleic acids and proteins was assessed by the methods of Spirin [4] and Lowry with the Folin reagent [4], respectively.

Identification of neutral monosaccharides was carried out after hydrolysis of the preparations in 2 N HCl (5 h, 100°C). Monosaccharides were analyzed as alditol acetates [5] on an Agilent 6890/5973N chromatography–mass spectrometry system with a DB-225mS column (30 m × 0.25 mm × 0.25 μm), with helium as a carrier gas, and a flow through the column of 1 mL/min. The temperatures of the evaporator, interface and thermostat were 250, 280, and 220°C, respectively (isothermal mode). Monosaccharides were identified by comparing the retention time of alditol acetates in the experimental and standard samples and using the ChemStation database. The quantitative ratios of individual monosaccharides were expressed as percentage of the total peak area.

Fatty acid composition was determined upon sample hydrolysis in 1.5% acetyl chloride solution in methanol (100°C, 4 h); fatty acid methyl esters were analyzed on an Agilent 6890N/5973 inert chromatography–mass spectrometric system with a HP-5MS column (30 m × 0.25 mm × 0.25 μm), at the temperature mode of 150–250°C and temperature gradient, 4°C, with helium as the carrier gas at the flow rate of 1.2 mL/min. The evaporator temperature was 250°C; flow distribution was 1 : 100. Fatty acids were identified using the PC database and the standard mixture of fatty acid methyl esters.

NMR spectroscopy. NMR spectra were recorded for the OPS solution in 99.95 D₂O at 30°C on a Bruker Avance II 600 spectrometer (Germany) using Ethyl-3-(trimethylsilyl)propionate-2,2,3,3-d₄ (δH 0 ppm) and acetone (δC 31.45 ppm) as internal standards. Before spectrum recording, the samples were lyophilized from 99.5% D₂O.

LPS pyrogenicity was determined in rabbits weighing 2.0–3.5 kg [4]. Thermometry was performed with an electronic thermometer (Omron Matsusaka Co.

Ltd., Japan), which was inserted into the rectum to a depth of 5–7 cm (depending on the weight of a rabbit). All rabbits were pretested for immunoreactivity by intravenous injection of 10 mL/kg of 0.9% sterile apyrogenic sodium chloride solution. The tested LPS preparations were dissolved in a sterile apyrogenic isotonic solution, incubated for 10 min at 37°C before the injection, and introduced intravenously (1 mL/kg of animal weight). The minimal pyrogenic dose of LPS preparations was determined in a series of dilutions from 0.5 to 1.0 × 10⁻² mg/mL. Each series of the solutions was tested in 3 rabbits of similar weight (at a difference not exceeding 0.5 kg).

Prior to injection of the LPS solution, the temperature of the rabbits was measured twice with a 30-min interval. Since the difference between temperature indices should not exceed 0.2°C, the animals not satisfying this criterion were not used for investigation. The result of the last measurement was taken as the initial temperature. LPS solution was injected not later than 15–20 min after the last temperature measurement. After the injection, the measurements were made three times with 1-h intervals. The tested LPS solution was considered apyrogenic if the total temperature increase during 3 h did not exceed 1.4°C.

LPS toxicity was determined in healthy white non-pedigreed mice of both sexes weighing 19–21 g, not used previously for any experiments and sensitized with galactosamine. For this purpose, 0.5 mL of 3.2% D-galactosamine hydrochloride solution in apyrogenic sterile 0.9% NaCl solution was injected intraperitoneally into all mice (both control and experimental ones). Immediately after that, 0.2 mL of LPS in isotonic sterile apyrogenic saline solution heated to 37°C was injected intraperitoneally at a rate of 0.1 mL/s. In the series of LPS dilutions, the preparation dose causing death of 50% tested animals (LD₅₀) was determined and its value was used for assessment of LPS toxicity. The control and experimental groups consisted of 10 mice each. The control mice were injected with 0.2 mL of sterile 0.9% NaCl solution. The animals were observed for 48 h [4].

Immunological studies. O-antiserum was obtained to heated *E. coli* cells (2.5 h, boiling water bath). The rabbits were immunized intravenously five times with 4-day intervals; cell concentration was 2 × 10⁹/mL (0.1 to 1 mL).

The antigenic activity of LPS was studied by the method of double immunodiffusion in agar according to Ouchterlony [6].

The effect of LPS on different lymphocyte populations was assessed by the level of expression of CD₃ and CD₂₂ receptors on T and B lymphocytes by the reaction of rosette formation with the monoclonal antibodies to the above receptors. CD-diagnosticum for the antibodies was introduced into round-bottom wells of immunological multiwell plate by 0.25 mL (25 μL), supplemented by an equal volume of lym-

Table 1. Fatty acid composition of the LPS from *E. coli* M-17

Acid	Amount (% of the total peak area)
C _{12:0}	14.5
C _{14:0}	21.6
3-OH-C _{14:0}	46.6
<i>i</i> -C _{16:0}	1.3
C _{16:1}	2.0
C _{16:0}	9.6
<i>c</i> -C _{18:1}	—
<i>t</i> -C _{18:1}	3.0
C _{18:0}	1.4

Note: “—” not revealed.

phocyte mixture, incubated for 25 min at 37°C, centrifuged at 500–1000g for 3 min, and placed into a refrigerator for 1 h at 4°C. Lymphocyte precipitates treated with the monoclonal antibodies were dried, fixed with alcohol, and stained by Romanowsky–Giemza so that the nuclei of the lymphocytes were clearly visible.

For the reaction of blast transformation of human blood lymphocytes (blast transformation is a transition of small lymphocytes from a dormant state into blast forms capable of proliferation and further differentiation), LPS of the tested strains was used as a mitogen in the following dilutions: 1 mL of LPS per 10 mL, and 1 mg of LPS per 100 mL of sterile saline solution. The reaction was performed with 0.2 mL each of the LPS preparation, medium 199 [7], antibiotic preparation, bovine serum, and human blood; the mixture was incubated for 2 days at 37°C. After incubation, acetic acid (10% solution) was added for precipitation of lymphoblasts, which were separated by centrifugation. The pellet was separated from the supernatant, transferred onto slides, and stained by Romanowsky–Giemza; the cells were counted by direct microscopy [7].

Statistic studies. The experimental results were statistically processed by the method proposed for pharmaceutical preparations [7].

RESULTS AND DISCUSSION

The studied LPS of *E. coli* isolated by the phenol–water method was characterized by a relatively high content of nucleic acids, which may be due to the method of isolation. LPS was purified from nucleic acids by ultracentrifugation (4 h, 105000g, 3 times). The purified LPS contained 52% carbohydrates and 7.5% nucleic acids; protein was not detected. The yield of LPS was 3.5%, being less than the average values typical of other representatives of *Enterobacteriaceae* (5%).

Analysis of the monosaccharide composition demonstrated that the predominant monosaccharides of the LPS from *E. coli* M-17 were rhamnose (45.4%), glucose (18.9%), and ribose (17.8%). The typical LPS components of gram-negative bacteria, heptose and KDO, were 7.9 and 0.25%, respectively.

Analysis of the fatty acid composition of the lipids A of the LPS (Table 1) showed the presence of fatty acids with 12 to 18 carbon atoms in their chains. Among hydroxy acids, only 3-hydroxy tetradecanoic acid was detected (46.2%); tetradecanoic (21.4%), dodecanoic (14.3%), and hexadecanoic (9.4%) acids were present as well.

The presence of only one hydroxy acid (3-hydroxy tetradecanoic acid, which acylates both amino and hydroxyl groups of glucosamine residues) is a characteristic feature of the whole family *Enterobacteriaceae*.

The generally known and best-studied role of LPS is its functioning as the main thermostable antigen of microbial cells. LPS introduction into the organisms of higher animals and humans results in production of complementary antibodies directed against specific structural regions in the LPS molecule is used for immunization [4]. The working titer of O-antisera to the heated culture of the tested strain *E. coli* M-17 determined by the ring precipitation reaction was 1 : 40000.

During the double immunodiffusion in agar by Ouchterlony, LPS of the tested *E. coli* strain in a homologous system exhibited an antigenic activity (Fig. 1). In cross-reactions, LPS of the tested strain did not interact with the antisera to representatives of other *Enterobacteriaceae* species: *Budvicia aquatica*, *Rahnella aquatilis*, and *Pragia fontium* (Fig. 2), which indicated the absence of common antigenic determinants.

Since O-specific polysaccharide is the molecular basis of serological affinity, its structure was investigated. O-specific polysaccharide was isolated by mild acid hydrolysis of the LPS.

The study of OPS monosaccharide composition showed the presence of rhamnose (Rha), N-acetylglucosamine (GlcNAc), and Fuc3NAc.

The ¹³NMR spectrum of the OPS (Fig. 3) contained the signals of five anomeric carbon atoms at 98.4–103.9 ppm, one hydroxymethyl group (C-6 GlcNAc) at 62.5 ppm, methyl groups at 18.0 ppm

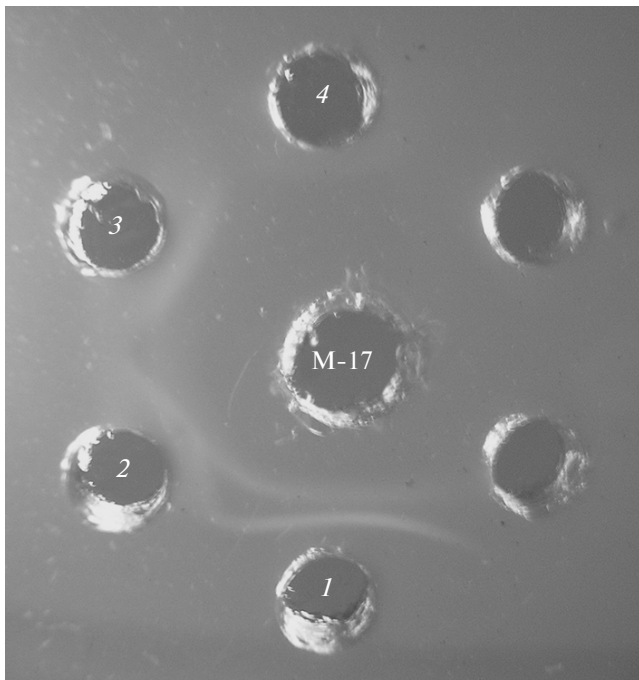


Fig. 1. Reaction of double immunodiffusion in agar by Ouchterlony between the LPS of *E. coli* M-17 and homologous O-antiserum. LPS dilutions: 1 : 100 (*I*), 1 : 5000 (*2*), 1 : 10000 (*3*), and 1 : 20000 (*4*).

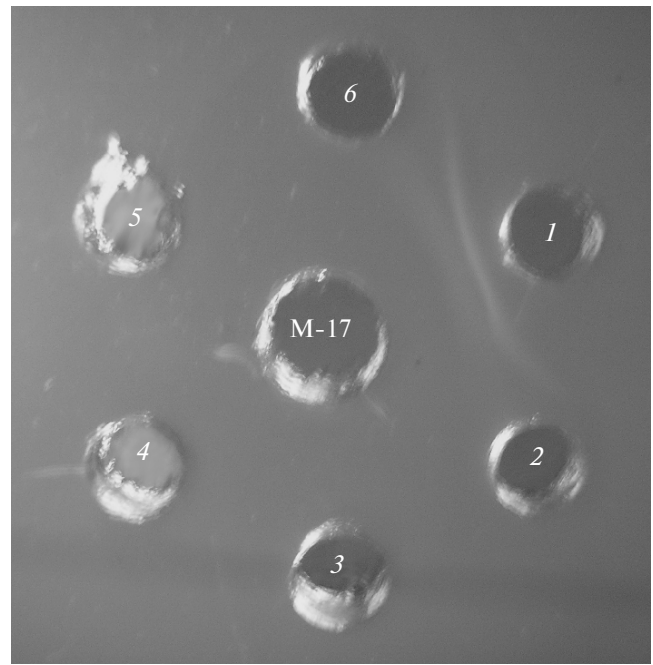


Fig. 2. Reaction of double immunodiffusion in agar by Ouchterlony between the LPS of *E. coli* M-17 (*I*), *E. coli* F-50 (*2*), *B. aquatica* 23270 (*3*), *B. aquatica* 124 (*4*), *P. fontium* 20125 (*5*), *R. aquitilis* 33071 (*6*), and O-antisera to *E. coli* M-17.

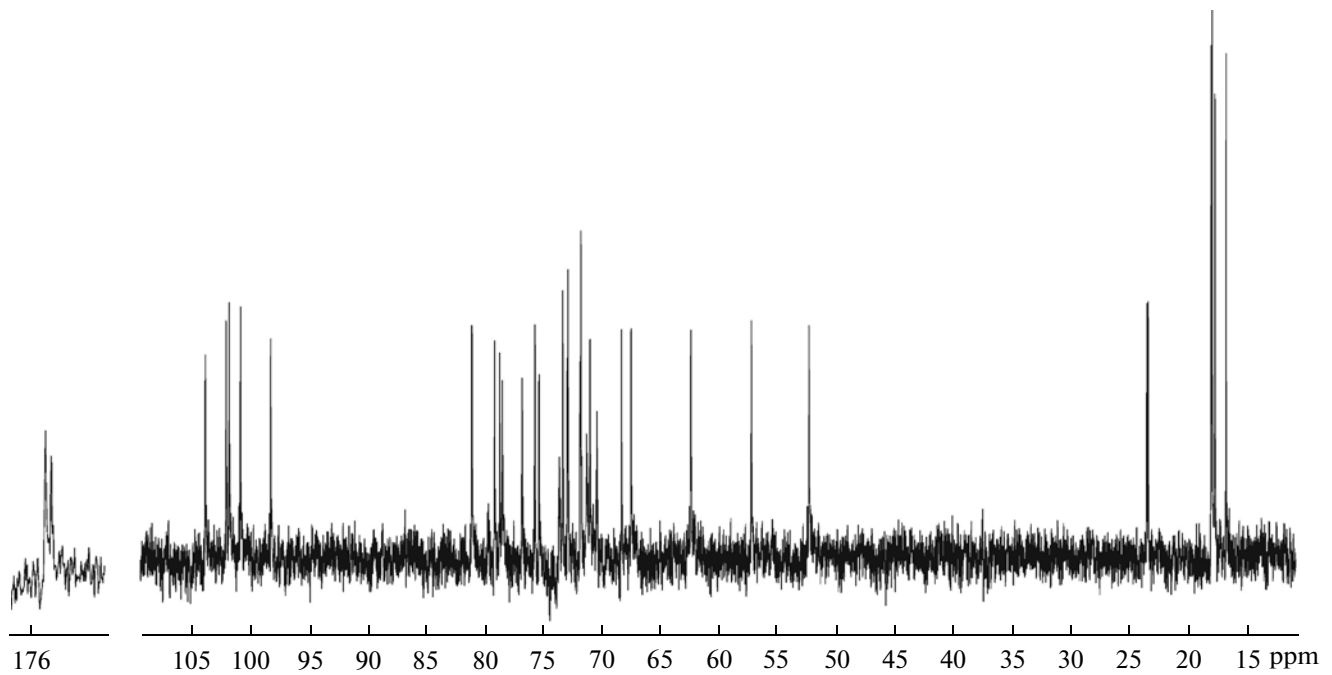


Fig. 3. The ^{13}C -NMR spectrum of the OPS from *E. coli* M-17.

Table 3. Effect of the LPS from *E. coli* M-17 on the expression of CD₃ receptors on T lymphocytes and CD₂₂ receptors on B lymphocytes

Preparation	Indices of expression of CD ₃ receptors	Indices of expression of CD ₂₂ receptors
	LSP dilution 1 : 10	
LPS of <i>E. coli</i> M-17	45.2 ± 0.8	24 ± 0.8
Control (intact lymphocytes)	50.6 ± 2.7	12 ± 0.5
	LSP dilution 1 : 100	
LPS of <i>E. coli</i> M-17	34.2 ± 1.3	10.7 ± 0.6
Control (intact lymphocytes)	38.6 ± 1.7	12 ± 0.5

mitogens or antigens into a culture of lymphocytes causes a series of morphological and biochemical changes in the cells. These include DNA synthesis and transformation of lymphocytes into blast forms (large dividing cells). RBTL determines the proliferative potential of the cells under study. Phytohemagglutinin, concanavalin A, bacterial lipopolysaccharides, enzymes, etc. are used as RBTL activators (mitogens).

The mitogenic activity of the LPS of the tested *E. coli* strain was studied in comparison with the commercial LPS preparation produced by the Diagnosticum Company at the Lviv Institute of Hematology and Blood Transfusion. The results showed (Table 2) that the commercial LPS preparation, irrespective of concentration, stimulated the formation of lymphoblasts to a somewhat higher extent. The tested LPS at a 1 : 100 dilution was more active than the LPS diluted 1 : 10.

The characteristic feature of B- and T-lymphocytes is the presence of a number of antigenic markers, in particular, CD₂₂ and CD₃ receptors, respectively. It is known that the CD₃ protein complex associated with an antigen-specific T cell receptor is a major functional marker of T lymphocytes. It promotes signal transduction from the cell membrane to the cytoplasm. Our studies have shown (Table 3) that the expression of CD₃ receptors on T lymphocytes

decreased in the presence of the LPS from *E. coli* M-17 in 1 : 10 and 1 : 100 dilutions.

The B lymphocyte receptor CD₂₂ is a carbohydrate-binding transmembrane protein from the lectin family. It is a regulatory molecule, which prevents over-activation of the immune system and development of autoimmune processes. Analysis of the results presented in Table 3 demonstrates that in the 1 : 10 dilution of the LPS of the tested *E. coli* strain, a two-

Table 4. Determination of LD₅₀ for *E. coli* LPS

LPS of the strain	LD ₅₀	
	µg/mouse	µg/kg
<i>E. coli</i> M-17	40.0	2000
<i>E. coli</i> O55:B5	0.14	7.0

Table 5. Pyrogenic activity of *E. coli* LPS

LPS of the strain	Pyrogenic dose of LPS, $\mu\text{g}/\text{rabbit}$	Total temperature rise ($^{\circ}\text{C}$) after LPS injection during 3 h
<i>E. coli</i> M-17	0.021	1.97
LPS of <i>Shigella typhi</i> (pyrogenal)	0.022	1.99

fold enhancement of the expression of CD₂₂ receptors on B lymphocytes occurred. At the same time, the LPS from *E. coli* M-17 diluted 1 : 100 suppressed the expression of CD₂₂ receptors on B lymphocytes by more than 80%.

Thus, the expression of CD₃ and CD₂₂ receptors on T and B lymphocytes depended on the LPS dilution.

Although LPS, as is known [2], show an immunomodulating effect, their application as therapeutic preparations is prevented by their properties such as toxicity and pyrogenicity.

Our experiments with nonpedigreed mice showed that the LPS of *E. coli* M-17 was characterized by a much lower toxic effect than the LPS from *E. coli*

O55:B5 (Table 4). Moreover, the toxicity of LPS of the tested strains was lower by an order of magnitude than the toxicity of the LPS of the new *Enterobacteriaceae* species: *R. aquatilis* [10] and *P. fontium* [11].

The study of pyrogenicity (Table 5, Fig. 5) showed that the LPS of *E. coli* M-17 lost its pyrogenic properties after 2 h of the experiment.

Thus, the LPS was isolated from *E. coli* M-17, chemically identified, and shown to be an apyrogenic compound of low toxicity. It is not surprising, since this strain is a component of the Bificol and Colibacterin probiotics. Investigation of the effect of the tested LPS on T and B lymphocytes suggests that it can be used as a mitogen in blast transformation reaction,

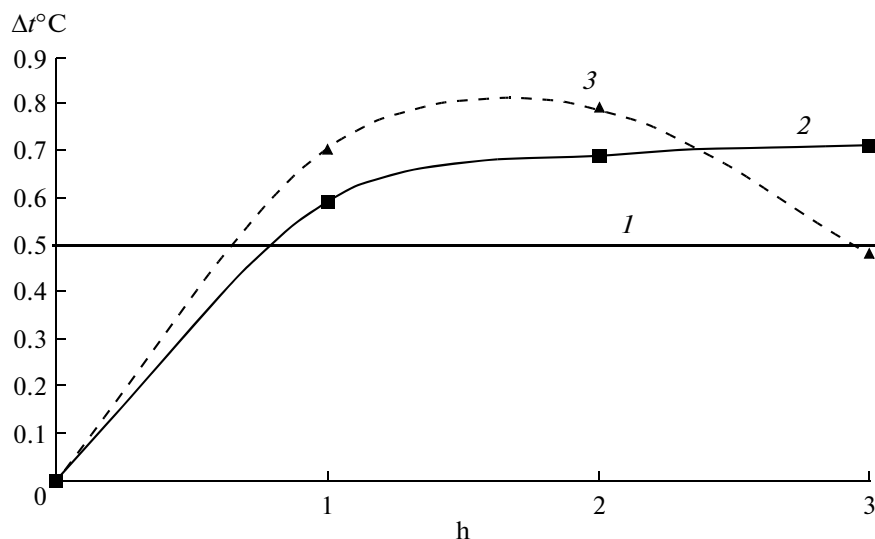


Fig. 5. Pyrogenic activity of the LPS from *E. coli* M-17: the time after LPS introduction, X-axis; the mean deviations of temperatures, Y-axis; pyrogenicity threshold (1), pyrogenal (2), LPS from *E. coli* M-17 (3).

because its activity is only insignificantly lower than that of the commercial preparation.

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