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Selected *Lactobacillus bulgaricus* and *Streptococcus thermophilus* Strains from Bulgarian Yogurt Demonstrate Significant Anti-Inflammatory Potential

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Abstract

In vitro tests for establishing the anti-inflammatory potential of twenty-two previously selected Lactic acid bacteria (LAB) strains were carried out. The aim was to compare the anti-inflammatory potential of *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* isolated from authentic homemade yogurt in non-industrial mountain villages in Bulgaria with selected strains originated from the intestinal tract of healthy volunteers. The assessed signal peptide associated with inflammation was IL-8, with and without stimulation of TNF-alpha, in order to simulate inflammatory shock *in vitro*. To establish the anti-inflammatory potential of the strains, two cytokines with predominant anti-inflammatory effects, IL-10 and TGF-beta were evaluated as well. The different cytokines were evaluated using three different analytical models, including mice splenocytes.

Among the presented strains of intestinal origin only two *L. gasseri*, one *Enterococcus faecium* and one *Bifidobacterium longum* possessed simultaneously the desirable optimal cytokine profile, where the highest induction of IL-10 was 1670.2 pg/ml (*E. faecium* 2092/2); the strongest reduction of IL-8 was 46.9 pg/ml (*B. longum* 10/48) and the induction of TGF-ss varied between 548.75 and 834.53 pg/ml. Among the strains of yogurt origin, three *Lactobacillus bulgaricus* and three *Streptococcus thermophilus* possessed concurrent or even better anti-inflammatory profiles, where the highest induction of IL-10 was 1584.1 pg/ml (*L. bulgaricus* b58); the strongest reduction of IL-8 was 46.0 pg/ml (*Str. thermophilus* T43) and the induction of TGF-ss varied between 499.96 and 841.50 pg/ml.

Keywords*:* probiotics, Bulgarian yogurt, cytokines, inflammation*, Lactobacillus bulgaricus, Streptococcus thermophilus*

Резюме

Бяха извършени *in vitro* тестове за установяване на противъзпалителния потенциал на двадесет и два щама, предварително подбрани, млечнокисели бактерии. Целта е да се сравни противъзпалителния потенциал на щамове *Lactobacillus delbrueckii subsp. bulgaricus* и *Streptococcus thermophilus*, изолирани от домашни кисели млека в неиндустриализирани планински райони на България с подбрани щамове с интестинален произход, изолирани от здрави доброволци. Оценените сигнални пептиди, които се свързват с процесите на възпалението са IL-8, при наличието на предварително стимулиране с TNF-alpha или без такова, с което се цели да се симулира инфламаторен шок в лабораторни условия. За да се установи противъзпалителния потенциал, също така бяха оценени и двата цитокина IL-10 и TGF-ss, които са с преобладаващ противовъзпалителен ефект. Различните цитокини бяха оценени чрез използването на три различни аналитични модела, включителни спленоцити от мишки.

Сред представените щамове с интестинален произход, само два *L. gasseri, Enterococcus faecium* и един *Bifidobacterium longum* притежават едновременно желания оптимален цитокинов профил, където най-високата индукция на IL-10 е 1670,2 pg/ml (*E. faecium* 2092/2); най-силната редукция на

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IL-8 е 46,9 pg/ml (*B. longum* 10/48) и индукцията на TGF-ss варира в стойностите между 548,75 и 834,53 pg/ml. Сред щамовете с киселомлечен произход, три *L. bulgaricus* и три *Str. thermophilus* притежават конкурентни или дори по-добри противовъзпалителни цитокинови профили, където най-високата индукция на IL-10 е 1584,1 pg/ml (*L. bulgaricus* b58); най-силната редукция на IL-8 е 46,0 pg/ml (*Str. thermophilus* T43) и индукцията на TGF-ss варира в стойностите между 499,96 and 841,50 pg/ml.

Introduction

Although the precise cause of inflammatory bowel disease (IBD) remains unknown, the most accepted hypothesis of pathogenesis is that dysbiosis triggered by environmental factors in modern way of living rather than any specific pathogens may have played the primary causative role (Tong *et al.,* 2013; Matsuoka and Kanai, 2015; Ni *et al.,* 2017). A better understanding of the mutual interactions between the microbiota and host immune system would shed light on disease prevention and treatment (Lin and Zhang, 2017). Cytokines are secreted proteins that regulate and determine the nature of immune responses. The functions of these proteins are diverse and include participation in normal T-cell-mediated immunity, the inflammatory response, cancer, autoimmunity, allergy, etc. Various pathologic conditions are accompanied by changes in cytokine levels (Borish and Rosenwasser, 1996) therefore the measurement of cytokine production is widely used (Katial *et al.,* 1998; Papadopoulos *et al.,* 2014). In some gastrointestinal infectious and inflammatory conditions, inflammatory cells, including monocytes and activated lymphocytes, secrete excessive inflammatory products such as cell-mediated T helper type 1 (TH-1) cytokines. Pro-inflammatory cytokines like tumor necrosis factor alpha (TNF-α), IL-1, IL-6 and interferons are among the first cytokines produced in response to pathogenic bacteria (Miettinen *et al.,* 1998). IL-8 may be induced by TNF-α. The intestinal epithelium is capable of releasing some pro-inflammatory chemokines such as IL-8 (Bai *et al.,* 2004), which is one of the most potent chemoattractants for neutrophils. Different populations of regulatory T cells exert their inflammation - modulating effect by secreting specific cytokines, including IL-10 or more regulatory cytokines, such as TGF-ss. IL-10 produced by macrophages and lymphocytes can inhibit the production of TNF-α and other TH-1 type cytokines, due to its function to limit and ultimately terminate immune and inflammatory responses, as pointed by Moore *et al.,* (2001). Certain probiotic strains help regulate inflammation and modulate the host immune system via their enhanced ability to influence the secretion of these important cytokines (Plaza-Diaz *et al.,* 2015, 2017). This effect has been demonstrated, *in-vitro* (Smits *et al.,* 2005; Hsieh *et al.,* 2013) and *in-vivo*, in various animal models (Di Giacinto *et al.,* 2005; Jeon *et al.,* 2012; Oksaharju *et al.,* 2013) and clinical studies (Timmerman *et al.,* 2004; Ringel *et al.,* 2009; Paramsothy *et al.,* 2017). Fermented food products like yogurt are important sources of transiting bacteria for the gastrointestinal tract (GIT), delivering approximately 10^8 live bacteria per gram (Bulgarian State Standard, 2010). While research on probiotics with immune modulation effects is mostly focusing on isolates of intestinal origin, relatively little is known about the effects of the dairy bacteria that takes part of the daily food intake of large group of the modern society. As mentioned by Rocha *et al.,* (2012), this contradicts to the present concept of probiotics, based on the hypothesis that the live bacteria in Bulgarian yogurt exert health-beneficial effects on the consumer and longevity (Metchnikoff, 1907). The aim of this study was to assess *in-vitro* strains of intestinal and yogurt origin and to compare the most promising among them in order to confirm or reject the possibility of anti-inflammatory abilities of strains suitable for production of dairy foods.

Materials and Methods

Bacterial strains and growth conditions

Lactobacillus bulgaricus and *Streptococcus thermophilus* strains were isolated from homemade yogurt and cultivated at 37°C in MRS broth (Oxoid, England) and M17 broth (Oxoid, England), respectively: *L. bulgaricus* b53/2; *L. bulgaricus* b144; *L. bulgaricus* b1997*; L. bulgaricus* b1972*; L. bulgaricus* b120*; L. bulgaricus* b2092*; L. bulgaricus* b58*; L. bulgaricus* b181*; S. termophilus* T69*; S. termophilus* T21*; S. termophilus* T43; *S. termophilus* T59; *S. termophilus* T109.

Nine bacterial strains were isolated from healthy volunteers (men and women, mean age 36 years) and used for comparison. Among them the *Bifidobacterium* strains (Gotova *et al.,* 2017) were cultivated in MRS broth with L-cysteine supplement under anaerobic conditions and the other strains (Gotova *et al.,* 2017) of intestinal origin were cultivated at 37°C in MRS broth.

For the purpose of the analyses the concen-

tration was adjusted to 1.10^9 CFU/mL by centrifugation at stationary phase and resuspended in PBS buffer. All strains are possession of the LB Bulgaricum PLC collection.

Cell cultures and preparation

The Cell lines Caco-2 and HT-29 (ATCC) - were cultured in media consisting of DMEM (Invitrogen, USA) with 10% fetal bovine serum (Invitrogen, USA) and in a CO_2-incubator (5% CO_2) at 37°C.

The Cell line THP-1 (ATCC) - was cultivated in media consisting of RPMI 1640 (Invitrogen, USA) with 10% fetal bovine serum and in a CO_2 - incubator (5% CO_2) at 37°C.

The two-component well-in-well cluster (Nunc™ Lab-Tek, Thermo Scientific™, USA) was prepared by using both HT-29 and THP-1 cell lines. A monolayer of HT-29 was cultivated in the upper wells, separated by a membrane (0.45 μm). After fifteen days of maturation of HT-29, the second cell line THP-1 was cultivated in the lower well of the cluster. The cells were differentiated for 24 h to macrophages in the presence of 1 μg/mL phorbol-myristate-acetate (PMA). Macrophages were washed with PBS (pH 7.4) to remove PMA and non-attached cells. To proceed, 200 μl suspension of the tested bacteria (concentration 1.10^9 CFU/mL) was added to 1.8 mL of RPMI media and then incubated in the lower well, together with the macrophages, and in the presence of the epithelium cell line (HT-29) in the upper well. After incubation for 20 h in the CO_2-incubator (5% CO_2) at 37°C, supernatants were collected and centrifuged for measurement of cytokines.

Splenocytes isolation and preparation

Spleens from BALB/c mice (eight weeks old) were cultivated in RPMI 1640 with 10% fetal bovine serum. The spleens were rubbed through a sterile sieve (mesh - 100 μm), washed with RPMI 1640 and centrifuged at 135 g for 10 min. The pellets were resuspended in 5 mL 0.87% of ammonium chloride for 2 min to remove erythrocytes. 100 μL of splenocyte suspension were transferred into each well and then 100 μL of bacterial suspension was added for evaluation after incubation in a CO_2 - incubator (5% CO_2) for 20 h at 37°C. The supernatants were collected and centrifuged for measurement of cytokines.

Cytokine assay in vitro

It was accomplished by the use of various enzyme-linked immunosorbent assay (ELISA) kits, according to the producer's instruction (Diaclone, USA). Murine ELISA kits were used for the supernatants obtained from splenocytes. Human ELISA kits were used for the supernatants obtained from combined human epithelial and monocytic cell lines. Each cytokine was evaluated in three separate analyses in the presence of control sample, containing only competent and/or epithelium cells and media, but no bacteria.

Results and Discussion

Considering the basic role of the anti-inflammatory cytokine IL-10, the first task was to select the strains with the highest induction regardless of their origin. Table 1 shows the concentrations of IL-10 evaluated on two different models, but corresponding to this cytokine analytical laboratory approaches. Table 1 also shows the concentrations of IL-8 together with the concentrations of IL-10 for each strain.

The combined cell line model with human HT-29 and THP-1 (differentiated to macrophages) represents *in vitro* the interaction between these two types of cells - epithelium and monocytic in the human body. The values evaluated on murine splenocytes are close but higher as splenocytes comprise a variety of immune cell types with particular interactions, which makes them more representative, considering the complicated immune signaling in the body. *L. gasseri* G4 strain demonstrates the highest induction of IL-10 and would be very appropriate for anti-inflammatory formulas, but the next task was to evaluate pro-inflammatory IL-8, because the optimal profile of the selected strain should not cause additional inflammation in the GIT.

Being the most powerful chemokine for activating neutrophils, the increased levels of IL-8 could result in lesions in the gut epithelium. IL-8 is a secondary and local marker of inflammation. It is released mostly by epithelial cells. The increased induction of pro-inflammatory cytokine TNF-α could be a triggering factor for abnormal induction of IL-8 and inflammatory shock in the body, although it is not an obligatory condition for increased levels of IL-8. Therefore, the evaluation was performed in the presence of TNF-α and compared with the values obtained without TNF-α, to check whether there are strains that could regulate this induction.

L. gasseri G4 demonstrates more than ten times higher levels of IL-8 when induced with TNF-α, compared with all the other selected strains. This is why it is inappropriate for consumption by people with intestinal inflammation. *E. faecium*

Table 1. Concentrations of IL-10 and IL-8 induced by LAB strains and evaluated with different analytical models, pg/ml

Cytokine / Strain	IL-10 HT29/THP1	IL-10 Splenocytes	IL8 HT29/THP1	IL8 with TNF-α Caco-2
L. gasseri G4	1860.6 ± 42,5*	1917.58 ± 48,2	271.6 ± 12.5	1482.0 ± 37.2
L. gasseri G7	1508.5 ± 39,8	1565.47 ± 38,4	52.9 ± 3.7	103.2 ± 5.8
L. gasseri G8	1489.9 ± 39,2	1546.86 ± 30,2	53.3 ± 3.9	107.0 ± 5.7
L. gasseri G11	1441.2 ± 35,4	1498.19 ± 31,6	63.7 ± 4.4	119.0 ± 5.8
E. facecium 2092/2	1670.2 ± 37,8	1727.21 ± 40,6	56.4 ± 4.2	123.4 ± 6.0
B. longum 1/2	1178.5 ± 29,8	1235.54 ± 35,0	43.5 ± 3.0	103.8 ± 5.5
B. longum 2/9	1258.0 ± 31,4	1314.98 ± 36,9	49.5 ± 3.3	110.3 ± 5.7
B. longum 3/15	1308.1 ± 30,8	1365.08 ± 41,6	58.7 ± 3.8	111.0 ± 5.9
B. longum 10/48	1449.1 ± 34,0	1506.07 ± 32,0	46.9 ± 3.2	90.2 ± 5.2
L. bulgaricus b53/2	1367.5 ± 32,6	1424.48 ± 30,6	65.7 ± 4.5	118.6 ± 5.7
L. bulgaricus b144	1400.4 ± 38,8	1457.40 ± 33,0	59.7 ± 4.7	116.3 ± 5.5
L. bulgaricus b1997	1276.6 ± 35,6	1018.69 ± 28,5	56.1 ± 4.7	132.1 ± 6.3
L. bulgaricus b1972	1067.6 ± 28,6	1333.61 ± 30,0	62.9 ± 4.9	123.6 ± 6.1
L. bulgaricus b120	930.2 ± 29,4	980.20 ± 26,3	66.6 ± 4.8	125.6 ± 6.0
L. bulgaricus b2092	945.2 ± 32,5	1002.23 ± 27,8	59.3 ± 4.5	119.8 ± 6.1
L. bulgaricus b58	1527.1 ± 40,2	1584.08 ± 36,4	78.1 ± 5.0	122.1 ± 6.2
L. bulgaricus b181	1041.8 ± 32,6	1098.84 ± 30,4	53.0 ± 3.6	110.3 ± 5.9
S. termophilus T69	1137.7 ± 38,0	1194.75 ± 38,2	46.4 ± 3.1	101.5 ± 4.8
S. termophilus T21	1331.7 ± 34,8	1388.70 ± 38,5	46.2 ± 2.9	133.0 ± 6.5
S. termophilus T43	1348.2 ± 38,6	1405.16 ± 40,2	46.0 ± 3.0	97.5 ± 5.1
S. termophilus T59	1597.9 ± 42,0	1659.70 ± 44,3	48.6 ± 3.2	103.6 ± 5.6
S. termophilus T109	973.9 ± 35,4	1033.20 ± 29,8	49.1 ± 3,5	123.0 ± 5,6
Control	4.8 ± 0.2	3.1 ± 0.1	50.4 ± 2.2	144.5 ± 4,9

Legend - *: mean values ± standard deviation

2092/2 is evaluated with the second highest result for IL-10 concentration, but strains- *S. thermophilus* T59 and *L. bulgaricus* b58, respectively, differ insignificantly from this result but also surpass the *L. gasseri* G7 strain and all selected *Bifidobacterium* strains. Furthermore, both yogurt strains are able to decrease the induction of IL-8 in the presence of TNF-α under the level of the control as well as *L. gasseri* G7, which possesses an optimal anti-inflammatory profile. Another two strains - *L. bulgaricus* b144 and *L. bulgaricus* C3/2 demonstrated significant concentrations of IL-10 compared to the selected *Bifidobacterium* strains, where only *B. longum* 10/48 is comparable, but it appears to be very difficult to cultivate in laboratory conditions. This is an additional issue that limits the potential industrial usage of strains of intestinal origin.

As the main target of this study was to select a representative *L. bulgaricus* and *S. thermophilus* with anti - inflammatory potential, it was equally important to measure the concentrations of IL-8. The assessment of this cytokine on one of the possible paths for production (epithelium/monocytic cell line model) shows a remarkably decreased level by all *S. thermophilus* strains. The results are competitive only with those of Bifidobacterium strains, where the absolute minimum measured belongs to *B. longum* 1/2.

In addition to their ability to affect intestinal epithelial cells and macrophages, probiotics also help influence the differentiation and function of a number of other immune cells associated with an inflammatory response, including dendritic cells as well as T cells (Thomas and Versalovic, 2010; Bermudez-Brito *et al.,* 2012). They also are an important source of cytokines that help promote differentiation of naive T cells into Th1, Th2, Th17 or T regulatory cells, thus the appropriate cell-mediated or humoral immune response. TGF-β is a regulatory cytokine which promotes the development of

regulatory T cells.

In order to receive a more complete profile of the anti- inflammatory potential of certain strains, evaluation of TGF-β which is released by both epithelial and immunocompetent cells, was performed. Therefore, it is appropriate to compare the results for some cytokines evaluated on different laboratory models. Table 2 presents the significant difference between TGF-β production on epithelial cell line Caco-2, combined cell lines (epithelium/monocytic) and splenocytes. Caco-2 synthesizes TGF-β even without being induced by bacteria, as shown by the high level of the control. TGF-β values obtained from splenocytes are many times higher than on epithelial and combined cell lines, which demonstrates the different power of these three analytical models. An exception of this observation are strains *S. thermophilus* T59 and *S. thermophilus* T109 that make a surprising difference with their very high values obtained on epithelial cell line. To summarize the results from Table 2, it is shown that none of the strains of intestinal origin are able to induce the production of TGF-β in concentrations above the control on both epithelial and combined cell lines, unlike the concentrations evaluated on splenocytes, where all strains demonstrate moderate induction of this cytokine.

Table 2. TGF-β concentrations analyzed by using different analytical models: epithelial cell line; combined (epithelium/monocytic) and splenocytes, pg/ml

Cytokine / Strain	TGF-β Caco-2	TGF-β HT-29/THP-1	TGF-β splenocytes
L.gasseri G4	72.6 ± 5.3*	259.0 ± 10.6	674.22 ± 16.8
L.gasseri G7	277.3 ± 11.2	279.3 ± 11.0	548.75 ± 15.2
L.gasseri G8	66.0 ± 5.6	313.2 ± 15.2	660.28 ± 16.4
L.gasseri G11	171.6 ± 10.6	262.4 ± 11.8	834.53 ± 16.9
B. longum 1/2	191.5 ± 11.0	279.3 ± 12.6	736.95 ± 16.8
B. longum 2/9	303.7 ± 14.8	330.2 ± 14.0	646.34 ± 15.8
B. longum 3/15	171.6 ± 11.5	341.1 ± 13.7	590.58 ± 15.7
B. longum 10/48	125.4 ± 8.4	306.4 ± 15.0	688.16 ± 16.0
E. facecium 2092/2	382.9 ± 15.0	550.4 ± 15.2	689.00 ± 16.0
*L. bulgaricus b*53/2	204.7 ± 13.2	309.8 ± 16.2	702.10 ± 17.5
L. bulgaricus b144	118.8 ± 8.2	289.5 ± 13.2	716.04 ± 17.4
L. bulgaricus b1997/4	230.1 ± 10.7	296.3 ± 15.4	653.31 ± 15.8
L. bulgaricus b1972/1	178.2 ± 11.8	289.5 ± 14.8	499.96 ± 14.6
L. bulgaricus b120	349.9 ± 15.0	303.1 ± 14.2	764.83 ± 16.5
L. bulgaricus b2092/13	244.3 ± 9.8	289.5 ± 13.9	702.10 ± 16.2
L.bulgaricus b58*/1*	257.5 ± 10.5	421.7 ± 15.8	716.04 ± 16.8
L. bulgaricus b181/10	165.0 ± 11.2	265.8 ± 12.0	618.46 ± 14.9
S. termophilus T69	204.7 ± 10.0	486.1 ± 16.5	813.62 ± 15.8
S. termophilus T21	99.0 ± 7.6	333.6 ± 14.7	757.86 ± 16.5
S. termophilus T43	244.3 ± 12.2	421.7 ± 15.0	841.50 ± 17.0
S. termophilus T59	1089.3 ± 21.8	306.4 ± 14.5	618.46 ± 16.2
S. termophilus T109	891.3 ± 17.0	496.2 ± 15.1	550.60 ± 15.6
Control	231.1 ± 10.9	343.7 ± 14.8	372.5 ± 9.4

Legend - *: mean values ± standard deviation

Conclusion

The novelty of this study is the ability of some LAB strains of yogurt origin not only to induce concurrent levels of the anti-inflammatory IL-10 concentration, but also to decrease the levels of IL-8, even additionally induced by TNF-α, to the values that are more likely to cause suppression of an already existing inflammation.

All probiotic properties are strain-specific, as it is well known and as the results clearly demonstrate. For example, the difference between *L. gasseri* G7 and *L. gasseri* G4, where the first one is suitable for developing anti-inflammatory formulas and the other is not. Another case is the difference between the intensity with which the strains in same

species are able to induce certain cytokine, like *L. bulgaricus* b144 and *L. bulgaricus* b120 strains.

In conclusion, it can be summarized that the selected LAB strains have the potential to reduce intestinal inflammation: - *L. bulgaricus* b58, *L. bulgaricus* b144, *L. bulgaricus* b53/2 and *S. thermophilus* T59, *S. thermophilus* T43, *S. thermophilus* T21. Due to the *in-vitro* results and their natural ability to produce yogurt, these strains could be directed to future clinical trials and eventually included in the diet of people with IBD.

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