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Original articles

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IMMUNOMODULATORY EFFECTS OF *LACTOBACILLUS PLANTARUM* AND *HELICOBACTER PYLORI* CagA$^+$ ON THE EXPRESSION OF SELECTED SUPERFICIAL MOLECULES ON MONOCYTE AND LYMPHOCYTE AND THE SYNTHESIS OF CYTOKINES IN WHOLE BLOOD CULTURE

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Helicobacter pylori (*H. pylori*) infections are usually superficial and clinically asymptomatic, but in approximately 10-20% cases it can be more aggressive and associated with other pathologies. The reason for weak or strong pro-inflammatory responses in gastric mucosa that occur during *H. pylori* infection is not understood. Combined treatment, including antibiotic therapy with administration of probiotic bacteria along, considerably improves the effectiveness of *H. pylori* eradication and reduces the relapse rate. Thus, the aim of this study was to analyze the effect of *Lactobacillus plantarum* (*L. plantarum*) and/or *H. pylori* CagA$^+$ on leucocytes in whole blood cultures. This study revealed how selected strains of *H. pylori* and *L. plantarum* modulate expression of chosen membrane markers of monocytes and lymphocytes, and the cytokine synthesis of *in vitro* cultures. The level of IFN-γ was higher in cultures stimulated with *L. plantarum* than in combination of this two examinated strains. We also observe the tendency to increase the level of IFN-γ by *L. planatrum* in relation to cells stimulated by *H. pylori*. In contrast, both *H. pylori* alone and in combination with *L. plantarum* had a strong modulatory effect on the synthesis of interleukin-10. Moreover lymphocytes with higher expression of CD25 and CD58 receptors was observed only in those cultures that were stimulated with *L. plantarum* strain alone or in combination with *H. pylori*. Effects exerted on the immune system, both in terms of natural and adaptive response, constitute the only functional criterion of probiotic bacteria. The immunostimulant effects documented in this study suggest that *Lactobacillus* spp. can restore immune function of mucosal membrane during symptomatic infection with *H. pylori*.

Key words: *Helicobacter pylori, immunomodulation, interleukin-10, Lactobacillus spp., interferon-γ, cytotoxin associated gene A, monocytes, lymphocytes*

INTRODUCTION

The immune homeostasis of the mucosa-associated lymphoid tissue (MALT) is determined by the tolerance to alimentary allergens, along with the induction and control of the immune response against bacteria, viruses and neoplastically transformed cells. Immunomodulatory effects of intestinal bacterial microflora constitute one of the mechanisms that maintain the balance within MALT. Furthermore, they can be applied in therapy or at least in the support of treatment of various disorders *e.g. Helicobacter pylori* (*H. pylori*) induced gastritis (1-3).

H. pylori infections are usually superficial and clinically asymptomatic (weak pro-inflammatory effect), but in approximately 10-20% cases it can be more aggressive and associated with other pathologies, such as gastric and duodenal ulcers, gastric malignancies, and MALT lymphomas (4). The reason for weak or strong pro-inflammatory responses gastric mucosa that occur during *H. pylori* infection is not understood. Combined treatment, including antibiotic therapy with administration of probiotic bacteria along, considerably improves the effectiveness of *H. pylori* eradication and reduces the relapse rate. Probably, the imbalance in the composition of gut microflora (bifidobacteria, lactobacilli *vs*. pathogenic bacteria) results in the development of inflammation and thus use of lactic acid bacteria (LAB) seem rational (5-9). The LAB strains, the physiological component of commensal intestinal microflora, are widely established bacterial probiotics that can modulate the pro- and anti-inflammatory responses of the immune system (10-11). Therefore, they are involved in the maintenance of MALT homeostasis. However, the exact mechanism behind this phenomenon is not understood and so far, studies, concerning that problem have not been carried out, nor any results published (12-13). Thus, the aim of this study was to analyze the effect of *Lactobacillus plantarum* (*L. plantarum*) and/or *H. pylori* CagA$^+$ on leucocytes in whole blood culture.

The *in vivo* study of the interaction between the cells of mucosa-associated lymphoid tissue and intestinal bacteria is complicated. Therefore, we used cell cultures in our model of *in vitro* study, which is a widely established and accepted alternative (14-16). Phenotypic analysis included monocytes - as the antigen presenting cells (APC), and T lymphocytes - the cells involved in the adaptive response.

MATERIAL AND METHODS

Blood samples and cell culture

The whole blood was obtained from healthy volunteers (blood donors, median age 30 years, range 20-35). The study was undertaken according to the Helsinki declaration with approval from the ethical committee of Collegium Medicum Nicolaus Copernicus University in Bydgoszcz, Poland. Venous blood samples (9 ml) have been drawn into tubes containing heparin (Medlab). Whole blood was diluted 1:5 with RPMI 1640 (PAA) in sterile, non-adherent, polypropylene tubes (Falcon, Becton Dickinson). Thereafter, specimens (1 ml of diluted whole blood per specimen) were incubated for 24 hours, at 37°C and 5% CO_2, with or without live bacteria. Human whole blood cells were cultured for 24 hours, resulting in less than 10% death cells by trypan blue exclusion (data not shown).

Bacteria

In this study we used Gram-positive bacteria: *L. plantarum* strain 0862 (strain collection from the Institute of Technology Fermentation and Microbiology, Faculty of Biotechnology and Food Sciences, Technical University of Lodz) isolated from fermented food, and Gram-negative *H. pylori* $CagA^+$ strain 95 (Department of Microbiology and Clinical Immunology, The Children's Memorial Health Institute). Live bacteria were used as a stimulating agent in all experiments in concentrations that induced optimal proliferative response of cells, for *L. plantarum* 4:10 bacteria/cells (*Fig. 1*) and for *H. pylori* 40:10 bacteria/cells (*Fig. 2*). Proliferation assay was performed as previously described (14).

Cell surface phenotype expression

The cells were labeled with fluorescein isothiocyanate (FITC) and phycoerithrine (PE)-conjugated mouse anti-human monoclonal antibodies (mAb) (IgG2a, IgG1, Becton Dickinson), specific for different cell membrane receptors of monocytes and lymphocytes surface. The panel of FITC-labeled MAbs (Becton Dickinson) involved: anti-CD14 (IgG2a, MϕP9), anti-HLA-DR (IgG2a, G46-6), anti-CD11a (IgG2a, G43-25B), and anti-CD25 (IgG1, M-A251). The panel of PE-labeled MAbs (Becton Dickinson) involved: anti-CD45 (IgG1, HI30), anti-CD16 (IgG1, 3G8), anti-CD18 (IgG1, 6.7), and anti-CD58 (IgG2a, 1C3). The staining was carried out following the manufacturer's instructions in accordance with the stain-and-then-lyse technique. Briefly, 20 µl of mAb was added to 100 µl of peripheral blood in accordance with staining panel. The samples were incubated for 15-20 minutes in dark conditions at room temperature (RT). Subsequently, 2 ml of lysing solution (Becton Dickinson) was added to each sample and incubated for 15-20 minutes in dark at RT. Then, 1 ml of cold (2-8°C) buffered saline (PBS) was added to each sample and centrifuged (300 x g) for 10 minutes at 4°C. After removing the supernatant, 2 ml of PBS (4°C) was added to the cellular sediment and centrifuged (300 x g) for 10 minutes at 4°C. After removing the supernatant, 200 µl of PBS (2-8°C) was added to the sediment and the cells were analyzed in FACScan flow cytometer (Becton Dickinson). Flow cytometry acquisition and analysis were performed on at least 20 000 acquired events. Obtained cytometric data was analyzed using FlowJo version 7.6.1 software (Tree Star). Monocytes were gated based on bright CD14 staining and intermediate levels of CD45 staining. Lymphocytes were gated based on bright CD45 staining and lack of CD14 staining. The percentage of cells expressing studied receptors and/or average density of receptors expressed as the mean fluorescence intensity (MFI) were analyzed in a population of monocytes and lymphocytes.

Cytokine assay

The assay was performed as previously described (17-18). Cytokine (IFN-γ, IL-10) concentrations in cell culture

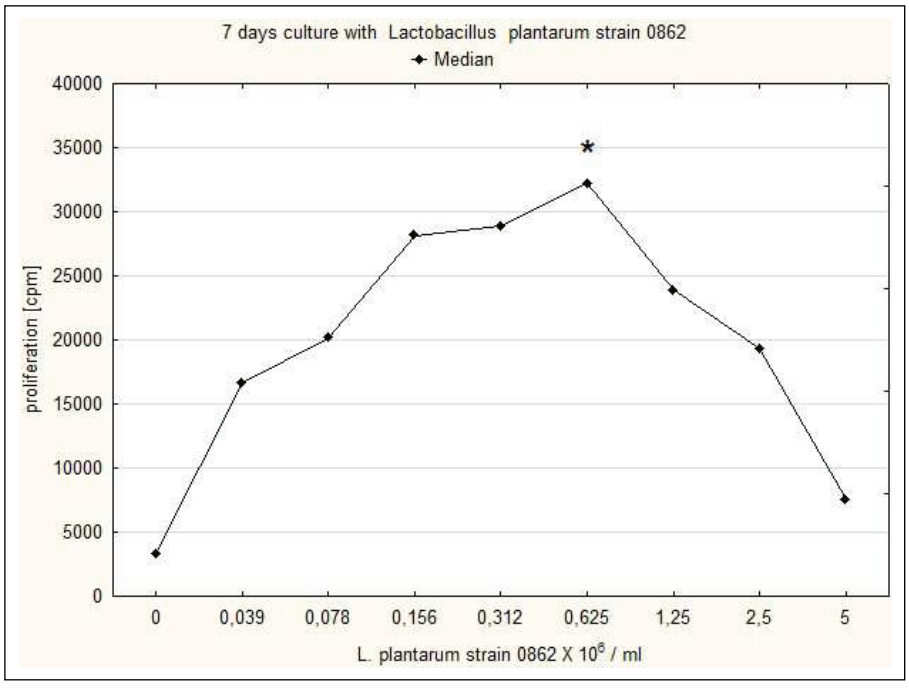

Fig. 1. The dose-response curve for *L. plantarum*.

supernatants were estimated following 24 hours of bacterial stimulation of whole blood cells, using ELISA (Becton Dickinson) according to the manufacturer's procedure.

Statistical analysis

The STATISTICA (version 9.0) computer software (StatSoft) was used for the statistical analysis. The Mann-Whitney *U*-test was used. Statistical significance was considered at $p<0.05$.

RESULTS

Cytokine assay

All analyzed stimulators induced cytokine synthesis effectively as compared to the control ($p<0.05$, *Figs. 3-4*). The LAB alone (108.72 pg/ml, range 93.22-292.79 pg/ml) was revealed as a stronger inducer of IFN-γ than *H. pylori+L. plantarum* mixture (87.98 pg/ml, range 69.71-91.27 pg/ml, $p<0.05$, *Fig. 3*). *H. pylori* also can stimulate the production of IFN-γ (52.09 pg/ml, range 46.10-164.04 pg/ml) and the level was lower than in culture stimulated with *L. plantarum* ($p<0.1$, tendency, *Fig. 3*). Furthermore, the combination of studied bacteria (60.34 pg/ml, range 34.17-161.76 pg/ml) as well as *H. pylori* alone (35.6 pg/ml, range 23.75-87.09 pg/ml) proved stronger inducers of IL-10 than the *L. plantarum* alone (10.20 pg/ml, range 9.85-12.07 pg/ml, $p<0.05$, *Fig. 4*).

Effect of bacteria on the expression of monocyte and lymphocyte surface receptors

As compared to the control, all single bacteria significantly increased the density of HLA-DR (in HLA-DR, HLA-DR/CD16

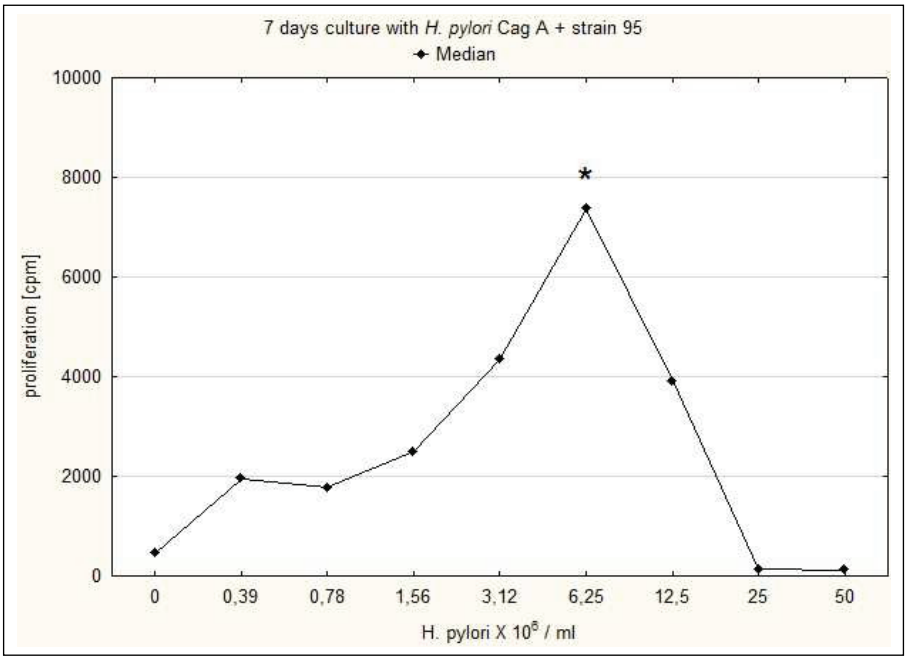

Fig. 2. The dose-response curve for *H. pylori*. Cell proliferation was determined by [^{3}H] thymidine incorporation at day 7 of culture. Values represent the medians (obtained from five independent experiments), * - concentrations that induced optimal cell proliferation.

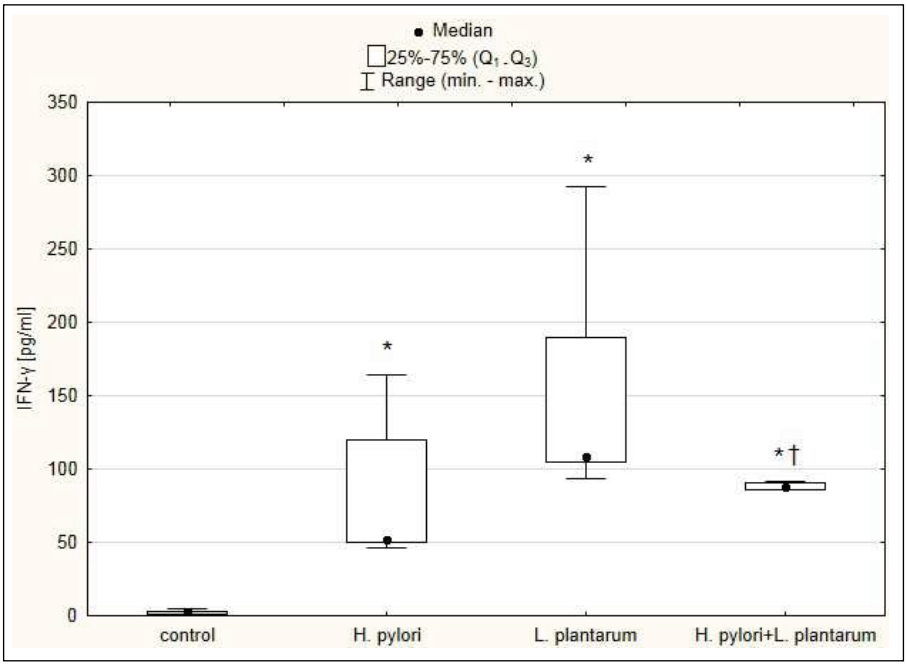

Fig. 3. Induction of IFN-γ in whole blood cells by *L. plantarum* strain 0862, *H. pylori* strain 95 CagA$^+$ and combination of these strains. Values represent the medians (obtained from five independent experiments), the first quartile and third quartile, range (min., max.); statistically significant differences: * - stimulators *vs*. control, † - stimulators *vs*. *L. plantarum*.

positive cells) and CD18 (in CD18/CD11a positive cells) in monocyte population (p<0.05, *Table 1*). *L. plantarum* proved stronger inducers of HLA-DR density in population of HLA-DR positive cells as compared to *H. pylori* and mixture of strains (p<0.05, *Table 1*). The mixture *L. plantarum+H. pylori* was the weakest inducer of HLA-DR density in HLA-DR positive monocytes (p<0.05, *Table 1*). Single LAB bacteria stimulated stronger this marker in population of HLA-DR/CD16 positive cells as compared to *L. plantarum+H. pylori* (p<0.05, *Table 1*). Stimulation with single bacteria (*L. plantarum* or *H. pylori*) was reflected by an increase in the percentage of CD16 positive cells in comparison to control cells (p<0.05, *Table 1*). In turn, exposure to the combination of bacteria decreased the percentage of HLA-DR positive monocytes and increased the population of HLA-DR/CD16, CD11a/CD18 positive cell as compared to the control (p<0.05, *Table 1*). All inducers increased MFI for CD25 receptors of the lymphocyte population (p<0.05, *Table 2*, *Fig. 5*). In contrast, only *L. plantarum*, alone or in the mixture, increased the percentage of CD25 and CD58 positive lymphocytes (p<0.05, *Table 2, Figs. 5, 6*). LAB bacteria was better inducer of CD25 positive cells than *L. plantarum+H. pylori* (p<0.05, *Table 2, Fig. 5*).

DISCUSSION

This study revealed that *H. pylori* and *L. plantarum* induce expression of selected membrane markers of monocytes and lymphocytes, and the cytokine synthesis of *in vitro* cultures, in a different manner. Single bacterial strains and their mixture induced the synthesis of IFN-γ. The level of IFN-γ was higher in cultures stimulated with *L. plantarum* than in combination of two examinated strains. We also observe the tendency to increase the level of IFN-γ by *L. planatrum* in relation to cells stimulated by *H. pylori*. In contrast *H. pylori* alone and in combination with *L. plantarum* had a modulatory effect on the

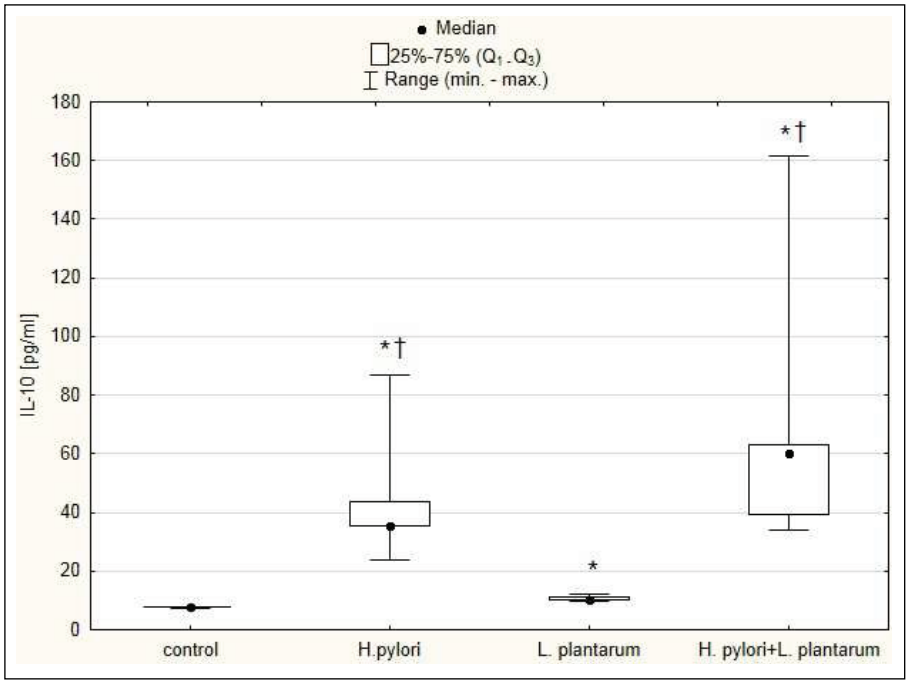

Fig. 4. Induction of IL-10 in whole blood cells by *L. plantarum* strain 0862, *H. pylori* strain 95 CagA$^+$ and combination of these strains. Values represent the medians (obtained from five independent experiments), the first quartile and third quartile, range (min., max.); statistically significant differences: * - stimulators *vs*. control, † - stimulators *vs*. *L. plantarum*.

Table 1. Effect of examinated bacteria and they mixture on the expression of monocyte markers (CD18, CD11a, HLA-DR, CD16). Values are expressed as the medians of four independent experiments and range (min.-max.); % - percentage of positive cells; MFI - mean fluorescence intensity; statistically significant differences: * - stimulators vs. control, † - stimulators *vs*. *L. plantarum*, ‡ - stimulators *vs*. *L. plantarum+H. pylori*, § - stimulators *vs*. *H. pylori;* p<0.05.

Receptors type on monocytes population		control	*H. pylori*	*L. plantarum*	*H. pylori+L. plantarum*
CD18/CD11	%	**88.4(86.8-89.3)**	**93.6(83.1-97.7)**	**95.2(86.6-98.6]**	**95.4*(93.8-97.6)**
CD18	MFI	**148.5(91.2-295.7)**	**629.5*(393.2-1027.4)**	**709.8*(455.5-1669.6)**	**606.2*(480.7-830.1)**
CD11a	MFI	**89.9(75.9-318.8)**	**99(34.9-180.3)**	**90.4(42-113.5)**	**49.6(34.2-93)**
HLA-DR	%	**59.8(53.9-62.3)**	**85.7(6.3-96.6)**	**51.3(14.5-86.9)**	**41.7*(31.2-53.0)**
	MFI	**204.3(196.6-289.7)**	**735.7*†‡(557.3-955.8)**	**1112.2*‡§(1016.7-1656)**	**553.2*†§(341.2-955.3)**
CD 16	%	**66.2(60.94-68.23)**	**82.2*(77.97-98.48)**	**95.4(83.85-97.52)**	**74.8(59.70-93.74)**
	MFI	**174.6(85.13-261.85)**	**139.4(81.19-180.11)**	**208.2(75.35-396.35)**	**104.7(90.93-112.40)**
HLA-DR/CD16	%	**26.9(24.2-29.1)**	**24.0(6.3-70.4)**	**44.2(14.7-91.9)**	**50.2*(47.3-52.3)**
HLA-DR	MFI	**211.1(176.8-218.4)**	**955.8*(567.1-1654.3)**	**906.2‡*(654.1-1412.8)**	**190.6†(122.7-580.4)**
CD 16	MFI	**66.7(33.6-76.6)**	**67.7(49.5-95.6)**	**70.4(56.3-126.7)**	**80.6(62-90.3)**

synthesis of IL-10, therefore limiting antibacterial activity of immune cells. Moreover, both studied strains alone or in combination stimulated expressions of HLA-DR and CD18 on monocytes. In turn, a higher percentage of lymphocytes showing expression of CD25 and CD58 receptors was observed only in those cultures that were stimulated with *L. plantarum* alone or in combination with *H. pylori*.

The *L. plantarum*-induced profile of cytokines was characterized by a high level of IFN-γ and lower concentration of IL-10. Such polarization of *L. plantarum*-induced response suggests activation of Th1 lymphocytes; which is consistent with previous data (19-21). An increase in IFN-γ level is an important factor determining the activation of cellular immune response. IFN-γ enhances expression of MHC class II, *i.e.* HLA-DR molecules, on the surface of APC, including monocytes. MHC class II is responsible for transmitting a signal to T lymphocytes during antigen presentation (21-22). Additionally, an enhanced expression of leukocyte adhesion molecule LFA-1 was observed on the surface of monocytes from all culture variants, along with a higher percentage of monocytes showing expression of this leukocyte integrin in the culture stimulated with bacterial mixture. LFA-1 comprises of two chains: alpha (CD11a) and beta (CD18); this molecule plays a vital role in cellular adhesion and extravasation at the site of ongoing inflammation (23). The LFA-1 mediated adhesion can be reflected by an enhanced expression of Fc receptors (FcR) on effector cells, which in turn stimulates such processes as opsonin-dependent phagocytosis and antibody-dependent cell-mediated cytotoxicity (ADCC) (24-25). Stimulation with *L. plantarum* was reflected by a marked enhancement of HLA-DR expression, suggesting expedited presentation of antigen. There is widely know that *H. pylori* could immunoescape. We observe in our study lower expression of these marker in culture stimulated with *H. pylori* and mixture of examined strains. It is, therefore, tempting to speculate that the reduced expression of HLA-DR antigens on monocytes might be the results in an increased susceptibility to *H. pylori* infection. Consequently, one can conclude that used *L. plantarum* 0862 shows characteristics of probiotics since it stimulates natural immunity, modulating the ability of antigen presentation to T lymphocytes.

Stimulation with single bacteria increased percentage of $CD16^+$ monocytes, and exposure to the strain mixture induced an increase in the percentage of monocytes representing $HLA\text{-}DR^+/CD16^+$ phenotype. Moreover, expression of this receptor

Table 2. Effect of examinated bacteria and they mixture on the expression of lymphocyte markers (CD25, CD58). Values are expressed as the medians of four independent experiments and range (min. - max.); % - percentage of positive cells; MFI - mean fluorescence intensity; statistically significant differences: * - stimulators *vs*. control, † - stimulators *vs*. *H. pylori;* $p<0.05$.

Receptors type on lymphocytes population		control	*H. pylori*	*L. plantarum*	*H. pylori*+*L. plantarum*
CD 25	**%** **MFI**	**5.6(4.2-10.4)** **19.0(17.1-20.1)**	**12.0(6.4-13.8)** **32.4*(18.2-23.1)**	**21.7*‡(11-26.4)** **29.5*(27.6-39.6)**	**16.7*†(12.4-24.1)** **37.9*(30.9-54.3)**
CD 58	**%** **MFI**	**51.8(43.8-54.7)** **18.8(18.2-23.1)**	**43.0(9-78.1)** **25.0(20.3-31.8)**	**85*(76.1-86.5)** **2 (19.4-31.1)**	**60.7*(56.4-96.4)** **27.7(19.9-30.8)**

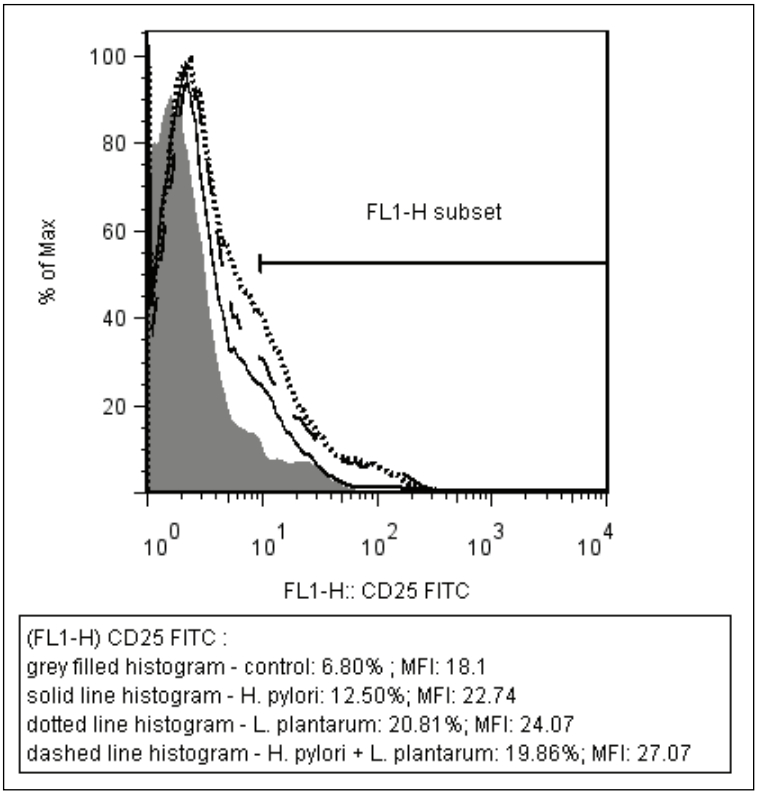

Fig. 5. The histogram represents the percentage of CD25 positive cells. Representative results selected from four independent experiments are shown. The whole blood cells were stimulated by *L. plantarum* strain 0862, *H. pylori* strain 95 $CagA^+$ and combination of these strains.

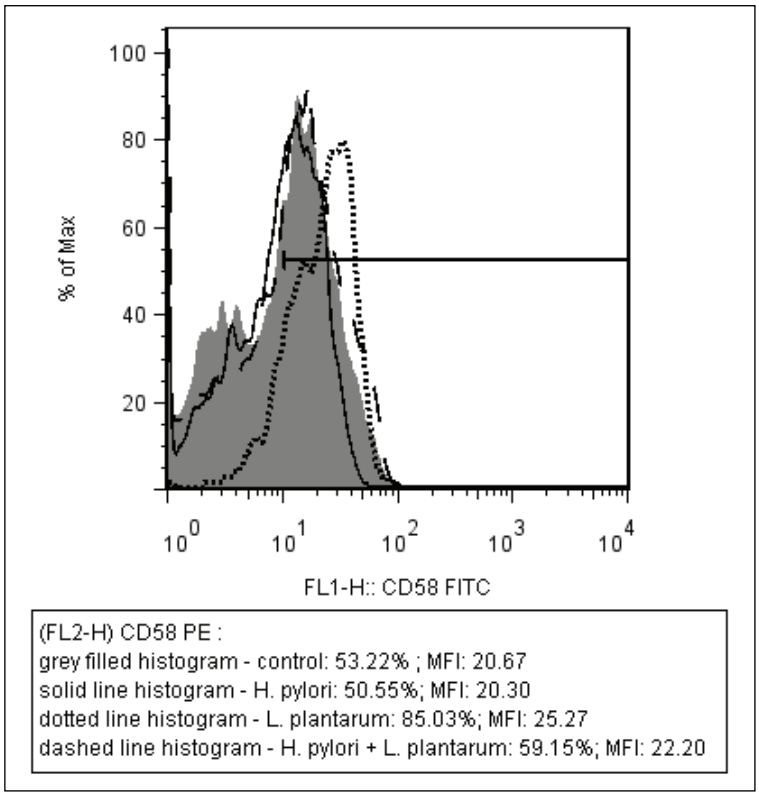

Fig. 6. The histogram represents the percentage of CD58 positive cells. Representative results selected from four independent experiments are shown. The whole blood cells were stimulated by *L. plantarum* strain 0862, *H. pylori* strain 95 $CagA^+$ and combination of these strains.

suggests a possible induction of non-specific cytotoxic mechanisms (26). Thus, one can conclude that stimulation with studied bacterial strains enhanced both the bactericidal and cytolytic properties of cells, characteristic for CD16-positive monocytes.

High levels of IL-10 documented after stimulation of peripheral blood cells with *H. pylori*, alone or in mixture, can exert unfavorable effects in the case of infections caused by this pathogen (27). Probably elevated concentrations of this cytokine enable *H. pylori* to escape the immune system response. This cytokine can inhibit pro-inflammatory activity of macrophages and T lymphocytes; furthermore, promotes development of Tregs. As widely known, *H. pylori* infection can lead to the suppression of immune response *via* the induction of Tregs formation and inhibit maturation of dendritic cells (28-31). Suppressive mechanism of *H. pylori* refers to direct interaction with target cell (*via* a receptor pathway) and/or synthesis and secretion of suppressor cytokines, such as IL-10. However, the immunosuppression associated with a higher fraction of Treg lymphocytes does not result in pathogen elimination in cases of *H. pylori* infection and thus does not protect against the development of symptomatic inflammation. Previous studies confirmed that Treg cells promote infection since they enhance bacterial colonization of the stomach (29-30).

This study revealed an increased percentage of lymphocytes showing expression of CD58 (LFA-3) in whole blood cultures after stymulation with the strain of *L. plantarum* alone or in the mixture. This surface molecule is specific for enhanced cytotoxic response. Binding CD58 to its ligand, that is CD2 molecule, can be reflected by the stimulation of cytotoxic lymphocytes (32). Interaction between T cells and APC in presence of IFN-γ cytokine, promotes differentiation of lymphocytes towards Th1 cells that synthesize IFN-γ, tumor necrosis factor -α and interleukin-2 (33). This latter cytokine can modulate cellular proliferation. Its receptor, interleukin-2R, comprises of three chains: α(CD25), β(CD122), and γ(CD132). In this study, an enhanced expression of the alpha chain (CD25) on lymphocyte surface, and a higher percentage of cells expresing this marker, were observed in cultures stimulated with *L. plantarum* alone and in those exposed to bacterial mixture (*L. plantarum+H. pylori*). Since *H. pylori* infection is associated with inhibition of lymphocyte proliferation, the enhanced expression of CD25 and the higher percentage of CD25-positive cells resulting from stimulation with *L. plantarum* (as an single strain or in mixture with *H. pylori*) can play a positive role during *H. pylori* eradication (34-35).

In summary, this study revealed that all analyzed bacterial strains caused considerable activation of monocytes - the antigen presenting cells that induce T cells activation. Furthermore, single strain of *L. plantarum* and its mixture with *H. pylori* enhanced expression of selected markers of lymphocyte activation. Moreover, stimulation with *L. plantarum* was reflected by a marked increase in the level of antibacterial cytokine, IFN-γ. One should not expect a potent activation of adaptive response potentially leading to inflammation, but rather cellular stimulation expediting faster reaction during contact with pathogen. Since *L. plantarum* belongs to commensal bacterial intestinal microflora, it is safe, could be effective in preventing and maintaining remission of some disease as probiotic. (2, 5, 14).

Effects exerted on the immune system, both in terms of natural and adaptative response, constitute the only functional criterion of probiotic bacteria. Optimal immune reactivity, providing both elimination of pathogen and the readiness of the body systems, is determined by activation of both abovementioned types of response. Plausibly, *L. plantarum* meets the criteria of probiotic bacterium due to its modulatory effects on both the natural and the specific immune response. Under physiological conditions, activation of lymphocytes is necessary for cellular regeneration and enhancement of effector and regulatory reactions (2). The immunostimulant effects documented in this study suggest that *Lactobacillus* spp. could

restore the immune function of mucosal membrane during a symptomatic infection with *H. pylori*. Moreover, the results of our experiments indicate a low activation of lymphocytes (CD25, CD58) after *H. pylori* induction. Inhibition of specific response was also observed in other studies (36). *H. pylori* immunoescape probably depends on APC (*e.g.* macrophage, dendritic cells), because they are the only cells capable of inducing specific immune response. This may be caused by down regulation of some receptors on their surface, for example MHC class II or co-stimulatory molecules (36-37). The exact mechanism is unknown. Further studies are needed to investigate the cause of diseases induced by *H. pylori* and possibilities for their prevention.

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Conflict of interests: None declared.

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