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(71) Applicants: **INSTITUTE OF MOLECULAR GENETICS AND GENETIC ENGINEERING, UNIVERSITY OF BELGRADE** [RS/RS]; Vojvode Stepe 444a, P.O. Box 23, 11010 Belgrade (RS). **VETERINARSKA STANICA "KOKER" D.O.O.** [RS/RS]; Branca Radičevića 7, 22244 Adaševci (RS).

(72) Inventors: **GOLIĆ, Nataša**; Milivoja Perovića 6, 11000 Belgrade (RS). **TERZIĆ-VIDOJEVIĆ, Amarela**; Borivoja Stevanovića 7, 11000 Belgrade (RS). **VELJOVIĆ, Katarina**; Zaplanjska 86b/36, 11000 Belgrade (RS). **MRVALJEVIĆ, Igor**; Gradski park 10, 22000 Sremska Mitrovica (RS).

(74) Agent: **PETOŠEVIĆ D.O.O.**; Knez Mihailova 1-3, 4th floor, 11000 Belgrade (RS).

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(54) Title: NEW PROBIOTIC STARTER CULTURE FOR HUMAN AND ANIMAL USE

(57) Abstract: The subject invention relates to the new starter probiotic culture that is composed of three thermophilic lactic acid bacteria (LAB) natural isolates: *Streptococcus thermophilus* LMG P- 28585 (BGVLJ1-44), *Lactobacillus fermentum* LMG P-28583 (BGHI14) and *Lactobacillus helveticus* LMG P-24226 (BGRA43), in specific ratios. The starter culture of the subject invention exhibits proteolytic and antimicrobial activity, as well as immunomodulatory effect. Moreover, it relates to the use thereof in prevention and/or treatment of intestinal infections in mammals, namely humans and animals and as an immunomodulator.



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## NEW PROBIOTIC STARTER CULTURE FOR HUMAN AND ANIMAL USE

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**Field of invention**

10 The subject invention belongs to the field of biotechnology and is referring to the new probiotic starter culture with unique combination of three bacterial strains within genus *Streptococcus thermophilus*, *Lactobacillus fermentum* and *Lactobacillus helveticus*. Also, the use thereof in prevention and/or treatment of intestinal infections in mammals, namely humans and animals, and use thereof as an immunomodulator is described.

15 **Background of the invention**

The technical problem that is solved by the subject invention can be defined as the provision of a new improved probiotic culture as an alternative to use of antibiotics in treatment of intestinal infections in humans and animals, whereby the improvement of overall health of the subject is achieved.

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It is well known that the probiotic characteristics are strain-dependent, not genus-dependent. Therefore, the probiotic starter culture of the subject invention is based on the optimal ratio of three unique natural isolates *Streptococcus thermophilus* LMG P-28585 (BGVL1-44), *Lactobacillus fermentum* LMG P-28583 (BGHI14) and *Lactobacillus helveticus* LMG P-24226 (25 (BGRA43) characterized on the strain level by 16S rDNA sequencing and pulsefield gel electrophoresis, for which it has been proven to express proteolytic and antimicrobial activity, as well as an immunomodulatory effect.

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Moreover, the use of this starter culture for producing the probiotics is described and compositions intended for the treatment and/or prophylaxis of disorders associated with the colonization of pathogens in mammalian gastrointestinal tract, as well as for obtaining the probiotics and compositions for colonization of mammalian gastrointestinal tract after antibiotic therapy.

Further, the subject invention is referring to the probiotics and the compositions based on this probiotic starter culture and to use thereof in prevention and/or treatment of intestinal infections in humans and animals, and use thereof as an immunomodulator in maintaining the native  
5 balance of the immune system and in the regulation of immune responses in humans and animals, including infants and cubs.

Moreover, the subject invention is also referring to the combination of probiotics based on this probiotic starter culture and the use thereof as an immunomodulator in prevention and/or  
10 treatment of inflammatory bowel disease in humans and animals.

Specifically, the subject invention is referring to a new probiotic starter culture comprising three thermophilic natural isolates of lactic acid bacteria (LAB): *Streptococcus thermophilus* LMG P-28585 (BGVL1-44), *Lactobacillus fermentum* LMG P-28583 (BGHI14) and *Lactobacillus*  
15 *helveticus* LMG P-24226 (BGRA43), in specific ratios, use thereof in prevention and/or treatment of intestinal infections in mammals and use thereof as an immunomodulator.

#### Prior art

The probiotic preparations based on the use of various LAB strains, *Bacillus sp.* and/or yeasts  
20 are well known in the prior art. In the prior art, probiotics such as mono- or mixed cultures of live microorganisms, which when administered in adequate amounts confer a health benefit on the host (humans or animals), improving thereby the existing features of gut microbiota, are well known.

The probiotic bacteria have a number of beneficial effects, among others, in prevention and  
25 treatment of diarrhea, the beneficial effect on the immune system (probiotic microorganisms form an intestinal ecosystem that inhibits the growth of pathogenic and opportunistic bacteria (*E. coli*, *Salmonella Typhimurium*, *Clostridium perfringens*, *Clostridium difficile*, *Pseudomonas*, *Listeria*, *Staphylococcus aureus*...) in prevention of candidiasis, etc.

Currently available compositions for treatment of intestinal infections in domestic animals are  
30 designed for feeding the new born animals (*Method for correction of immunobiological indices with piglets in period of weaning - RU2471359 (C1) — 2013-01-10*), prepared in the form of dry feed with addition of various active substances such as zeolite (*Complex probiotic preparation*

for beef strains - RU2011153643 (A) — 2013-07-10), medicinal plants (Chinese herbal medicine composite probiotic feed additive for pigs and preparation method of additive - CN103549141 (A) — 2014-02-05), prebiotics (Feed additive for veterinary drug solid fermentation and preparation method of feed additive - CN103039700 (A) — 2013-04-17), vitamins (Pig feed capable of preventing piglets from diarrhoea and traditional Chinese medicine composition - CN102835609 (A) — 2012-12-26) etc.

US patent **US 5,656,268** describes the process for producing of biological product in liquid form or as cream, comprising pure milk or milk ingredients and the ferments or bacterial cultures of *Lactobacillus fermentum*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus helveticus*, *Lactobacillus casei* and *Streptococcus thermophilus* species. This invention can be considered as the closest prior art document since the biological product comprises milk and at least two of the bacteria as previously stated. By the described process the product of defined acidity is obtained, which can further comprise an oil (olive oil and aromatic oil) as well as fruits, vegetables, plants or honey, and based on milk or whey that can be used as an additive for improving users' health, especially when in dry or sublimed form. Moreover, the product can be used as a cosmetic cream, cream and liquid for body and massage, as cream for gum treatment and in spa for taking baths.

The probiotic starter culture of the subject invention substantially differs from the stated patent, among other things, in that it is based entirely on optimal ratios of three clearly defined bacterial strains within the genus of *Streptococcus thermophilus*, *Lactobacillus fermentum* and *Lactobacillus helveticus* stated in the subject invention, namely: *Streptococcus thermophilus* LMG P-28585 (BGVLJ1-44), *Lactobacillus fermentum* LMG P-28583 (BGHI14) and *Lactobacillus helveticus* LMG P-24226 (BGRA43), that is prerequisite for use thereof in humans and animals. In the prior art document, the ratio of components in the biological product is not defined, only the process for obtaining the product, which is substantially different from the process for obtaining the probiotic starter culture of the subject invention.

Moreover, as described below, probiotic starter culture expresses proteolytic and antimicrobial activity and immunomodulatory effect, compared to the prior art document wherein such activity is not shown in any way. The probiotic starter culture of the subject invention does not contain any oils, nor other ingredients as in stated prior art document, and differs by the forms of use. It's well known that the probiotic characteristics are strain-dependent, not genus-dependent. Therefore, the combination of strains expresses its probiotic characteristics, and is supported by the description and as such can be used, compared to the product of the prior art document that

refers to a species comprising broader (all strains), but biologically is absolutely inapplicable.

European patent **EP1359816** relates to a combination of probiotics comprising lactobacilli, propionic acid bacteria and bifidobacteria. The probiotics are preferably combined with a suitable prebiotic to produce a symbiotic. The combination of the invention may be consumed as such or combined with a suitable foodstuff, such as a dairy product or a drink, and it is therapeutically useful for example for stimulating the immune system and for general health improvement. The given combination is prepared in unit dosage form, precisely in the form of a capsule.

European patent **EP1541673** relates to novel lactic acid bacteria and particularly microorganisms of the genera *Lactobacillus*, *Bifidobacterium* and *Streptococcus* (*Enterococcus*) that have been isolated and used thereof for preparing the composition for use in the preparation of pet food compositions intended to improve the health of pets (mainly canine and feline). Given compositions are selected for the regulation of animal's immune response primarily for its ability to colonize the gastrointestinal tract of a pet and to exert a beneficial probiotic activity on pet health.

Moreover, European patent **EP2219658** relates to a composition based on probiotic bacteria and a substance having probiotic properties and the use thereof to prevent and/or treat respiratory pathologies and/or infections and simultaneously improve intestinal functionality. A symbiotic composition comprising the following bacterial strains *Lactobacillus plantarum* (LMG P-21020), *Lactobacillus plantarum* (LMG P-21021), *Lactobacillus rhamnosus* (DSM 16605), *Lactobacillus rhamnosus* (DSM 19739), *Bifidobacterium lactis* (LMG P-21384) and prebiotic component comprising i.e. fructo-oligosaccharides or galacto-oligosaccharides.

European patent **EP2101796** discloses the strain of microorganism *Bacillus smithii* TBM12 MSCL P737, and the compositions comprising the said strain, compositions for use, as a probiotic for antibacterial purposes, for colonizing the gastrointestinal tract and for stimulating the immune system, as well as use of said strain as a food supplement and as a component in therapeutic and non-therapeutic probiotic composition.

European patent **EP0856259** describes the composition, comprising lyophilized live active lactic acid bacteria in combination with oligosaccharides for use as food supplement, intended for balancing intestinal flora. Stated lyophilizate comprises the strains of genus *Bifidobacterium*, *Lactobacillus*, *Streptococcus*. Moreover, the invention is disclosing a kit comprising two containers, wherein one is holding a liquid, creamy or pasty foodstuff, and the other one is holding the stated composition for the purpose of supplementing said foodstuff with the composition held therein, at the moment of consuming the foodstuff. The foodstuff in this case is a milk, a yoghurt, or another type of fermented milk, a milk-based dessert, a milk-based beverage or a beverage based on milk serum or milk permeate enriched with fruit, a fruit juice, a tomato juice, a carrot juice, a tea, or a beverage based on vegetable extracts.

Therefore, the vast number of various probiotic starter cultures and probiotics are known, as mono- culture as well as the combination of different bacterial strains. As can be seen, from previously described documents from patent and non-patent literature, among others, the combinations of various strains of microorganisms that can be used in various forms, and that have a broad scope of different effects on humans and animals, are well-known. However, according to our knowledge, so far no probiotic product containing the suggested combination of bacteria in the given ratios and with such probiotic potential for use in mammals has been described.

#### **Brief description of the invention**

Microorganisms ingested with food have a positive effect on human and animal health. While the use of probiotics for humans is extensive, the use of probiotics in livestock is negligible. The latest studies are intensively engaged in the development of probiotic preparations for animals, based on the use of lactic acid bacteria (LAB), *Bacillus* sp. or yeasts. Increased interest in use of probiotics in livestock has arisen from tight European regulations related to use of the antibiotics in livestock production. Therefore, probiotics are considered as an appropriate alternative especially in terms of intestinal infections in animals, especially cubs. Establishing good microflora by colonisation with the LAB in the intestines of newborn animals immediately after birth results in healthier animals.

In the first days after birth the animals are prone to bacterial infections from the environment, which is extremely important for development of their immune system. Nevertheless, it often occurs that newborn animals are infected by enterobacterias (*Enterobacteriaceae*), which

causes the intestinal infections accompanied with diarrhea, weight loss, and often with high mortality rate in animals, which represents a significant economic loss to the livestock industry. Given that the use of antibiotics is often the first choice in treating intestinal infections, this represents an additional problem in the livestock and food industry.

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Fermented dairy products with probiotic bacteria are products obtained by fermentation of the milk by probiotic starter culture activity or by combining the starter cultures with other lactic acid bacteria.

#### 10 **Detailed description of the invention**

The subject invention is specifically related to the new probiotic starter culture for human and animal use, the technological process for preparation thereof and use of this probiotic culture in prevention and/or treatment of intestinal infections of various ethyology in humans and animals as well as use thereof as an immunomodulator.

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The basic unit of product is the probiotic culture, which represents a unique combination of bacteria with specific probiotic potential, for prevention and/or treatment of intestinal infections and other gut immunological diseases in humans and animals. Probiotic culture is particularly effective in therapy of intestinal infections of various ethyology and reduces the number of pathogenic bacteria with unique wide range of antimicrobial activity.

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Probiotic culture exhibits exceptional probiotic potential, has the ability of immunomodulation as well as specific antimicrobial effect against large number of bacterial strains. The new probiotic culture represents an alternative to use of antibiotics in treatment of intestinal infections, based on wide range of antimicrobial activity, especially for severe hardly curable infections e.g. infections caused by *Clostridium perfringens* and *Clostridium difficile*, and thereby the improvement of overall health of humans and animals is achieved.

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In one embodiment, the subject invention is related to a new probiotic starter culture comprising three thermophilic LAB natural isolates: *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226 in a specific ratio that provides desired effects, use thereof for producing probiotics and compositions intended for prevention and/or treatment of disorders related to colonisation of mammalian gastrointestinal tract by pathogenic microorganisms and have an immunomodulator effect.

30

In the next embodiment, probiotic starter culture of the subject invention comprises three natural thermophilic LAB isolates: *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226 in optimal ratio. Specific ratio thereof is the key factor of effect of this starter culture in prevention and/or treatment of intestinal infections in mammals, both in humans and animals, and will be described in details below.

By *in vitro* experiments, various combinations of strains were tested: *Streptococcus thermophilus* LMG P-28585 from 10% to 40%, *Lactobacillus fermentum* LMG P-28583 from 10% to 50% and *Lactobacillus helveticus* LMG P-24226 from 40% to 80%. Results showed that in all combinations tested, strains expressed probiotic potential.

In the technological sense, for preparation of fermented milk probiotic product the optimal ratio of strains *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226 in probiotic starter culture is 30%: 30%: 40% (3:3:4), as well as 30%: 20%: 50 (3:2:5). These ratios provide sufficient number of all strains in the culture, and do not lower the pH of fermented product below 4.2-4.3 (threshold level of acidic taste tolerance).

Moreover, in one embodiment, the preferred ratio of strains *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226 is 10%: 10%: 80% (1:1:8) or 10%: 20%: 70% (1:2:7). This ratio provides larger number of *Lactobacillus helveticus* LMG P-24226 bacterial strain for which it has been proven to show broad antimicrobial range of action on difficult to cure pathogenic bacteria, comprising *Staphylococcus aureus* ATCC25923, *Staphylococcus* sp. ATCC Met<sup>R</sup> (clinical isolate) Torlak collection, *Yersinia enterocolitica* O3 (clinical isolate) Torlak collection, *Shigella sonnei* (Hamburg IO5) Torlak collection, *Shigella flexneri* (London 9950) Torlak collection, *Shigella dysenteriae* (serotype 5 and 7) Torlak collection, *Streptococcus pneumoniae* ATCC276, *Streptococcus pneumoniae* ATCC496, *Pseudomonas* sp., *Escherichia coli* C600, *Escherichia coli* ATCC25922, *Clostridium sporogenes* Torlak collection and *Clostridium difficile* (clinical isolate).

Ratio of *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226 of 40%:20%:40% (4:2:4) has a potential use in the immunocompromised group of patients, since in this combination the strain present in least

amount is *Lactobacillus fermentum* LMG P-28583, which expresses mild proinflammatory activity.

In the next embodiment, probiotic starter culture comprises *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226 in the ratio of 30%: 40%: 30% (3:4:3). In this case, the most present strain is *Lactobacillus fermentum* LMG P-28583 that by its characteristics bursts immune response.

As it is described in the specification, depending on stated ratios of given bacterial isolates in probiotic starter culture, the latter will express its proteolytic activity (acidification) and antimicrobial activity.

Stated combinations provide exceptional results in treatment of intestinal infections in mammals, especially in animals. Namely, the subject starter culture shows exceptional outcome in the treatment of infections caused by bacteria *Clostridium perfringens* and *Clostridium difficile*, as well as by families of *Enterobacteriaceae*, when the numbers of live bacteria are in the following ranges: *Streptococcus thermophilus* LMG P-28585 from  $6 \times 10^7$  to  $1.25 \times 10^{11}$ , *Lactobacillus fermentum* LMG P-28583 from  $1.1 \times 10^8$  to  $1.25 \times 10^{11}$  and *Lactobacillus helveticus* LMG P-24226 from  $4 \times 10^6$  to  $2.5 \times 10^9$ .

It is well known that ingestion of certain microorganisms with food has a beneficial effect on human and animal health. Namely, cubs during the first few days of life get infected with bacteria from the immediate environment, which is extremely important for immunity development. However, during that period it occurs that cubs are infected with pathogenic intestinal bacteria (*Enterobacteriaceae*), which leads to intestinal infections accompanied by diarrhoea, weight loss and often by death of cubs. Taking into account that in such cases, for the treatment of intestinal infections the use of antibiotics is usually the first choice of the practitioner, this represents an additional problem in the livestock breeding and the food industry. Therefore, this probiotic culture, aside from solving a problem of intestinal infections in domestic animals, also contributed to reduction of antibiotic use in livestock.

Further, majority of probiotics from the prior art are intended for feeding of the cubs, wherein this probiotic culture (due to safe status of lactic acid bacteria) in one embodiment is designated for

gravid animals wherein cubs receive the passive immunity from their mothers and are therefore already immunised during their first days of life. Also, during the last 15 days of gravidity, the immunity in animals lowers and the number of enterobacterias in mother's intestines increases, and immediately after the birth they are transmitted to offspring that becomes infected.

- 5 Treatment of gravid animals with the new probiotic culture results in decreased number of enteropathogens in the intestines of mother and infections of newborn cubs is significantly reduced.

10 In another embodiment, probiotic starter culture comprising *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226 wherein the range of number of live bacteria for *Streptococcus thermophilus* LMG P-28585 is from  $4 \times 10^9$  to  $8 \times 10^{10}$ , for *Lactobacillus fermentum* LMG P-28583 is from  $3 \times 10^9$  to  $1 \times 10^{10}$  and for *Lactobacillus helveticus* LMG P-24226 is from  $4 \times 10^6$  to  $9 \times 10^8$  CFU/ml.

- 15 The new probiotic culture of the subject invention was tested in farm trials and was proven to be extremely effective in the treatment of intestinal infections of various ethyology, and specifically successfully reduced (below detection level) the number of *Clostridium perfringens*, as well as *Enterobacteriaceae*.

20 All previously described combinations can be prepared in the form of a fermented dairy product, suspension, lyofilisate, premix, spray-dry, tablets, capsules and other forms that are know to a person skilled in the art and as such are for human and animal use. All these forms comprise probiotic starter culture of the subject invention obtained by known methods.

- 25 Method for preparation of probiotic starter culture comprises: 1) the cultivation of natural isolates included in the composition of a probiotic starter culture under optimal conditions for growth of these bacteria; 2) combination of natural isolates that form a part of a probiotic starter culture in an optimal ratio.

30 Due to the presence of strain *Streptococcus thermophilus* LMG P-28585 in the probiotic culture, the one can be also used for the preparation of a fermented dairy product that can be used for prevention of intestinal infections in mammals including humans and animals (pigs, canine, feline, etc.).

Method for preparation of a fermented probiotic product for human and animal use (non ruminants) which includes the milk fermentation process by use of a defined starter and probiotic bacteria, that includes the following stages: 1) Pre-cultivation of natural isolates that form a probiotic starter culture under the optimal conditions for growth of these bacteria; 2) Milk is inoculated with natural isolates prepared in stage 1, in optimal ratio; 3) Incubation of milk inoculated in stage 2 under optimal conditions for fermentation.

The obtained fermented product is stable, the number of live probiotic bacteria is reproducible and the technology of producing it is easily scaled up. Moreover, such product exhibits better probiotic effect probably due to presence of specific bioactive peptides incurred by degradation of milk protein by proteolytic enzyme activity of probiotic bacteria.

The use of probiotic culture in other mammals (ruminants) as well as other animals is recommended to be used as the probiotic culture prepared without milk (as previously described).

#### Brief description of figures

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Figure 1. Genotyping of the strains included in the probiotic composition by pulse-field gel electrophoresis (PFGE).

- Results indicate that bacteria of the probiotic culture represent unique strains within the stated species.

Figure 2. Antimicrobial activity of individual strains of the probiotic composition and probiotic combination (synergism) on *Clostridium difficile*. 1. Control *C. difficile* + MRS; 2. *C. difficile* + LMG P-28585; 3. *C. difficile* + LMG P-28583; 4. *C. difficile* + LMG P-24226; 5. *C. difficile* + probiotic combination.

- Testing of the individual cultures and the probiotic combination indicate that the combination (synergism) successfully suppresses the growth of *C. difficile* – there is no gas production nor cracking of growth media, while in the case of individual cultures the growth of *C. difficile* is just partially suppressed– slight gas production and partially cracking of growth media. Control – the growth of *C. difficile* induces gas production and cracking of growth media.
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- Figure 3. Survival of strain LMG P-28585, in simulated conditions of the gastrointestinal tract in a saline buffer and in the dairy matrix.
- Tested strain successfully survived in the simulated gastrointestinal tract conditions
- Figure 4. Survival of strain LMG P-28583 in simulated conditions of the gastrointestinal tract in a saline buffer and in the dairy matrix.
- Tested strain successfully survived in the simulated gastrointestinal tract conditions
- Figure 5. Survival of strain LMG P-24226 in simulated conditions of the gastrointestinal tract in a saline buffer and in the dairy matrix.
- Tested strain successfully survived in the simulated gastrointestinal tract conditions
- Figure 6. The adhesion of strains LMG P-28585, LMG P-28583 and LMG P-24226. to Caco-2 epithelial intestinal cells. The values given in the graph represent mean values of 3 measurements and are expressed in percentages. Statistically significant values compared to the control are marked with asterisks (\*p <0.5, \*\*p <0.1).
- The percentage of adhesion showed success of binding of individual strains to epithelial intestinal cells.
- Figure 7. Adhesion of *E. coli* ATCC25922 in the presence and the absence of strains LMG P-28585, LMG P-28583 and LMG P-24226. The values given in the graph represent mean values of 3 measurements and are expressed in percentages. Statistically significant values compared to the control are marked with asterisks (\*p <0.5, \*\*p <0.1).
- The percentage of adhesion *E. coli* ATCC25922 in the presence of individual strains and the probiotic combination indicate that the probiotic combination (synergism) is the most successful in blocking of the binding of *E. coli* ATCC25922 to epithelial intestinal cells.
- Figure 8. Adhesion of *Salmonella* 654/7E (veterinary isolate) in the presence and the absence of strains LMG P-28585, LMG P-28583 and LMG P-24226. The values given in the graph represent mean values of 3 measurements and are expressed in percentages. Statistically significant values compared to the control are marked with asterisks (\*p <0.5, \*\*p <0.1).
- The percentage of adhesion *Salmonella* 654/7E in the presence of individual strains and the probiotic combination indicate that the strain LMG P-28585 is the most successful in blocking of the binding of *Salmonella* 654/7E to intestinal epithelial cells.
- Figure 9. Proliferation of rat's GALT cells in the presence of UV-inactivated strains LMG P-28585, LMG P-28583 and LMG P-24226, live strains LMG P-28585, LMG P-28583 and LMG P-24226 in milk and probiotic combination prepared in milk. The values given in the graph represent the mean values of 4 measurements and are expressed in percentages. Statistically significant values compared to the control are marked with asterisks (\*p <0.5, \*\*p <0.1).
- The results showed that the live strains significantly increase the GALT lymphocytes proliferation compared to the control.
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- Fig. 10. Reduction of the number of pathogenic bacteria after the treatment of animals in the farm trial, in this case reduction of the number of *Enterobacteriaceae* in the neonatal piglets after the treatment of pregnant cows with the probiotic fermented dairy product.
- The results indicate that the number of bacterias of *Enterobacteriaceae* family significantly decreases after the probiotic treatment of animals, while the number of same bacteria in untreated animals remained unchanged.
- Fig.11 Reduction of the number of pathogenic bacteria after the treatment of animals in the farm trial, in this case rReduction of the number of *Clostridium perfringens* in goats after the probiotic treatment.
- Results indicate that the number of bacteria of *Clostridium perfringens* genus is reduced below the detection treshhold after the probiotic treatment of animals
- Fig. 12. The body weight of the laboratory animals
- The results indicate that individual strains partially affect the increase in appetite of the animals, which affects the increase of the bodymass thereof
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#### Natural isolates included in the probiotic culture

The probiotic starter culture is composed of three thermophilic LAB natural isolates *Streptococcus thermophilus* BGVLJ1-44 (natural isolate from an artisanal yogurt produced from uncooked cow's milk, the strain from laboratory collection of Laboratory of Molecular Microbiology, Institute of Molecular Genetics and Genetic Engineering, University of Belgrade - LMM-IMGGE), *Lactobacillus fermentum* BGHI14 (natural isolate from the newborn feces, the strain from laboratory collection of LMM-IMGGE) and *Lactobacillus helveticus* BGRA43 (natural isolate from the human gastrointestinal tract, from the laboratory strain collection of LMM-IMGGE).

The strains were isolated by classical microbiological methods described previously (Terzic Vidojevic et al., 2007). For the cultivation of BGVLJ1-44 liquid and solid M17 medium (Merck GmbH, Darmstadt, Germany), supplemented with 0.5% glucose (GM17) was used, while for the cultivation of strains BGHI14 and BGRA43 liquid and solid MRS medium (Merck, GmbH) was used. The cultures were stored at -80°C in a suitable growth medium (GM17 or MRS supplemented with 15% glycerol) in cryovials.

#### Deposit of biological materials

All strains were deposited in Belgian Co-ordinated Collection of Microorganisms (BCCM), Laboratory for Microbiology, University of Ghent, Belgium, K.L. Ledeganckstraat 35, Ghent, Belgium.

The strain BGRA43 was deposited on July 31, 2007 by Ljubisa Topisirovic, the Head of Laboratory for Molecular Genetics of Industrial Microorganisms (former name of Laboratory for Molecular Microbiology), IMGGE, as the depositor. Strains BGVLJ1-44 and BGHI14 were deposited on October 28, 2014 by Milan Kojic, the Head of the Laboratory of Molecular Microbiology, IMGGE, as the depositor. The strain *Streptococcus thermophilus* BGVLJ1-44 was deposited under LMG P-28585 number, the strain *Lactobacillus fermentum* BGHI14 under LMG P-28583 number, and the strain *Lactobacillus helveticus* BGRA43 has a deposit number LMG P-24226.

The strain ***Lactobacillus helveticus* BGRA43** was deposited on 31/07/2007; the strain assigned number is **LMG P-24226**. The depositor is Ljubisa Topisirovic, the head of Laboratory for Molecular Genetics of Industrial Microorganisms (former name of Laboratory for Molecular Microbiology), IMGGE, Belgrade, Serbia.

The strain ***Streptococcus thermophilus* BGVLJ1-44** was deposited under the number **LMG P-28585**, on 28/10/2014. The depositor is Dr. Milan Kojic, the Head of Laboratory of Molecular Microbiology, IMGGE, Belgrade, Serbia.

The strain ***Lactobacillus fermentum* BGHI14** was deposited under the number **LMG P-28583**, on 28/10/2014. The depositor is Dr. Milan Kojic, the Head of Laboratory of Molecular Microbiology, IMGGE, Belgrade, Serbia.

#### **Characterization and identification of LMG P-28585, LMG P-28583 and LMG P-24226 strains**

Natural isolates *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226 were characterized in details, both by biochemical and molecular genetics methods.

Phenotypic characteristics of natural isolates LMG P-28585, LMG P-28583 and LMG P-24226 are given in the Table 1.

Identification of LMG P-28585, LMG P-28583 and LMG P-24226 strains to the species level was performed by nucleotide sequencing of 16S rDNA (Jovcic et al., 2009).

Additional identification of the strain level was performed by PFGE (pulse-field gel electrophoresis) fingerprint method (Figure 1). The total DNA of strains *Streptococcus thermophilus* LMG P-28585, *Lactobacillus helveticus* LMG P-24226 and *Lactobacillus fermentum* LMG P-28583 were digested *in situ* by *NotI* restriction enzyme. The obtained restriction profiles represent unique fingerprint profiles of the analyzed strains.

#### **Proteolytic activity**

Due to importance of proteolytic activity of the strains in the process of preparation of fermented probiotic dairy product, the proteolytic activity of strains *Streptococcus thermophilus* LMG P-28585, *Lactobacillus helveticus* LMG P-24226 and *Lactobacillus fermentum* LMG P-28583 was tested as previously described (Kojic et al., 1991). The ability of  $\alpha$ -,  $\beta$ - and  $\kappa$ -casein hydrolysis was tested.

Strains LMG P-28585 and LMG P-24226 completely hydrolysed all three casein fractions ( $\alpha$ -,  $\beta$ - and  $\kappa$ -casein), whereas the strain *Lactobacillus fermentum* LMG P-28583 did not exhibit any proteolytic activity towards any type of casein (see Table 1).

**Table 1**

Phenotypic characteristics of the strains *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226.

| Feature tested                             | <i>Streptococcus thermophilus</i><br>LMG P-28585 | <i>Lactobacillus fermentum</i><br>LMG P-28583 | <i>Lactobacillus helveticus</i><br>LMG P-24226 |
|--------------------------------------------|--------------------------------------------------|-----------------------------------------------|------------------------------------------------|
| Growth at 15°C                             | –                                                | +                                             | –                                              |
| Growth at 30°C                             | +                                                | +                                             | +                                              |
| Growth at 45°C                             | –                                                | +                                             | –                                              |
| Growth in broth with 2% NaCl               | –                                                | –                                             | –                                              |
| Arginine hydrolysis                        | –                                                | +                                             | –                                              |
| Esculin hydrolysis                         | –                                                | ±                                             | ±                                              |
| Citrate utilization                        | –                                                | –                                             | –                                              |
| Production of CO <sub>2</sub> from glucose | –                                                | +                                             | –                                              |
| Production of acetoin                      | +                                                | –                                             | –                                              |
| Production of diacetyl                     | +                                                | –                                             | –                                              |
| Curdling (h)                               | 5                                                | No curdling                                   | 5                                              |
| Litmus milk                                | A <sup>1</sup> C <sup>2</sup> R <sup>3</sup>     | R                                             | ACR                                            |
| Survival at 63.5°C for 30 min              | ±                                                | +                                             | +                                              |
| Proteolytic activity                       | +                                                | –                                             | +                                              |
| Antimicrobial activity                     | –                                                | +                                             | –                                              |
| Aggregation                                | –                                                | –                                             | –                                              |
| Production of exopolysaccharides           | +                                                | –                                             | –                                              |

5 <sup>1</sup>A-acid production (red ring); <sup>2</sup>C-curdling (milk curdled due to lactose fermentation); <sup>3</sup>R-reduction of H<sup>+</sup> ions (white color).

+ positive reaction; – negative reaction; ± weak reaction.

### Antimicrobial activity

Antimicrobial activity was tested by the deferred antagonism method using overnight cultures of *Streptococcus thermophilus* LMG P-28585, *Lactobacillus helveticus* LMG P-24226 and *Lactobacillus fermentum* LMG P-28583 and various indicator strains. Briefly, soft GM17 and MRS agars (0.7% w/v) containing indicator strains were overlaid onto GM17 and MRS plates, respectively. The plates were incubated overnight at 37°C. A clear zone of inhibition of indicator strain growth around the well was taken as a positive signal for production of antimicrobial compound.

In the case of anaerobic bacteria (*Clostridium* sp.) antimicrobial activity was tested as follows: the glass tubes were filled with overnight culture of *C. difficile* or *C. sporogenes* (1 ml), followed by a soft agar of growth medium for *C. difficile*, and on top of it with the soft GM17 containing 200 µl of overnight culture of *Streptococcus thermophilus* LMG P-28585, or MRS with 200 µl of overnight cultures of *Lactobacillus helveticus* LMG P-24226 or *Lactobacillus fermentum* LMG P-28583 or 200 µl of mixed probiotic culture. Production of gas by *Clostridium sporogenes* and *Clostridium difficile*, manifested in the form of cracking agar, represents the signal for the growth of *Clostridium sporogenes* and *Clostridium difficile* and absence of antimicrobial activity. The antimicrobial activity of probiotic strains on *Clostridium sporogenes* and *Clostridium difficile* is scored as the absence of gas production, i.e. agar remains intact.

*L. helveticus* LMG P-24226 exhibits antimicrobial activity at various strains of the genera *Lactococcus* sp. and *Lactobacillus* sp., *Bacillus subtilis* ATCC8, *Bacillus cereus* ATCC11778, *Bacillus mycoides*, as well as the pathogenic bacteria, *Staphylococcus aureus* ATCC25923, *Staphylococcus* sp. ATCC (clinical isolate) Collection Torlak, *Yersinia enterocolitica* O3 (clinical isolate) Collection Torlak, *Shigella sonnei* (Hamburg IO5) Collection Torlak, *Shigella flexneri* (London 9950) Collection Torlak, *Shigella dysenteriae* (serotype 5 and 7) Collection Torlak, *Streptococcus pneumoniae* ATCC276, *Streptococcus pneumoniae* ATCC496, *Pseudomonas* sp., *Escherichia coli* C600, *Escherichia coli* ATCC25922, *Clostridium sporogenes* Collection Torlak and *Clostridium difficile* (clinical isolate). The strains of *Streptococcus thermophilus* LMG P-28585 and *Lactobacillus fermentum* LMG P-28583 exhibited weak antimicrobial activity on *Clostridium difficile*, while probiotic combination shows a synergistic effect and exhibits the strongest antimicrobial effect on *Clostridium sporogenes* and *Clostridium difficile* (Figure 2).

**Antibiotic resistance**

Resistance to antibiotics is tested by methods prescribed by the European Food Safety Authority (EFSA, 2012). The results showed that strains LMG P-24226, LMG P-28583 and LMG P-28585 are sensitive to the recommended levels of antibiotics on the basis of the recommendations of EFSA (2012).

**Survival of probiotic strains in simulated conditions of the gastrointestinal tract (GIT)**

Survival of probiotics in the gastrointestinal tract is critical for their activity. Survival of probiotic strains in simulated GIT conditions is performed as previously described (Uroic et al., 2014).

Simulated GIT conditions included the growth of bacteria in artificial gastric juice (NaCl [7.31 g/l], KCL [0.52 g/l], NaHCO<sub>3</sub> [3.78 g/l], pH 2 using concentrated HCl, with addition of filtered sterilized pepsin [3 g/l]), and intestinal fluids A (simulation of the conditions in the duodenum: 0.6% (w/v) bile salts, pH 8.0 using 10 N NaOH), and B (simulation of the conditions in the ileum: 0.3% (w/v) bile salts and pancreatin [1 g/l]). Samples were taken during the period from t = 0 min - CFU/ml of the overnight culture; t = 90 min - CFU/ml of culture incubated 90 min in the gastric juice; t = 100 min - CFU/ml of culture incubated 10 min in intestinal juice A, t = 160 min and t = 220 - CFU/ml of culture incubated for 60 and 120 min in intestinal juice B.

Results showed that the strain LMG P-28585 successfully survived in simulated GIT conditions in a saline buffer, while strains LMG P-28583 and LMG P-24226 successfully survived simulated GIT conditions when administered in milk (Figure 3).

**Adhesion of the probiotic strains to Caco-2 epithelia intestinal cell lines and pathogen exclusion**

Adhesion to epithelial intestinal cell (EIC) lines is an essential prerequisite for the probiotic strain showing positive effects on the host's health. A higher percentage of adhesion enables better antagonistic activity against enteropathogens by competing for the same binding sites at EIC (Coconniere et al., 1993). On the other hand, the strain that shows a greater degree of adhesion to EIC retains longer in the gastrointestinal tract and makes better contact with the EIC stimulating the immune system of the host (Schiffrin et al., 1995).

Adhesion to Caco-2 EIC was performed as previously described (Nikolic et al., 2012). The results showed that the strain LMG P-24226 exhibited the highest level of adhesion, while

strains LMG P-28583 and LMG P-28585 exhibited a slightly lower level of adhesion to Caco-2 cells (Figure 4A).

Moreover, adhesion of *Escherichia coli* ATCC25922 (Figure 4B) and *Salmonella* 654/7E (veterinary isolate, the Collection of Scientific Institute of Veterinary Medicine, Novi Sad) (Figure 4C) to Caco-2 cells in the presence and absence of probiotic bacteria LMG P-24226, LMG P-28583 and LMG P-28585, as well as in the presence of probiotic fermented dairy product (mixed culture of probiotic strains). The results showed that the strain LMG P-24226 significantly reduced the number of attached *E. coli* ATCC25922 ( $p < 0.5$ ). The probiotic combination in milk exerts a synergistic effect and a significantly ( $p < 0.1$ ) reduced the number of attached *E. coli* ATCC25922. Also, strains LMG P-28585 ( $p < 0.1$ ) and LMG P-24226 ( $p < 0.5$ ) significantly reduce the number of attached *Salmonella* 654/7E.

#### **The proliferation of GALT (Gut Associated Lymphoid Tissue) cells of rats in the presence of probiotic strains LMG P-24226, LMG P-28583 and LMG P-28585 and mixed probiotic culture**

The effect of probiotic UV-inactivated strains LMG P-28585, LMG P-28583 and LMG P-24226, live strains of LMG P-28585, LMG P-28583 and LMG P-24226 grown in milk and mixed probiotic culture prepared in milk on proliferation of GALT primary culture isolated from rats was monitored. Isolation and proliferation of GALT cells from rats was performed as previously described (Hidalgo-Cantabrana et al., 2014).

The results showed that live LMG P-28585, LMG P-28583 and LMG P-24226 strains grown in milk and mixed probiotic culture prepared in milk exhibit stimulatory effect on the proliferation of lymphocytes, indicating that the probiotic strains and the combination (synergism) have potential immunomodulatory effect (Figure 5).

#### **Farm trial: *in vivo* testing of the probiotic combination**

The effect of the probiotic combination (including strains LMG P-28585, LMG P-28583 and LMG P-24226) on the health status of farm animals was monitored *in vivo* in farm trial. Out of 50 pregnant sows, 25 were treated with fermented dairy probiotic product prepared as given above for 7-15 days before farrowing (at a time when the natural immunity of pregnant sows usually declines). Pregnant sows have been received the probiotic every day during the first morning feeding. In parallel, 25 control sows, not treated with the probiotic, were followed. Before and

after the probiotic treatment, the general health of treated and control sows and the total number of bacteria (LAB and *Enterobacteriaceae*) in pigs' faeces was followed as described previously (Terzic-Vidojevic et al., 2007; Anonymous, 1996) (Figure 5a).

5 The results showed that after probiotic treatment the number of *Enterobacteriaceae* in faeces of piglets was significantly lower compared to the same number of bacteria in the faeces of sows before treatment, while the number of enteropathogenic bacteria in the control sample was higher in faeces after farrowing than in faeces of pregnant sows before farrowing (Figure 6a). Also, it was observed that piglets of sows treated with a probiotic combination did not have  
10 diarrhoea, while in the control group diarrhoea occurred sporadically in piglets.

In parallel, the effect of probiotic bacteria on the goats spontaneously infected with *Clostridium perfringens* was tested. Ten goats spontaneously infected with *Clostridium perfringens* were treated with mixed probiotic culture for 10 days before giving birth (at a time when the natural  
15 immunity of the pregnant goats declines).

The results showed that the probiotic culture successfully reduced the number of *Clostridium perfringens* below the detection level (Figure 6b). In addition, it was noticed that the number of other spore-forming bacteria decreased after treatment with probiotic culture, and general health  
20 of goats was improved.

### Toxicology analysis

For the safe use of the probiotic combination for humans and animals, acute and subacute toxicology analysis of individual probiotic strains and probiotic combination studies was conducted. Acute and subacute toxicology analysis was performed on 12 mice of NMRI HAN.  
25 The analysis was conducted in accordance with the Guidelines of Good Laboratory Practice (Official Gazette no. 28/2008) with respect to the general requirements for the test specified in OECD GUIDELINES FOR TESTING OF CHEMICALS NO 423.

The results showed that not a single strain, or a probiotic combination expressed toxic effects  
30 (see Table 2-6, Figure 7).

**Table 2**

Summary of clinical observation of animals (mice) treated with probiotic strain *Lactobacillus helveticus* LMG P-24226 at a dose of 0.5 ml/mouse for a 15-day period.

| Clinical observations |                                        | Ratings |   |   |   |
|-----------------------|----------------------------------------|---------|---|---|---|
|                       | parameter                              | 0       | 1 | 2 | 3 |
| appearance            | eyes, nostrils,<br>natural<br>openings | X       |   |   |   |
|                       | skin and fur<br>(coat)                 | X       |   |   |   |
|                       | body posture                           | X       |   |   |   |
| behavior              | natural forms<br>of behavior           | X       |   |   |   |
|                       | provoked<br>behavior                   | X       |   |   |   |

Legend: 0 - normal response; 1 - slight change; 2 - marked change; 3- a terminal change

**Table 3**

Summary of clinical observation of animals (mice) treated with probiotic strain *Streptococcus thermophilus* LMG P-28585 at a dose of 0.5 ml/mouse for a 15-day period.

| Clinical observations |                                        | Ratings |   |   |   |
|-----------------------|----------------------------------------|---------|---|---|---|
|                       | parameter                              | 0       | 1 | 2 | 3 |
| appearance            | eyes, nostrils,<br>natural<br>openings | X       |   |   |   |
|                       | skin and fur<br>(coat)                 | X       |   |   |   |
|                       | body posture                           | X       |   |   |   |
| behavior              | natural forms<br>of behavior           | X       |   |   |   |
|                       | provoked<br>behavior                   | X       |   |   |   |

5 Legend: 0 - normal response; 1 - slight change; 2 - marked change; 3- a terminal change

**Table 4**

Summary of clinical observation of animals (mice) treated with probiotic strain *Lactobacillus fermentum* LMG P-28583 at a dose of 0.5 ml/mouse for a 15-day period.

| Clinical observations |                                        | Ratings |   |   |   |
|-----------------------|----------------------------------------|---------|---|---|---|
|                       | parameter                              | 0       | 1 | 2 | 3 |
| appearance            | eyes, nostrils,<br>natural<br>openings | X       |   |   |   |
|                       | skin and fur<br>(coat)                 | X       |   |   |   |
|                       | body posture                           | X       |   |   |   |
| behavior              | natural forms<br>of behavior           | X       |   |   |   |
|                       | provoked<br>behavior                   | X       |   |   |   |

Legend: 0 - normal response; 1 - slight change; 2 - marked change; 3- a terminal change

**Table 5**

Summary of clinical observation of animals (mice) treated with probiotic combination at a dose of 1 ml/mouse for a 15-day period.

| Clinical observations |                                        | Ratings |   |   |   |
|-----------------------|----------------------------------------|---------|---|---|---|
|                       | parameter                              | 0       | 1 | 2 | 3 |
| appearance            | eyes, nostrils,<br>natural<br>openings | X       |   |   |   |
|                       | skin and fur<br>(coat)                 | X       |   |   |   |
|                       | body posture                           | X       |   |   |   |
| behavior              | natural forms<br>of behavior           | X       |   |   |   |
|                       | provoked<br>behavior                   | X       |   |   |   |

5

Legend: 0 - normal response; 1 - slight change; 2 - marked change; 3- a terminal change

**Table 6**

Summary of clinical observation of animals (mice) treated with a mixture of probiotic daily at a dose of 0.5 ml/mouse for a 15-day period.

5

| Clinical observations |                                        | Ratings |   |   |   |
|-----------------------|----------------------------------------|---------|---|---|---|
|                       | parameter                              | 0       | 1 | 2 | 3 |
| appearance            | eyes, nostrils,<br>natural<br>openings | X       |   |   |   |
|                       | skin and fur<br>(coat)                 | X       |   |   |   |
|                       | body posture                           | X       |   |   |   |
| behavior              | natural forms<br>of behavior           | X       |   |   |   |
|                       | provoked<br>behavior                   | X       |   |   |   |

Legend: 0 - normal response; 1 - slight change; 2 - marked change; 3- a terminal change

**Technological process for preparation of probiotic fermented dairy product**

The overnight culture of individually grown strains LMG P-28585, LMG P-28583 and LMG P-24226 in milk was added to pasteurized skim milk in a total amount of 3%, preferably in ratio of LMG P-28585: LMG P-28583 : LMG P-24226 = 1: 0.5: 1, and optimally in ratio 1: 1: 1.3. Inoculated milk was incubated under optimal conditions for bacterial growth while the total number of bacteria ( $10^8$ - $10^{10}$ CFU/ml) was not achieved, after which the resulting fermented product can be stored in a refrigerator at 4-6°C up to 28 days, without losing activity and the total number of live bacteria remains the same as immediately after preparation.

**Industrial applicability**

Probiotic starter culture of the subject invention will, according to ratios thereof of bacterial isolates in probiotic culture, exert its proteolytic activity, antimicrobial and/or immunomodulatory activity. Therefore, according to needs and intended purposes this probiotic starter culture find its human and animal use in prophylaxis and/or treatment of intestinal pathologies of various ethologies and as an immunomodulator.

This probiotic starter culture can be prepared as such and in forms suitable for target group of mammals, namely humans or animals. Special use of the probiotic starter culture is as food supplement for humans and animals.

For veterinary use, the probiotic starter culture can be prepared as standard pharmaceutical forms that find the veterinary use, but not limited to, fermented probiotic dairy product, lyophilisate, premix, granules, *spray-dry* (sprinkled over the food), powders that can be mixed with water or food, suspensions, tablets, capsules and other forms known to the person skilled in art. As the case may be, the probiotic starter culture can be used preferably as forage additive, and especially preferable as *spray dry*.

For use in humans, the probiotic starter culture/probiotic/composition of the subject invention comprising three thermophilic natural bacterial isolates, as previously described, can be prepared in the form of fermented probiotic product, lyophilisate, powder, oral suspension, pastilles and chewable tablets, tablets, capsules and other forms known to the person skilled in art.

## Literature

1. Terzic-Vidojevic, A., Vukasinovic, M., Veljovic, K., Ostojic, M., and Topisirovic, L. 2007. Characterization of microflora in homemade semi-hard white Zlata Cheese. *International Journal of Food Microbiology* 114, 36–42.
2. Harrigan, W.F., McCance, M.E. 1976. *Laboratory Methods in Food and Dairy Microbiology*. Academic Press, New York.
3. Kojic, M., Fira, D., Banina, A., Topisirovic, L., 1991. Characterization of the cell wall-bound proteinase of *Lactobacillus casei* HN14. *Applied and Environmental Microbiology* 57, 1753–1757.
4. Tagg, J.R., McGiven, A.R. 1971. Assay system for bacteriocins. *Applied Microbiology* 21, 943.
5. Jovicic, B., Begovic, J., Lozo, J., Topisirovic, L., Kojic, M., 2009. Dynamic of sodium dodecyl sulfate utilization and antibiotic susceptibility of strain *Pseudomonas* sp. ATCC19151. *Archives of Biological Sciences* 61, 159–165.
6. *Performance Standards for Antimicrobial Susceptibility Testing, Vol. 24*, Approved Standard M7-A6 of National Committee for Clinical Laboratory Standards (NCCLS), Vellore, Tamil Nadu, India (2004).
7. Uroic, K., Nikolic, M., Kos, B., Lebos Pavunc, A., Beganovic, J., Lukic, J., Jovicic, B., Filipic, B., Miljkovic, M., Golic, N., Topisirovic, L., Cadez, N., Raspor, P., Suskovic, J.: Functional probiotic properties of lactic acid bacteria isolated from Croatian fresh soft cheese and Serbian white pickled cheese. *Food Technol. Biotechnol.* 52(2)(2014): 232–241.
8. Hidalgo-Cantabrana, C., Nikolic, M., López, P., Suárez, A., Miljkovic, M., Kojic, M., Margolles, A., Golic, N. and Ruas-Madiedo, P., 2014. Response elicited by exopolysaccharide-1 producing *Bifidobacterium animalis* subsp. *lactis* strains and their polymers upon immune cells isolated from rat blood and gut associated lymphoid tissue. *Anaerobe* 26, 24–30.
9. Nikolic, M., López, P., Strahinic, I., Suárez, A., Kojic, M., Fernández-García, M., Topisirovic, L., Golic, N. and Ruas-Madiedo, P., 2012. Characterisation of the exopolysaccharide 1 (EPS)-producing *Lactobacillus paraplantarum* BGCG11 and its non-EPS producing derivative strains as potential probiotics. *International Journal of Food Microbiology*, 158, 155–162.
10. Anonymous, 1996. ISO 11290-1:1996. Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Listeria monocytogenes* – Part 1: Detection method.
11. European Food Safety Authority (EFSA), 2012. Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP). *EFSA Journal* 10(6):2740.

**Patent claims**

1. Probiotic starter culture comprising the following thermophilic natural isolates of lactic acid bacteria (LAB): *Streptococcus thermophilus* LMG P-28585,  
5 *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226.
2. Probiotic starter culture according to claim 1, comprising *Streptococcus thermophilus* LMG P-28585 in the amount of 10% to 40%, *Lactobacillus fermentum* LMG P-28583 in the amount of 10% to 50% and *Lactobacillus helveticus* LMG P-24226 in the amount of 40% to 80%.  
10
3. Probiotic starter culture according to claim 1, characterized in that, the ratio between *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and  
15 *Lactobacillus helveticus* LMG P-24226 is 30%:30%:40%.
4. Probiotic starter culture according to claim 1, characterized in that, the ratio between *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and  
20 *Lactobacillus helveticus* LMG P-24226 is 10%:10%:80%, preferably 10%:20%:70%, more preferably 30%:20%:50%, preferably 30%:40%:30% and more preferably 40%:20%:40%.
5. Probiotic starter culture according to claim 1, characterized in that, the number of live bacteria is: *Streptococcus thermophilus* LMG P-28585 from  $6 \times 10^7$  to  $1.25 \times 10^{11}$ ,  
25 *Lactobacillus fermentum* LMG P-28583 from  $1.1 \times 10^8$  to  $1.25 \times 10^{11}$  and *Lactobacillus helveticus* LMG P-24226 from  $4 \times 10^6$  to  $2.5 \times 10^9$ .
6. Probiotic starter culture according to any one of previous claims for use as replacement  
30 for antibiotics usage, for prophylaxis and/or treatment of bacterial infections of gastrointestinal tract, disorders associated with colonization of gastrointestinal tract, for alleviation and/or elimination of side effects caused by usage of antibiotics and for use as an immunomodulator, in humans and animals.

7. Probiotic starter culture according to claim 6, wherein the bacterial infections are caused by bacteria of genus *Clostridium perfringens*, *Clostridium sporogenes*, *Clostridium difficile*, bacteria of family *Enterobacteriaceae*, bacteria of species *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus*, *Staphylococcus* sp. Met<sup>R</sup>, *Yersinia enterocolitica*, *Shigella sonnei*, *Shigella flexneri*, *Shigella dysenteriae*, *Streptococcus pneumoniae* and *Pseudomonas* sp., in humans and animals.
8. Probiotic starter culture according to any one of previous claims, which is in the spray-dry form (sprinkled over the food), lyophilisate, suspension and fermented probiotic dairy products.
9. Probiotic starter culture according to claims 1-8 for human and animal use as a probiotic and as food and feed additive for humans and animals.
10. Composition comprising the probiotic starter culture according to one of previous claims and a pharmaceutically acceptable vehicle which is in form of capsules, tablets, lyophilizate, *spray-dry*, suspensions, fermented probiotic dairy products.
11. Composition according to claim 10 for human and animal use.

## AMENDED CLAIMS

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1. Probiotic starter culture comprising the following thermophilic natural isolates of lactic acid bacteria (LAB): *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226, wherein the probiotic starter culture comprises *Streptococcus thermophilus* LMG P-28585 in the amount of 10% to 40%, *Lactobacillus fermentum* LMG P-28583 in the amount of 10% to 50% and *Lactobacillus helveticus* LMG P-24226 in the amount of 40% to 80%.
2. Probiotic starter culture according to claim 1, characterized in that, the ratio between *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226 is selected from:
  - a) 10%:10%:80%;
  - b) 10%:20%:70%;
  - c) 30%:20%:50%;
  - d) 30%:40%:30%;
  - e) 40%:20%:40%; and
  - f) 30%:30%:40%.
3. Probiotic starter culture according to claim 1 or claim 2, characterized in that, the ratio between *Streptococcus thermophilus* LMG P-28585, *Lactobacillus fermentum* LMG P-28583 and *Lactobacillus helveticus* LMG P-24226 is 30%:30%:40%.
4. Probiotic starter culture according to claim 1, characterized in that, the number of live bacteria is: *Streptococcus thermophilus* LMG P-28585 from  $6 \times 10^7$  to  $1.25 \times 10^{11}$ , *Lactobacillus fermentum* LMG P-28583 from  $1.1 \times 10^8$  to  $1.25 \times 10^{11}$  and *Lactobacillus helveticus* LMG P-24226 from  $4 \times 10^6$  to  $2.5 \times 10^9$ .

5. Probiotic starter culture according to any one of the previous claims for use as replacement for antibiotics usage, for prophylaxis and/or treatment of bacterial infections of gastrointestinal tract, disorders associated with colonization of gastrointestinal tract, for alleviation and/or elimination of side effects caused by usage of antibiotics and for use as an immunomodulator, in humans and animals.
6. Probiotic starter culture according to claim 5, wherein the bacterial infections are caused by bacteria of genus *Clostridium perfringens*, *Clostridium sporogenes*, *Clostridium difficile*, bacteria of family *Enterobacteriaceae*, bacteria of species *Escherichia coli*, *Salmonella* sp., *Staphylococcus aureus*, *Staphylococcus* sp. Met<sup>R</sup>, *Yersinia enterocolitica*, *Shigella sonnei*, *Shigella flexneri*, *Shigella dysenteriae*, *Streptococcus pneumoniae* and *Pseudomonas* sp., in humans and animals.
7. Probiotic starter culture according to any one of previous claims, which is in the spray-dry form (sprinkled over the food), lyophilisate, suspension and fermented probiotic dairy products.
8. Probiotic starter culture according to claims 1-7 for human and animal use as a probiotic and as food and feed additive for humans and animals.
9. Composition comprising the probiotic starter culture according to one of previous claims and a pharmaceutically acceptable vehicle which is in form of capsules, tablets, lyophilizate, spray-dry, suspensions, fermented probiotic dairy products.
10. Composition according to claim 9 for human and animal use.

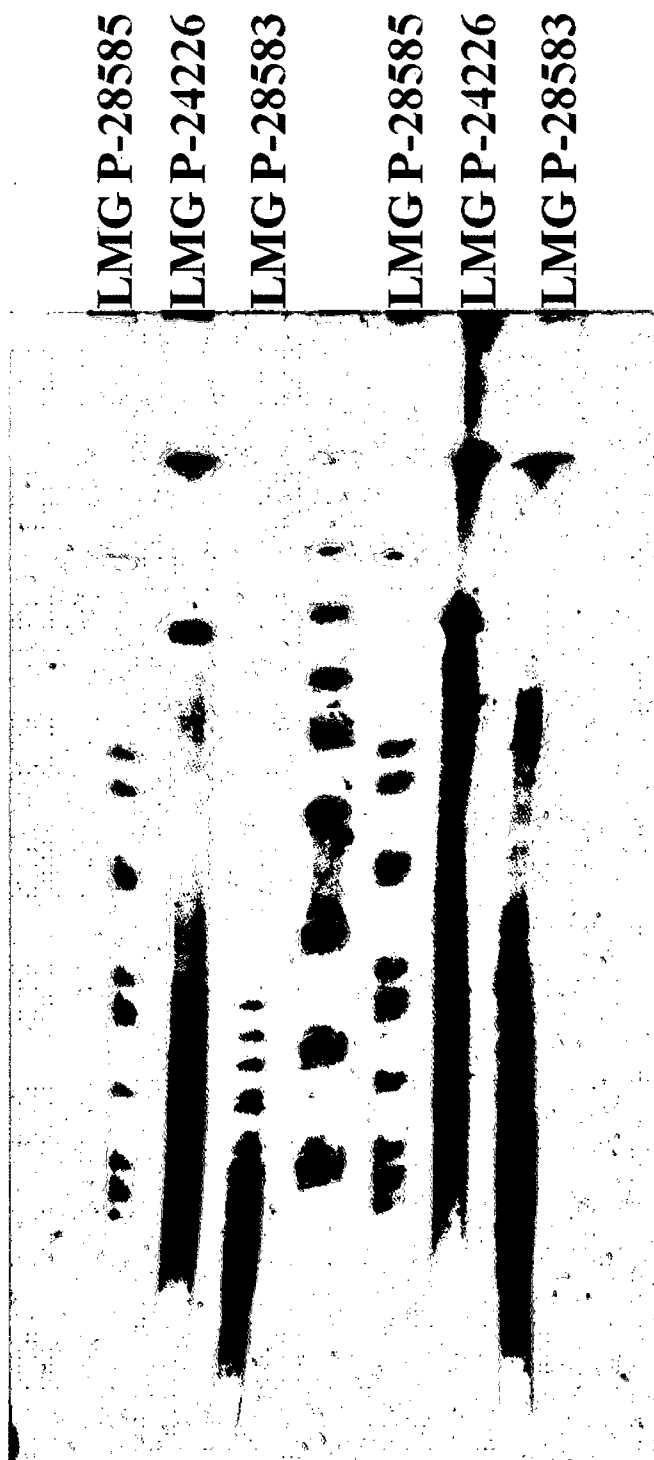
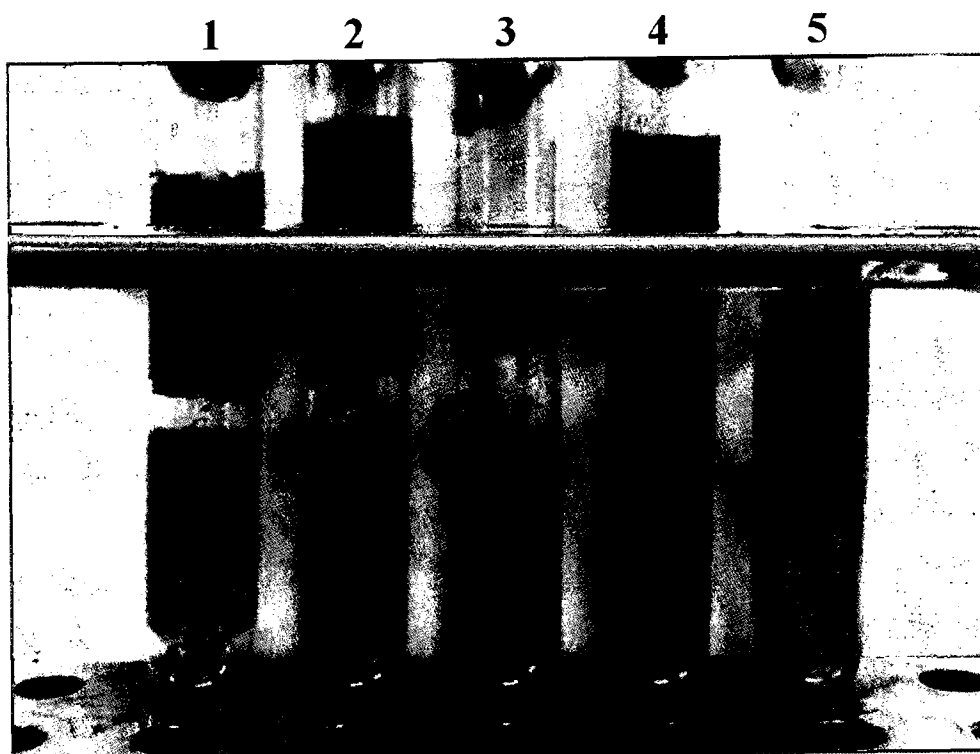


Fig.1

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**Fig.2**

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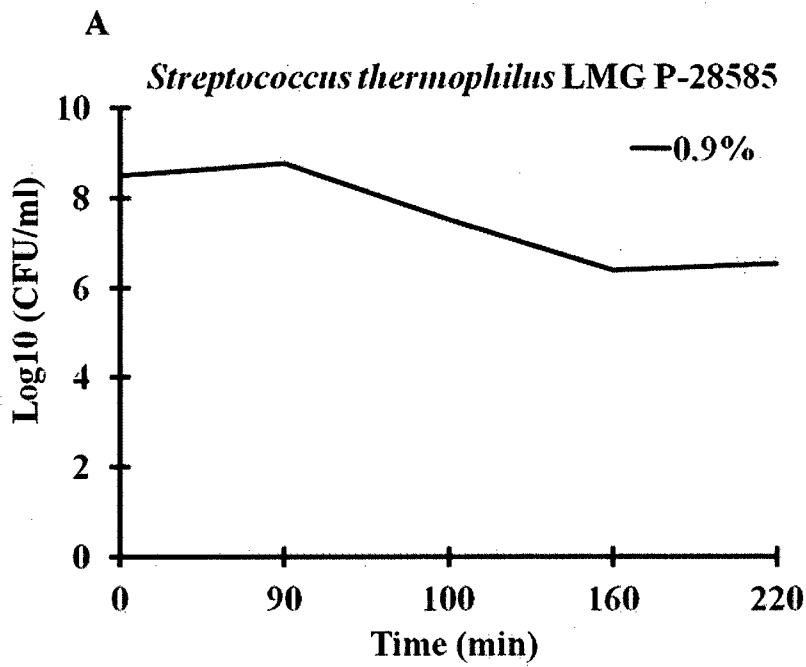


Fig.3

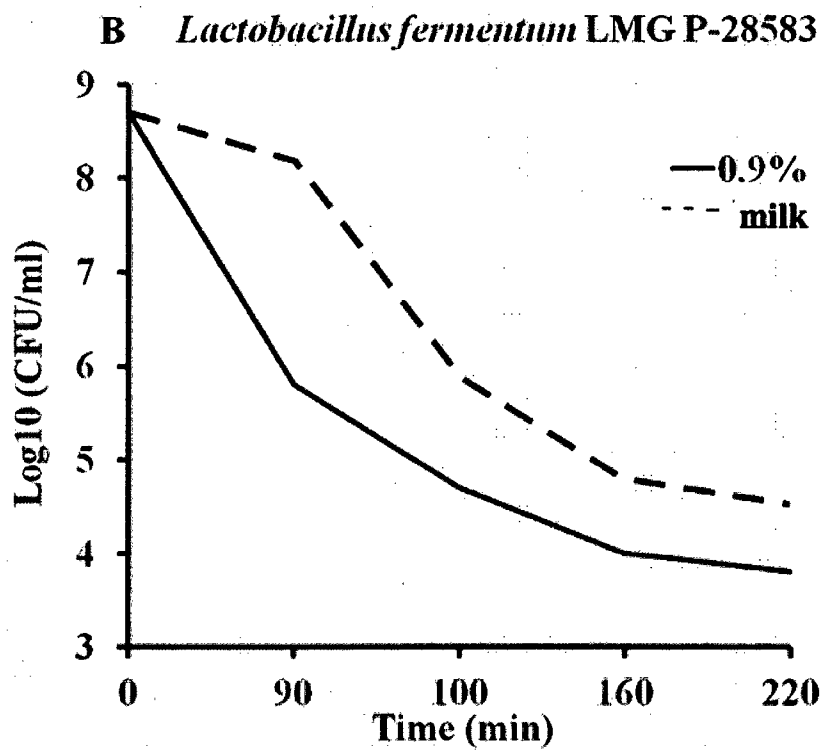


Fig.4

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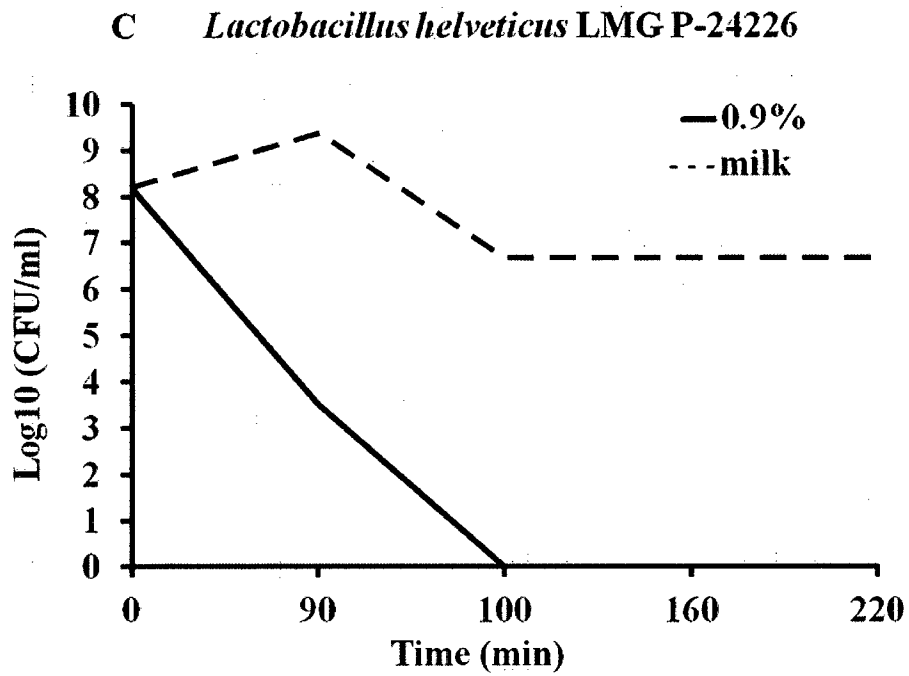


Fig.5

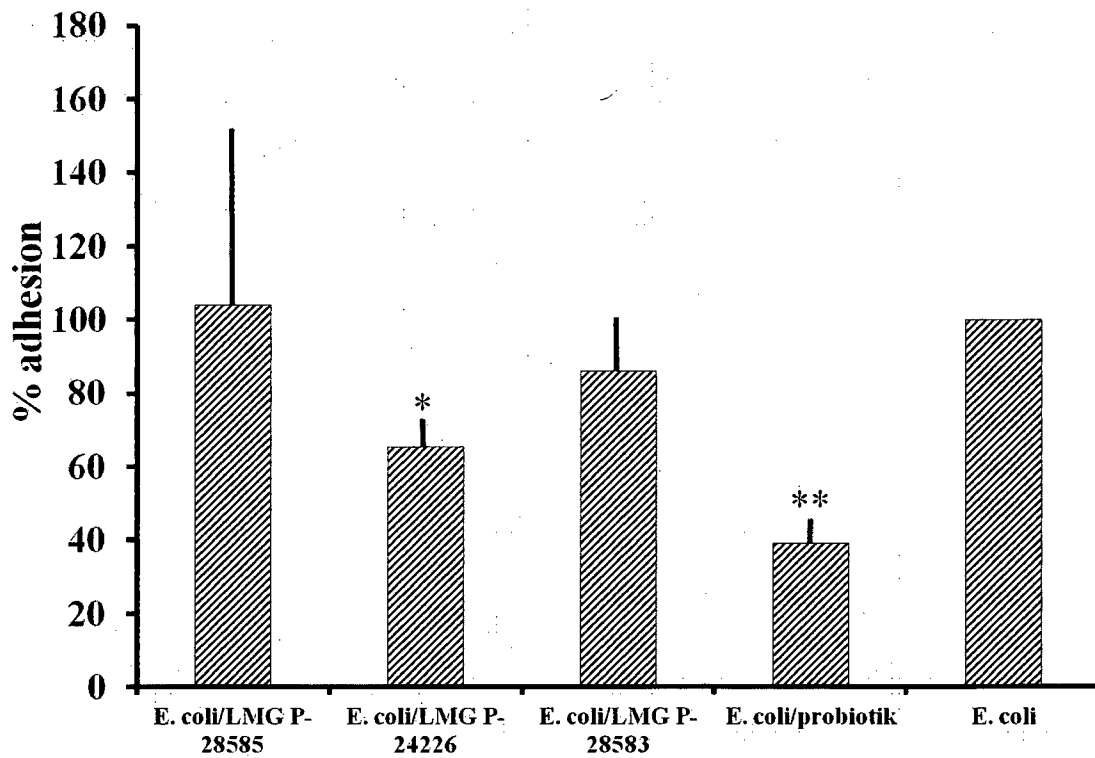


Fig.6

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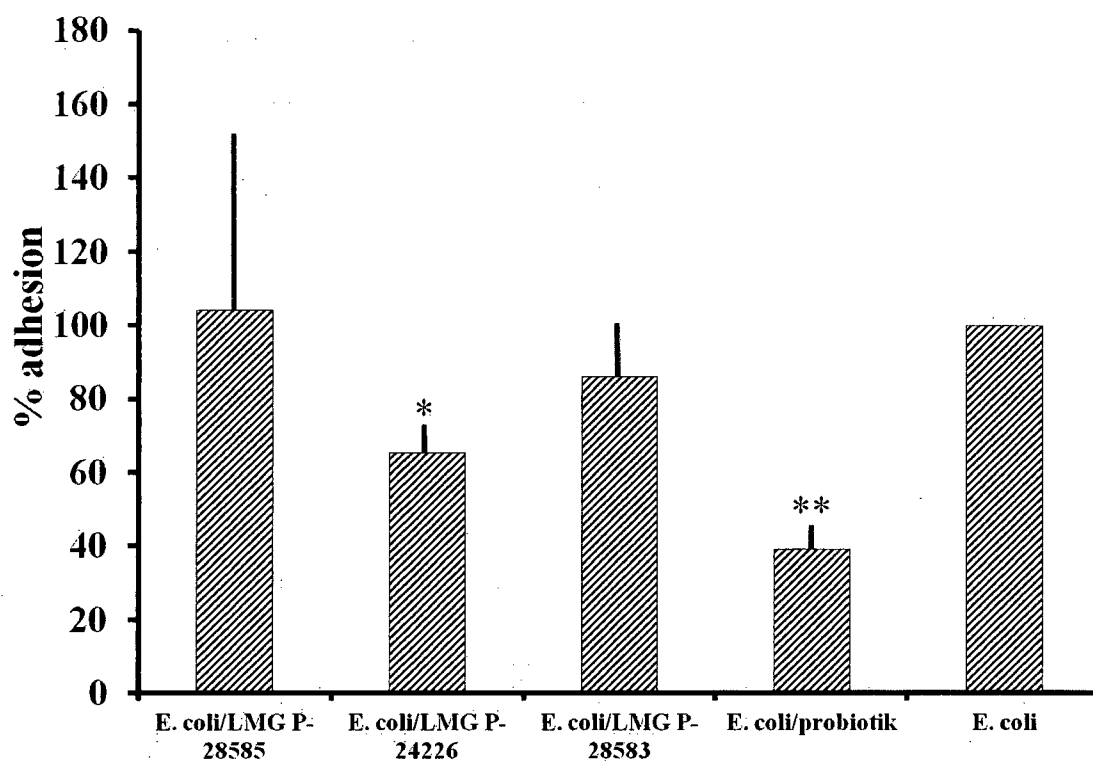


Fig.7

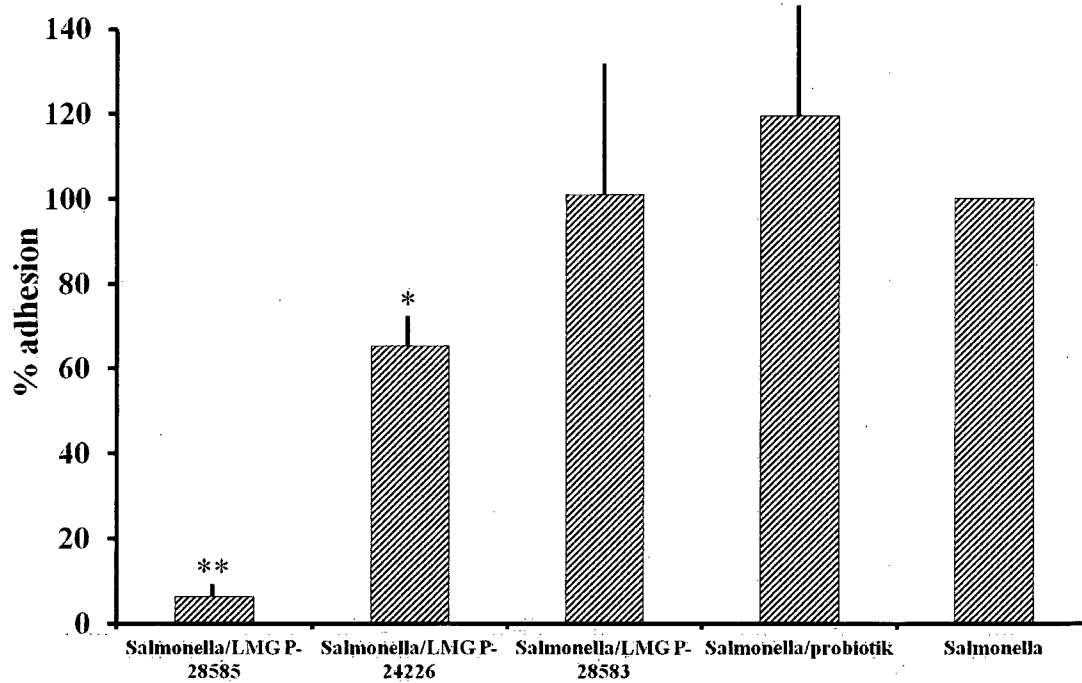


Fig.8

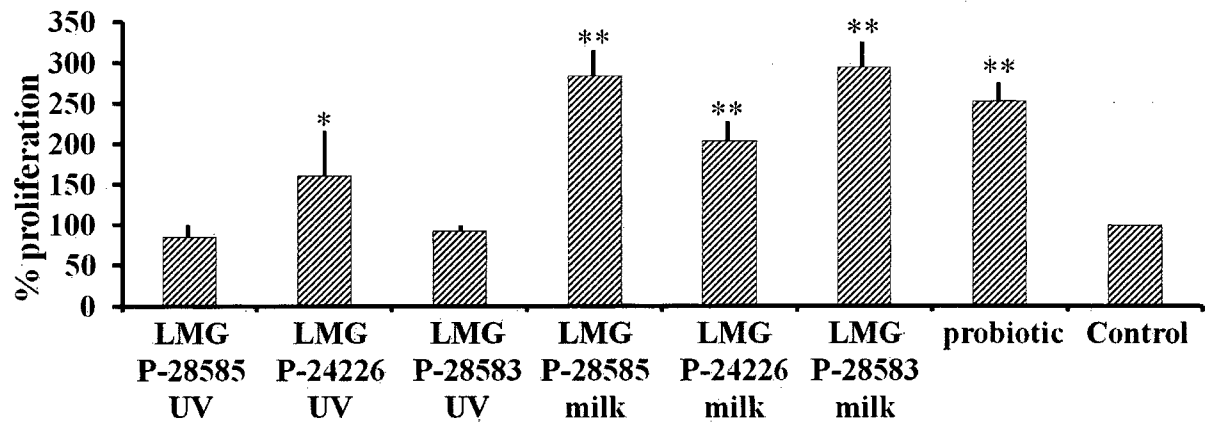


Fig.9

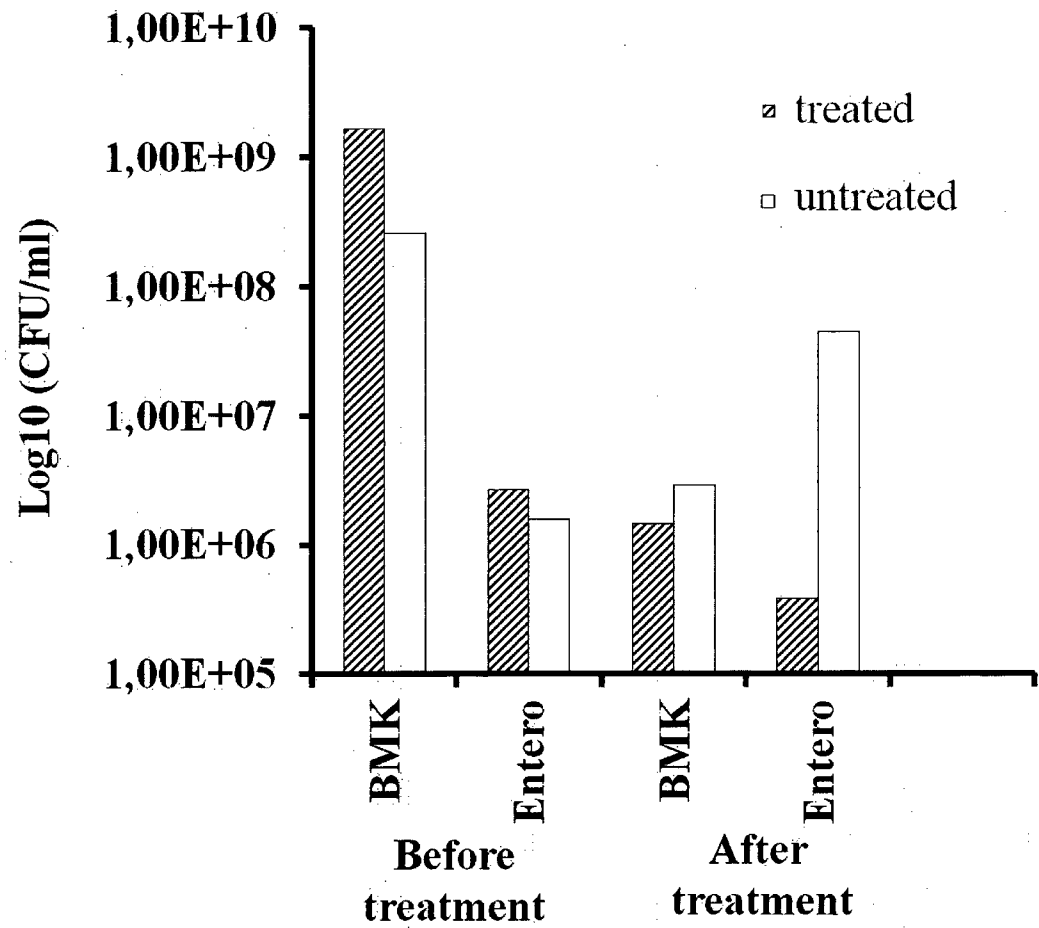


Fig.10

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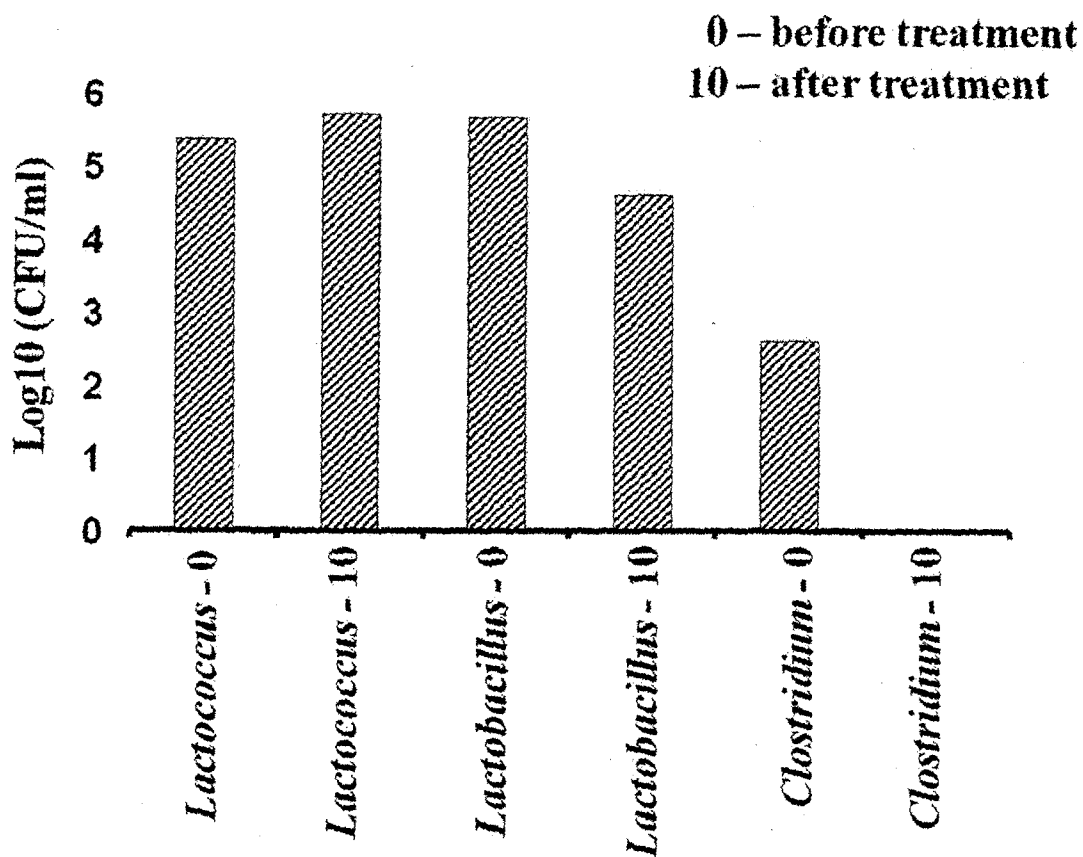


Fig.11

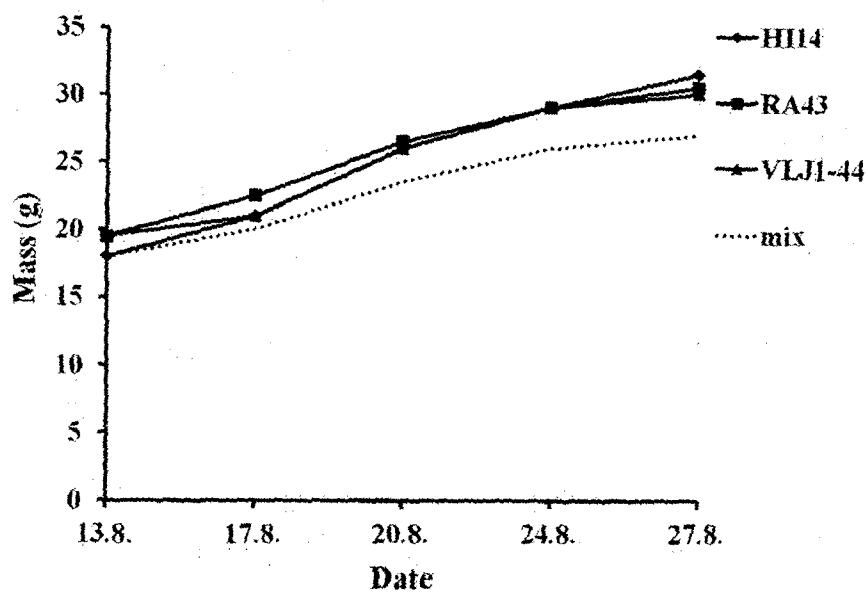


Fig.12

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/RS2015/000027

## A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K35/747 A61K35/744 A61P1/00 A23L33/135  
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                             | Relevant to claim No. |
|-----------|--------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | US 5 656 268 A (SORODSKY MICHAEL [US])<br>12 August 1997 (1997-08-12)<br>cited in the application<br>the whole document        | 1-11                  |
| A         | -----<br>EP 0 856 259 A1 (SITIA YOMO SPA [IT])<br>5 August 1998 (1998-08-05)<br>cited in the application<br>the whole document | 1-11                  |
| A         | -----<br>EP 1 359 816 A1 (VALIO LTD [FI])<br>12 November 2003 (2003-11-12)<br>cited in the application<br>the whole document   | 1-11                  |
|           | -----<br>-/-                                                                                                                   |                       |



Further documents are listed in the continuation of Box C.



See patent family annex.

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Date of the actual completion of the international search

9 September 2016

Date of mailing of the international search report

27/09/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040,  
Fax: (+31-70) 340-3016

Authorized officer

Greif, Gabriela

## INTERNATIONAL SEARCH REPORT

International application No  
PCT/RS2015/000027

| C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                   |                       |
|------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| Category*                                            | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                | Relevant to claim No. |
| A                                                    | WO 03/002131 A1 (UNIV TARTU [EE]; MIKELSAAR MARIKA [EE]; ZILMER MIHKEL [EE]; KULLISAAR) 9 January 2003 (2003-01-09)<br>the whole document                                                                                                                                                                                                         | 1-11                  |
| A                                                    | -----<br>TEMMERMAN R ET AL: "Identification and antibiotic susceptibility of bacterial isolates from probiotic products", INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY, ELSEVIER BV, NL, vol. 81, no. 1, 25 February 2003 (2003-02-25), pages 1-10, XP002346520, ISSN: 0168-1605, DOI: 10.1016/S0168-1605(02)00162-9<br>the whole document<br>----- | 1-11                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/RS2015/000027

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|-------------------------------------------|---------------------|----------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| US 5656268                                | A                   | 12-08-1997                 | NONE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |
| EP 0856259                                | A1                  | 05-08-1998                 | AR 010802 A1 12-07-2000<br>AT 169456 T 15-08-1998<br>BR 9706278 A 01-06-1999<br>CA 2213113 A1 23-06-1998<br>CN 1185904 A 01-07-1998<br>DE 69600525 D1 15-10-1998<br>DE 69600525 T2 10-12-1998<br>EP 0856259 A1 05-08-1998<br>JP H10286078 A 27-10-1998<br>US 5895648 A 20-04-1999                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
| EP 1359816                                | A1                  | 12-11-2003                 | AT 297664 T 15-07-2005<br>BR 0206689 A 03-02-2004<br>CA 2434337 A1 08-08-2002<br>CN 1487798 A 07-04-2004<br>CZ 20031915 A3 14-01-2004<br>DE 60204662 D1 21-07-2005<br>DE 60204662 T2 18-05-2006<br>DK 1359816 T3 15-08-2005<br>EE 200300351 A 15-12-2003<br>EP 1359816 A1 12-11-2003<br>ES 2243697 T3 01-12-2005<br>FI 20010157 A 26-07-2002<br>HK 1061501 A1 24-12-2008<br>HR P20030586 A2 30-06-2005<br>HU 0401017 A2 30-08-2004<br>JP 3984546 B2 03-10-2007<br>JP 2004524836 A 19-08-2004<br>KR 20040012717 A 11-02-2004<br>NO 20033283 A 24-09-2003<br>NZ 527172 A 26-11-2004<br>PL 363366 A1 15-11-2004<br>PT 1359816 E 31-10-2005<br>RU 2291899 C2 20-01-2007<br>US 2004062758 A1 01-04-2004<br>WO 02060276 A1 08-08-2002<br>ZA 200305458 B 15-10-2004 |
| WO 03002131                               | A1                  | 09-01-2003                 | AT 489959 T 15-12-2010<br>DK 1401457 T3 17-01-2011<br>EE 200100356 A 17-02-2003<br>EP 1401457 A1 31-03-2004<br>ES 2356119 T3 05-04-2011<br>RU 2284354 C2 27-09-2006<br>US 2004151708 A1 05-08-2004<br>WO 03002131 A1 09-01-2003                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |