

Strain of exopolysaccharide-secreting *Lactobacillus plantarum* and application thereof

Field of invention

The present invention relates to the field of microbial technology. More specifically, the invention relates to a strain of exopolysaccharide-secreting *Lactobacillus plantarum* and application in foods thereof.

Prior arts

Lactic acid bacteria (LAB) is a generic term which refers to a kind of bacteria that can use fermentable sugar to produce a large amount of lactic acid. It has been found that this kind of bacteria include at least 23 genera in taxonomy in nature at present. In the fields of food and medicine, the widely applied *Lactic acid bacteria* mainly include *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Lactococcus*, *Pediococcus* and *Leuconostoc*, etc. *Lactic acid bacteria* are the main source of probiotics. Many *Lactic acid bacteria*, which are inherent in human gut, have already been proved to have important physiological activities, such as improving the human intestinal flora, regulating the body's immunity, suppressing tumors, reducing serum cholesterol and regulating blood pressure, etc..

Significant progress has been made on the application of *Lactic acid bacteria* in fermented dairy products and microecologies. Current research is focused on how to develop new microbial starter cultures which can endow specific functional properties to fermented milk. The mechanism that the known *Lactic acid bacteria* display their functions include colonization, improving the intestinal environment by main metabolites such as lactic acid, besides these, some secondary metabolites such as bacteriocin and exopolysaccharides etc., also plays a very important role. Among these metabolites, exopolysaccharides of *Lactic acid bacteria* (LAB EPS) which have theoretical and practical application value have aroused the interest of many scholars at home and abroad.

Polysaccharide refers to carbohydrates consisting of more than 20 monosaccharides and can be divided into plant polysaccharides, animal polysaccharide and microbial polysaccharide according to different sources. LAB EPS is a kind of polysaccharide produced and secreted into the extracellular space by *Lactic acid bacteria*. LAB EPS has important functions in technology as it has significant influence on the rheological properties, texture, taste and flavor of yogurt, cheese, and the vast majority of the of fermented dairy products. LAB EPS can improve the rheological properties and textural properties of the dairy products. Compared with yogurt formed by strains with stickiness produced, the yogurt formed by both strains with stickiness produced and strains with no stickiness produced has

smoother taste and increased viscosity because of the natural thickening effect. As exopolysaccharide-secreting *Lactic acid bacteria* can enhance the water-retaining capacity of yogurt, it can avoid whey separation. Moreover, the enhancement of water-retaining capacity of yogurt caused by exopolysaccharide can help improve the yield of products like cheese, etc. EPS also has good physiological functions, such as enhancing mucosal absorption, anti-tumor, anti-ulcer, immune regulation, lowering cholesterol and lowering blood pressure, etc. Therefore, the study on LAB which could produce extracellular polysaccharide, is of great significance and economic value to improve dairy production and develop fermented dairy products that bear specific functional properties. To exploit LAB EPS bearing probiotic functions is becoming a hot spot of current researches.

Since the 1960s, the polysaccharide has been considered as a non-specific promoter with broad spectrum. In human immunity, the polysaccharide can enhance the cellular immunity and humoral immunity of the host cells, such as activating macrophages, T cells, B cells, and NK cells, activating complement and inducing to produce interferon. The polysaccharide can activate the body's non-specific defense mechanisms and has good effect on anti-virus, anti-tumor, anti-radiation and so on (Zhou Shiwen, Xu Chuanfu, "Immunopharmacological Action of Polysaccharides", Chinese Journal of Biochemical Pharmaceutics, 1994, 15 (2): 143-147).

Content of the present invention

The technical problem to be solved in the present invention is for overcoming the shortcomings that there is no *Lactic acid bacteria* produces high content of exopolysaccharides in prior art to provide a high-productive strain of *Lactobacillus plantarum*.

The technical solutions of the present invention are as follows.

The first technical solution of the present invention is a strain of exopolysaccharide-secreting *Lactobacillus plantarum*, and it is deposited in China General Microbiological Culture Collection Center (CGMCC) with an accession number of CGMCC No.5222.

The second technical solution of the present invention is a bulk starter culture of *Lactobacillus plantarum* CGMCC No.5222 which is prepared according to a method comprising the following step (a) or (b):

(a) inoculating the strain of *Lactobacillus plantarum* CGMCC No.5222 into sterilized milk added with whey protein and culturing till the milk begin to curd, continuing the activated culture for two generations to obtain the mother starter culture; inoculating said mother culture with a ratio of 3-5% (v/v) into new sterilized milk added with whey protein and culturing till the new milk begins to curd, and then

obtaining said bulk starter culture;

(b) inoculating the strain of *Lactobacillus plantarum* CGMCC No.5222 into liquid medium to culture for activation, continuing the activated culture for two generations, inoculating the activated culture obtained with a ratio of 2-4% (v/v) into new liquid medium and culturing for 16-18h, obtaining the cell sediment through solid-liquid separation, and suspending the cell sediment in sterilized milk, and then obtaining said bulk starter culture.

In procedure (a), preferably, the content of whey protein which is added into the sterilized milk is 0.5-5% (wt), and more preferably, the content of whey protein which is added into the sterilized milk is 1% (wt). The method of culturing till the milk begin to curd is a conventional method in the art, and preferably refers to culture at the temperature of 37°C for 14-16h.

In procedure (b), the method of culturing for activation in liquid medium is a conventional method to activate *Lactobacillus plantarum* in the art, and preferably refers to culture at the temperature of 37°C for 12-16h. Said liquid medium is conventional liquid medium for culturing *Lactobacillus plantarum* in the art, and preferably refers to MRS liquid medium. The method of solid-liquid separation is a conventional solid-liquid separation method to separate thalli of fermentation liquor in the art including centrifugation and filtration, etc.; preferably the method of solid-liquid separation in the present invention refers to centrifugation, and more preferably refers to centrifugation at the temperature of 4°C for 15min with the speed of 4000r/min.

Preferably, the viable count of bacteria in said bulk starter culture of *Lactobacillus plantarum* CGMCC No.5222 in the present invention is no less 10^9 cfu/mL.

The third technical solution of the present invention is use of *Lactobacillus plantarum* CGMCC No.5222 in fermented foods.

Therein, said fermented foods refer to conventional fermented foods, preferably *Lactic acid bacteria*-containing milk beverage or fermented milk.

Preferably, said *Lactic acid bacteria*-containing milk beverage is prepared according to a method comprising the following steps: cooling raw milk after sterilization, mixing with said bulk starter culture of *Lactobacillus plantarum* CGMCC No.5222 uniformly to make the concentration of *Lactobacillus plantarum* CGMCC No.5222 up to 10^6 cfu/mL or more, and then obtaining said *Lactic acid bacteria*-containing beverage.

Therein, the method of said sterilization refers to conventional methods for raw

milk sterilization, preferably refers to sterilization with UHT such as at the temperature of 140°C for 2s. Said bulk starter culture of *Lactobacillus plantarum* CGMCC No.5222 is added after cooling the sterilized milk, therein, the cooling temperature is conventional, preferably is 40°C.

Preferably, said fermented milk is prepared according to a method comprising the following steps: cooling raw milk after sterilization,, inoculating a ratio of 3-5% (v/v) of bulk starter culture of *Lactobacillus plantarum* CGMCC No.5222 as well as a ratio of 3-5% (v/v) of mutualistic commercial starter cultures into the sterilized milk, mixing uniformly and fermenting till the titration acidity reaches to 0.6-0.7 calculated by lactic acid, and then obtaining the fermented milk containing said *Lactobacillus plantarum*.

Therein, the method of sterilization refers to conventional methods for raw milk sterilization, preferably refers to sterilization with UHT such as high at the temperature of 140°C for 2s, and more preferably refers to sterilization at the temperature of 95°C for 20min. Said bulk starter culture of *Lactobacillus plantarum* is added after cooling the sterilized milk, therein, the cooling temperature is conventional, preferably 37°C. The temperature for fermentation is conventional, preferably 37°C. The mutualistic commercial starter cultures refer to conventional commercial starter cultures, such as *Lactobacillus Bulgarica*.

The forth technical solution of the present invention is the exopolysaccharides extracted from strain of *Lactobacillus plantarum* CGMCC No.5222.

Therein, the method of extraction refers to conventional method for extracting microbial exopolysaccharides, preferably refers to a method comprising the following steps: centrifuging the fermentation liquor of *Lactobacillus plantarum* CGMCC No.5222 after boiling in order to remove thalli and condensed protein, precipitating protein from the supernatant via trichloroacetic acid method to remove the protein, obtaining a precipitate by adding alcohol, dissolving the precipitate in water and then dialyzing with water.

Therein, the fermentation liquor of *Lactobacillus plantarum* CGMCC No.5222 refers to conventional fermentation liquor of *Lactobacillus plantarum* CGMCC No.5222, preferably refers to the fermentation liquor that is obtained by the fermentation comprising the steps of inoculating 5%(v/v) *Lactobacillus plantarum* CGMCC No.5222 into the 12% (w/w) skimmed milk containing 1% (w/v) glucose *plantarum* and ferment; preferably refers to the fermentation liquor that obtained by the fermentation at 30°C for 30h.

Therein, the preferred condition of centrifugation is 10000 g at 4°C for 20min. Trichloroacetic acid method is a conventional method of removing protein. Preferably, trichloroacetic acid is added to a final concentration of 4% (w/v) and stand overnight,

then centrifugate with the speed of 10000g at 4°C for 20min to remove the precipitated proteins. The method of precipitating with alcohol is also a conventional method, preferably with ethanol, i.e., add ethanol to a final concentration of 75% (v/v) and stand at 4°C for 24h, then centrifuge with the speed of 10000g at 4°C for 20min to obtain the precipitate.

The fifth technical solution of the present invention is use of the exopolysaccharides extracted from strain of *Lactobacillus plantarum* CGMCC No.5222 for use in immune-enhancing medicament, healthcare product or food product.

Unless otherwise indicated, all materials or reagents used in the present invention are commercially available.

Compared to the prior art, the advantageous effects of the present invention are as follows: The present invention provides a strain of *Lactobacillus plantarum* CGMCC No.5222, which, proved by tests, is a newly isolated and identified strain of *Lactobacillus plantarum* that produces exopolysaccharides and the amount of exopolysaccharides is different from other strains of *Lactobacillus plantarum*. The exopolysaccharide secreted is capable of eliciting B lymphocyte proliferation to enhance immunity, and has broad application prospects in medicaments, healthcare products and food products for immunity enhancement.

Deposit Information of the Biological Material Sample

The strain of *Lactobacillus plantarum* BDLP0001 provided by the present invention is deposited in China General Microbiological Culture Collection Center (CGMCC) which is located in No.1 Yard, West Beichen Road, Chaoyang District, Beijing with the postal code 100101 on September 6th, 2011. The accession number of the strain is CGMCC No.5222.

Brief description of the drawings

Combined with the description of figures, the feature and the advantageous effects of the present invention are illustrated as follows.

Fig.1 is the colony morphology of *Lactobacillus plantarum* CGMCC No.5222.

Fig.2 is the cellular morphology of *Lactobacillus plantarum* CGMCC No.5222 (×1000).

Fig.3 is the growth curves of *Lactobacillus plantarum* CGMCC No.5222.

Fig.4 is the optimum growth temperature of *Lactobacillus plantarum* CGMCC No.5222.

Fig.5 is the optimum pH of *Lactobacillus plantarum* CGMCC No.5222.

Detailed description of the preferred embodiment

The present invention collected samples from habitats of *Lactic acid bacteria* and screened wild strains of *Lactic acid bacteria* which produced exopolysaccharides, and by *in vitro* T/B lymphocyte proliferation test, the immunological activity of the exopolysaccharides is preliminary identified.

The present invention isolates a *Lactic acid bacteria* strain BDLP0001 from naturally fermented pickles. By use of microbial characteristics like morphological characteristics, culture characteristics, physiological and biochemical traits and genetic characteristics 16s rDNA, the *Lactic acid bacteria* strain BDLP0001 is identified as *Lactobacillus plantarum*, which is deposited in China General Microbiological Culture Collection Center (CGMCC) with accession number of CGMCC No.5223 on September 6th, 2011.

The morphological characteristics of said *Lactobacillus plantarum* CGMCC No.5222 in the present invention are described as below:

Colony characteristics: the strain grows well after streaked on MRS agar plate and incubated anaerobically at 37°C for 48h, and the colony morphology is shown in Figure 1. The colonies are rounded with neat edge, slightly raised, milky white and mixed up with little yellowish color, non-transparent, with humid and smooth surface, and could be present the stringy state if be picked.

Strain characteristics: the bacterium is rod-shaped (see Figure 2), and the strain mainly exist in form of chain with varying lengths and sometimes also in form of dispersed single cells. Being gram positive, the bacteria do not generate spores with general size of $0.6\mu\text{m}\times 1.5\mu\text{m}$.

The culture characteristics of said *Lactobacillus plantarum* CGMCC No.5222 in the present invention are described as below:

The minimum growth temperature of *Lactobacillus plantarum* BDLP0001 is 15°C while the maximum growth temperature is 40°C, and the optimum growth temperature is between 30°C and 40°C. The highest initial pH for growth is 9.0, the lowest initial pH for growth is 4.0, and the optimum initial pH for growth is 5.0. Said *Lactobacillus plantarum* BDLP0001 has a relatively short lag phase and grows into the logarithmic phase at the time of 2h and the stationary phase at the time of 10h. The strain of *Lactobacillus plantarum* BDLP0001 has a good bile salt tolerance and could grow well with the bile salt concentration ranging from 0.1% to 0.4%; and also the strain BDLB0001 grows well when the concentration of NaCl is not more than 7% and the strain could tolerate 8% NaCl.

Said *Lactobacillus plantarum* BDLP0001 in the present invention is derived from traditional fermented food and belongs to the Generally Recognized As Safe (GRAS) strains, so it can be used in *Lactic acid bacteria*-containing foods.

Therefore, the present invention also relates to an application of said *Lactobacillus plantarum* BDLP0001 in fermented foods. Said fermented foods refer to *Lactic acid bacteria*-containing beverage or fermented milk.

The present invention also provides bulk starter culture of said *Lactobacillus plantarum* BDLP0001.

Preferably, the bulk starter culture in the present invention is prepared according to the following preparation method: The strain of *Lactobacillus plantarum* CGMCC No.5222 is inoculated into sterilized 12% (w/v) skimmed milk which is sterilized at the temperature of 115°C for 15min added with 1% (wt) whey protein to culture at 37°C for 14-16h till the milk begin to curd, and the activated culture is continued for two generations to obtain the mother starter culture; then said mother starter culture is inoculated into new sterilized 12% (w/v) skimmed milk which is sterilized at the temperature of 95°C for 15min added with 1% (wt) whey protein with a ratio of 3-5% (v/v) to culture till the new milk begin to curd, then said bulk starter culture is obtained with a viable bacteria count of about 10^9 cfu/mL in the curdled milk. Alternatively, the strain of *Lactobacillus plantarum* CGMCC No.5222 is inoculated into MRS liquid medium to culture for activation at the temperature of 37°C for 14-16h, and the activated culture is continued for two generations, then the activated culture obtained is inoculated into new MRS liquid medium with a ratio of 2-4% (v/v) to culture for 16-18h, and then the cell sediment is obtained via centrifugation at the temperature of 4°C for 15min with the speed of 4000r/min, and the cell sediment is suspended in a certain amount of sterilized skimmed milk, then said bulk starter culture is obtained.

Preferably, said *Lactic acid bacteria*-containing milk beverage in the present invention is prepared by the following procedure: Raw milk is cooled to 40°C after pasteurization at the temperature of 95°C for 20min or high temperature sterilization at the temperature of 140°C for 2s and said bulk starter culture of *Lactobacillus plantarum* BDLP0001 is added to make the concentration of living bacteria up to more than 10^6 cfu/mL, then the product is kept in cold storage at 4°C, i.e., said *Lactic acid bacteria*-containing milk beverage is obtained.

Preferably, said fermented milk in the present invention is prepared by the following procedure: Raw milk is sterilized via pasteurization at the temperature of 95°C for 20min or high temperature sterilization at the temperature of 140°C for 2s and cooled to 37°C, then a ratio of 3-5% (v/v) of the bulk starter culture of *Lactobacillus plantarum* BDLP0001 is added and then a ratio of 3-5% (v/v) of

mutualistic commercial starter cultures which are used for preparing fermented milk is added into the sterilized milk, and after being mixed uniformly, the inoculated milk is fermented till the titration acidity reaches to 0.6-0.7 calculated by lactic acid, then cool the product to 37°C and kept it in cold storage, i.e., the fermented milk containing *Lactobacillus plantarum* BDLP0001 is obtained.

The following embodiments further illustrate the invention, but the present invention is not limited thereto. Unless otherwise indicated detailedly, the experimental methods of the present invention are usually in accordance with conventional conditions, or in accordance with the conditions recommended by the manufacturer. Said "room temperature" in the embodiments refers to the temperature of the operating room used for experiments, generally is 25°C.

Embodiment 1: Collection and isolation of the strain of *Lactobacillus plantarum* BDLP0001

(1) Sample collection

Collect samples from naturally fermented pickles, traditional fermented dairy products (yogurt, sour horse milk, etc.), raw milk, raw dough, fermented sausages, salami, kefir grains (Tibetan kefir), silage and baby feces, etc. The collected samples are kept storing in ice box and brought back to the laboratory at low temperatures, and then placed in a refrigerator at 4°C, then separate the *Lactic acid bacteria* as early as possible.

(2) Sample pretreatment

Put 20g solid sample (or 20mL liquid sample) into 250mL flask (with glass beads) containing 180mL sterilized 0.1% peptone water (peptone 1g, distilled water 1000g), and stand 20min after shaking for reserve.

(3) Preliminary isolation of exopolysaccharide-secreting strains

Serial dilutions of the samples are carried out using sterilized 0.1% peptone water with a ratio of 1:10 in volume, and in each dilution, 0.1mL diluted sample is coated on to MRS agar plate, M17 agar plate, SM agar plate, Modified MRS agar plate and ESM plate, respectively. The plates are placed for anaerobic culture at a constant temperature of 37°C for 24-48h, and then pick single colonies which is sticky and presents stringy state obviously by sterilized toothpicks. Then the single colonies are streaked onto corresponding agar plates to obtain purified single colonies for Gram stain and catalase test. And the purified strains obtained are deposited in corresponding isolation mediums supplemented with 20% glycerol as protective agent and cryo-preserved at -20°C for cryopreservation.

Therein, the medium formulations are as follows:

SM medium (g/L): 120g skimmed milk powder, 10g glucose, 880g water.

ESM medium (g/L): 90g skimmed milk powder, 3.5 g yeast extract, 3.5g peptone, 20g glucose (Van den Berg, D.J.C., A. Smits, B. Pot, A.M. Ledeboer, K. Kersters, J.M.A Verbakel, and C.T. Verrips. 1993. Isolation, screening and identification of *Lactic acid bacteria* from traditional food process and culture collections. Food Biotechnol. 7:189-205.).

MRS medium (selective medium for *Lactobacillus*, Merck, Germany)

M17 medium (medium for *Lactococcus*, BD Difco)

Modified MRS medium: the glucose content of the MRS medium is changed into 50g/L and other components remain unchanged.

700 strains are isolated from different samples on MRS agar plate, M17 agar plate, Modified MRS agar plate, ESM plate and SM agar plate. These strains present stringy, sticky and mucoid states on the isolation plates.

(4) Exopolysaccharide-secreting strains

The isolated strain obtained from the plate is inoculated into MRS liquid culture medium and culture for 18h, then 1% (V/V) of the inoculum is inoculated into MRS liquid medium containing 50g/L glucose and ferment at 30°C for 24h. Take 20mL of the culture medium obtained for boiling water bath for 10 min, then after cooling to the room temperature, the supernatant is added trichloroacetic acid with the mass fraction of 80% to a final concentration of 4% (m/v). Stand overnight at 4°C and centrifuge with the speed of 10000g for 20min, then slightly pour the supernatant into dialysis bag with the MWCO (Molecular Weight Cut Off) of 14000 for dialysis using deionized water for 72h. Change the water once every 8 h in the process of dialysis and keep in constant volume finally. The content of exopolysaccharide is measured by sulfuric acid-phenol method (Dubois M, Gilles KA, Hamilton JK, Pebers PA, Smith F. (1956). Colorimetric method of determination of sugars and related substances. Analytical chemistry. 28(3):350-356.). The experimental results are shown in Table 1. The strain 9-9, as can be seen from Table 1, produces relatively high content of crude polysaccharides and is selected and named as BDLP0001.

Table 1. Preliminary isolation of exopolysaccharide-secreting *Lactic acid bacteria* strains (culture at 30°C for 24h)

strain number	EPS(mg/L)
4-21	60.98
5-28	81.6
9-9	131.96

17-5	109.03
17-15	135.79
17-16	126.79
18-9	125.48
19-16	117.61
19-19	115.87
26-9	81.53
26-8	158.91
22-22	161.88
27-2	93.12
33-4	107.79
33-10	100.23

Embodiment 2: Identification of the strain of *Lactobacillus plantarum* BDLP0001

(1) Physiological and biochemical tests

The BDLP0001 strain, a kind of bacillus, which is Gram-positive, peroxidase-negative and with no mobility, can grow at the temperature of 15°C and 40°C. The strain does not hydrolyze starch and liquefy gelatin and neither produce hydrogen sulfide. Acid is produced and no gas is produced when the strain is fermented with glucose. The strain is negative in benzidine test and indole test, and positive in methyl red test.

(2) Identify the strain by sugar fermentation tests

Pick up a small amount of culture medium of the BDLP0001 strain to streak onto the MRS solid plate for anaerobic culture at 37°C for 24-48h. Single colony picked from the plate is inoculated into API 50 CHL liquid medium (bioMérieux China Ltd., API 50 CHL Medium) to prepare bacterial suspension, then the bacterial suspension is placed at 37°C for anaerobic culture for 24-48h after adding API 50 CHL identification reagent strips (bioMérieux China Ltd.). The fermentation results of 49 kinds of carbohydrates of the strain are recorded and input into the Merieux authentication software API LAB PLUS, and results are shown in Table 2. Via database queries, the BDLP0001 strain in the present invention shows a homology of 99.9% with the *Lactobacillus plantarum*, thus the BDLP0001 strain in the present invention is identified as *Lactobacillus plantarum* preliminarily.

Table 2. Carbon source utilization of the BDLP0001 strain

carbohydrate	result	carbohydrate	result
Glycerin	-	Salicin	+
Erythritol	-	D-cellobiose	+
D-arabinose	-	D-maltose	+
L-arabinose	-	D-lactose	+
D-ribose	+	D-melibiose	+
D-xylose	-	D-sucrose	+
L-xylose	-	D-trehalose	+
D-adonitol	-	Inulin	-
Methyl- β -D-xylopyranoside	-	D-melezitose	+
D-galactose	+	D-raffinose	-
D-glucose	+	Starch	-
D-fructose	+	Glycogen	-
D-mannose	+	Xylitol	-
L-sorbose	-+	D-gentiobiose	+
L-rhamnose	-	D-Toulon sugar	-
Dulcitol	-	D-lyxose	-
Inositol	-+	D-tagatose	-
Mannitol	+	D-fucose	-
Sorbitol	+	L-fucose	-
Methyl- α -D-mannopyranoside	+	D-arabitol	-
Methyl- α -D-glucopyranoside	-	L-arabitol	-
N-acetylglucosamine	+	Potassium gluconate	-
Laetrile	+	2-keto-potassium gluconate	-
ARBULIN	+	5-keto-potassium gluconate	-
Esculin ferric citrate	+		

Note: "+" means positive reaction. "-" means negative reaction. "- +" means the carbon source utilization cannot be determined.

(2) 16s rDNA sequence analysis of the BDLP0001 strain

Genomic DNA extraction method of the BDLP0001 strain: Single colony of purified BDLP0001 is picked and inoculated into 1 mL MRS liquid medium, and after cultured at 37°C for 14h, centrifugate (5000g, 10min) the bacterial liquid to collect the thalli cells. Extract the genomic DNA by using the genomic DNA extraction kit (TIAN GEN company). PCR amplification is carried out by using two synthetic universal primers (16s 27F: GAGAGTTTGATCCTGGCTCAG; 16s 1492R: CGGCTACCTTG TTACGACTT). The PCR products are recovered by using extraction kit (BioFlux) after cutting gel, then purifies the recovered PCR products for sequencing (Invitrogen biotechnology companies). The 16s rDNA nucleotide sequence of the BDLP0001 strain obtained is 1446bp in length (shown as SEQ ID NO: 1 in the sequence listing) and the sequence is input into GenBank for Blast analysis with an accession number of JN86879. The BDLP0001 strain has the highest homology of 100% with *L.plantarum* IMAU 80597 (GenBank accession number: HM958789).

According to the argument of Goodfellow and O'Donnell, for the DNA, if G+ C (mol %) $\leq 0\% \sim 12\%$, and meanwhile for the 16S rRNA, if the sequence homology $\geq 95\%$, the strains can be classified as a genus. And according to the argument of Embley and Stackebrandt, for the 16S rRNA, if the sequence homology $\geq 97\%$, the strains can be classified as a species. It can be inferred that the *L.plantarum* IMAU 80597 strain and the BDLP0001 strain belong to the same species. According to these arguments, the BDLP0001 strain is identified as *Lactobacillus plantarum*.

Based on microbial characteristics like morphological characteristics, physiological-biochemical characteristics and genetic characteristics, i.e., 16s rDNA, strain of *Lactic acid bacteria* BDLP0001 is identified as *Lactobacillus plantarum*. The strain is deposited in China General Microbiological Culture Collection Center (CGMCC) with an access number of CGMCC No.5222.

Embodiment 3: Growth characteristics of the BDLP0001 strain

(1) Drawing the growth curve of the BDLP0001 strain

The activated strain of *Lactobacillus plantarum* BDLP0001 is inoculated into MRS liquid medium with a ratio of 1% (V/V) and cultured at 37°C for 24h. And at 620nm, the viable bacteria count and pH value of the culture medium is measured every 2h. The pH value of the culture medium is measured with a pH meter, while the viable bacteria count is measured by using the plate count method. The logarithm of the viable bacteria count and pH value are plotted against time, and the growth curve of the BDLP0001 strain in MRS liquid medium is obtained. The results (Figure 3) show that: The strain of *Lactobacillus plantarum* BDLP0001 grows rapidly in MRS liquid medium, and it grows into the logarithmic phase at the time of 2h and the

stationary phase at the time of 10h. With prolonged incubation time, the pH lower continuously as the strain begins to produce acid and the degree of pH decline slows after entering the stable stage. At the end of 24h's culture, the pH value of the culture medium is 3.89 and the viable bacteria count in the culture medium can reach 10^8 CFU/mL.

(2) Measuring the optimum growth temperature of the BDLP0001 strain

The activated strain of *Lactobacillus plantarum* BDLP0001 is inoculated into 10mL MRS liquid medium with a ratio of 1% (V/V) and cultured at 15°C, 37°C, 40°C, 45°C and 65°C for 16h, with the uninoculated MRS liquid medium as a control, then the OD values of the culture medium at different culture temperatures are measured at 620nm, and the optimum growth temperature is determined according to the OD values. The results shows (Figure 4) that the strain of *Lactobacillus plantarum* BDLP0001 has a wide temperature range for growth from 15°C to 45°C and can grow well at the growth temperature between 30°C and 40°C, and the optimum growth temperature is 35°C.

(3) Measuring the optimum growth pH of the BDLP0001 strain

The activated strain of *Lactobacillus plantarum* BDLP0001 is inoculated into MRS liquid medium with different initial pH of 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, respectively and then cultured at 37°C for 16h and the uninoculated MRS liquid medium with same initial pH is as a control, then the OD values of the culture medium are measured at 620nm, and the optimum growth pH is determined according to the OD values. The results (Figure 5) show that the BDLP0001 strain grows well with the initial pH of 4.0-8.0 and the optimum pH is determined to be 6.0.

(4) Bile tolerance test of the strain of *Lactobacillus plantarum* BDLP0001

The activated strain of *Lactobacillus plantarum* BDLP0001 is inoculated into MRS liquid medium with different concentrations of sodium taurocholate (TCA) (with mass fraction of 0.0%, 0.05%, 0.1%, 0.15%, 0.2%, 0.25%, 0.3%, 0.35% and 0.4%, respectively), and then cultured at 37°C for 24h, then the OD values of the culture medium are measured at 620 nm, and determine the bile tolerance according to the OD values. The bile salt content in human intestinal is between 0.03%-0.3% with fluctuations, and strains which could grow and metabolite in normal physiological concentration of bile salts in the intestinal transit was likely to survive in the process of intestinal transit. As shown in Table 3, along with the increasing concentration of bile salts, the tolerance to bile salts of the strain declines. The strain of *Lactobacillus plantarum* BDLP0001 exhibits good tolerance to bile salts as it can grow well with the bile salt concentration ranging from 0.1% to 0.4%. Especially, in the medium with 0.4% bile salt, the OD value of the strain can still reach more than 1.5. The results suggest that the strain of *Lactobacillus plantarum* BDLP0001 can

survive, grow normally and reproduce in the small intestine and there is potential for the development of being probiotics.

Table 3. The growth conditions of strain of *Lactobacillus plantarum* BDLP0001 in the medium with different concentrations of bile salt

	Mass fraction of TCA (%)						
	0	0.1	0.15	0.2	0.25	0.3	0.4
OD620	3.4785	3.1617	3.0336	2.7342	2.3217	1.8309	1.6635

(5) NaCl tolerance test of the strain of *Lactobacillus plantarum* BDLP0001

The activated strain of *Lactobacillus plantarum* BDLP0001 is inoculated into MRS liquid medium with different concentrations of NaCl (with mass fraction of 0%, 2%, 4%, 6%, 7%, 8%, 9%, 10% and 11%, respectively), and then cultured at 37°C, then with bromcresol purple as an indicator, the NaCl tolerance is observed. The results are shown in Table 4. The strain of *Lactobacillus plantarum* BDLP0001 grows well when the growth medium contains no more than 7% NaCl, grows slowly when the growth medium contains 8% NaCl, and no growth can be observed when the growth medium contains more than 9% NaCl. The results suggest the BDLP 0001 strain has good NaCl tolerance.

Table 4. NaCl tolerance of the strain of *Lactobacillus plantarum* BDLP0001

Concentrations of NaCl (%)	Growth condition
0	++
2	++
4	++
6	++
7	++
8	+
9	-
10	-
11	-

Notes: “++” means grows well, “+” means growth can be observed, “-” means no growth can be observed

Embodiment 4: Extraction of the exopolysaccharides produced by the strain of *Lactobacillus plantarum* BDLP0001

(1) Strain activation: The strain of *Lactobacillus plantarum* BDLP0001 is inoculated into MRS liquid and cultured at 37°C for 12-16h for activation, the activated culture is continued for two generations.

(2) Seed culture: The activated strain of *Lactobacillus plantarum* BDLP0001 is inoculated into 12% (w/w) skimmed milk which is sterilized at 115°C for 15min containing 1% (w/v) glucose, and cultured at 37°C for 14-16h till the milk begin to curd, the activated culture is continued for two generations, and the products obtained is used as a mother starter culture.

(3) Fermentation: The strain of *Lactobacillus plantarum* BDLP0001 is inoculated into 12% (w/w) skimmed milk containing 1% (w/v) glucose with a ratio of 5% (v/v) and cultured at 30°C for 30h.

(4) Extraction and Purification of EPS: Take the fermentation liquor prepared above for boiling water bath for 10min first to inactivate the enzyme which could degrade the polysaccharides, then centrifuge (20min, 10000g, 4°C) to remove the thalli cells and the coagulated proteins. The supernatant is concentrated to 1/2 of the original volume, and add 80% (w/v) trichloroacetic acid to a final concentration of 4% (w/v), stand overnight, then centrifuge (20min, 10000g, 4°C) to remove the precipitated proteins. 95% (v/v) ethanol is added into the concentrated solution to a final concentration of 75% (v/v), stand at 4°C for 24h, then centrifuge (20min, 10000g, 4°C). The precipitate obtained is dissolved in deionized water, centrifuge (20min, 10000g, 4 °C) to remove the precipitate, and the supernatant is dialyzed in deionized water for 72h. Change the water once every 8 h in the process of dialysis and via freeze drying the crude polysaccharide sample is obtained.

Embodiment 5: Immune activity of the polysaccharides *in vitro*

Cytotoxicity assay and the proliferative response of T/B lymphocytes of EPS

Aseptically removed BALB/C mouse spleen and make into spleen cell suspension. Lymphocytes are isolated by lymphocyte separation medium (Shanghai SABC) and washed twice with PBS buffer solution (containing 0.144g KH₂PO₄, 9.0g NaCl, 0.795g Na₂HPO₄ 7H₂O per liter, pH=7.4), then adjust the cell concentration to 1×10⁶/mL to obtain a splenic lymphocytes suspension with RPMI1640 medium (Biosharp Amresco Company). 150μL splenic lymphocytes suspension and 50μL exopolysaccharides samples of different concentrations (10μg/mL, 100μg/mL, 1000μg/mL) are added to a 96-well culture plate. Elicit T lymphocyte proliferation with mitogen concanavalin A (ConA, 5μg/mL, Sigma) and elicit B lymphocyte proliferation with lipopolysaccharide (LPS, 10μg/mL). Negative control group (containing only splenic lymphocytes suspension) and positive control group (added with mitogen) is also setup parallelly. In cytotoxicity assays, do not add mitogen. Three repeats are set up for each experimental group and then cultured at 37°C for

72h with 5% CO₂ and saturated humidity.

(1) Cytotoxicity test: Use MTT assay (Xu Deyi, Jia HongBin “5-HT₃ receptors in amygdala mediate neuroimmunomodulation in rats” [J] *Acta Physiologica Sinica*, 2001, 53 (5): 349-354) to take the test. 4h before the end of culturing, each well is added 20μL MTT (5g/L, Sigma) and continued to culture for 4h. After culturing, 150μL DMSO is added in. Then A₅₇₀ values is measured at 570nm via the ELISA apparatus. Therein, the MTT solution is prepared by the following process: MTT is dissolved with D-hank's solution. And stir to dissolve completely, then keep in constant volume to make the concentration of MTT be 5mg/mL.

MTT assay develops rapidly and is widely applied because of its short experimental period, simple operation, high sensitivity and good reproducibility, and it is very important in the fields of cell biology, radiation biology and immunology. The principle of MTT assay is that the succinate dehydrogenase of mitochondria in the living cells can make the yellow MTT restore to poorly soluble blue-violet product and deposite the product in cells (dead cells have no such function). After dissolution with dimethyl sulfoxide (DMSO), the absorbance at a certain wavelength, which is positively correlated with the metabolic capacity of the living cells and reflects the proliferation of cells, is measured via the ELISA apparatus. Through the MTT assay, it is found that there is no significant differences of the OD values between the *in vitro* culture medium of mouse spleen lymphocyte with different concentrations of polysaccharides and the control group, the results are shown in Table 6. The results suggest that the exopolysaccharides display no cytotoxicity.

Table 6. Cytotoxicity test of the crude exopolysaccharides

	Concentration μg/mL	OD value	P value	cytotoxicity
control	-	0.124±0.001		
	10	0.109±0.016	0.2614	no
crude	100	0.143±0.026	0.3304	no
exopolysaccharides	1000	0.267±0.000	0.0000	no

(2) Cell proliferation test: Use ³H-TdR incorporation method (Guo Qu-lian, Zhang Yang-de, Zou Wang-yuan, et al. “Effects of fintrathecal morphineonspleen T-lymphocyte proliferation and NK cell activity in rats”, [J] *Chinese Journal of Anesthesiology*, 2005, 25 (2). : 118-121). 8h after cultivation, 20μL, each well is added with 20μL ³H-TdR (370kBq/mL). After cultivation, cells are harvested onto the 49 glass-fiber filter paper, and the paper is dried and placed in the PPO-POPOP (Sigma) scintillation solution overnight, and measure CPM value of each tube by the liquid scintillation spectrometer.

Therein, the ^3H -TdR working solution is prepared by the following procedure: The stock solution is 37 MBq/mL, the specific strength of radioactivity is 0.925 TBq/mmol. Dilute to the final concentration of 370kBq/mL with RPMI 1640 culture medium before use.

The scintillation solution is prepared by the following procedure: After being added with a small amount of xylene, the POPOP (0.1-0.3g) is dissolved in water bath at 37°C and PPO (5.0g) is added in, then xylene is added in to 1L. Scintillation solution prepared should be kept away from light.

The ConA solution is prepared by the following procedure: 10mg accurately weighed ConA is dissolved with RPMI 1640 medium sufficiently, make the volume to 100mL with the concentration of 100 $\mu\text{g/mL}$.

The LPS solution is prepared by the following procedure: 10mg accurately weighed LPS is dissolved with RPMI 1640 medium sufficiently, make the volume to 100mL with the concentration of 100 $\mu\text{g/mL}$.

Compared with the MTT assay method, the ^3H -TdR method has higher sensitivity, better stability and more affordable. For the ^3H -TdR method, based on DNA, RNA synthesis in the cell cycle, ^3H -TdR can be absorbed into the cells as starting material. Measurement the intracellular radioactivity of ^3H -TdR could reflect cell proliferation.

Spleen lymphocytes include T lymphocytes and B lymphocytes with basically similar content of both. As T lymphocyte mitogen, ConA only promotes the proliferation of T lymphocytes and do not work on the B lymphocytes. On the contrary, LPS can only induce proliferation of B lymphocytes. Crude exopolysaccharides can significantly promote ($P<0.05$) (Table 7) the proliferation of LPS-activated B lymphocyte, and has a significant dose-dependent manner.

Crude polysaccharide does not promote the proliferation of *in vitro* ConA-activated T lymphocyte on mice.

Table 7. Effects on T/B lymphocyte proliferation of the crude exopolysaccharides

	concentration	T lymphocyte		B lymphocyte	
	$\mu\text{g/mL}$	CMP value	percentage of promotion (%)	CMP value	percentage of promotion (%)
negative control	-	332 $\pm$ 35	-	277 $\pm$ 59	-
positive control	-	24911 $\pm$ 822	-	11984 $\pm$ 1287	-

	10	28601±2515	15	14021±282	17
crude	100	28167±3162	13	15355±722	28
exopolysaccharides	1000	25658±3674	3	21243±2971	77

In vitro lymphocytes culturing experiment shows that exopolysaccharides produced by the strain of *Lactobacillus plantarum* BDLP0001 has no cytotoxicity. *In vitro* immune activity experiment shows that exopolysaccharides produced by the strain of *Lactobacillus plantarum* BDLP0001 can significantly promote the proliferation of B lymphocytes and demonstrate a strong immune-enhancing activity.

Applied Embodiment 1: Bulk starter culture of *Lactobacillus plantarum* BDLP0001

The strain of *Lactobacillus plantarum* BDLP0001 is inoculated into sterilized 12% (w/v) skimmed milk which is sterilized at the temperature of 95°C for 15min added with 1% whey protein for activation at the temperature of 37°C for 14-16h till the milk begin to curd, and the activated culture is continued for two generations to obtain the mother culture; then said mother culture is inoculated into new sterilized 12% (w/v) skimmed milk which is sterilized at the temperature of 95°C for 15min added with 1% (wt) whey protein with a ratio of 3-5% (v/v) to culture for 14-16h till the new milk begin to curd, then said bulk starter culture (1) is obtained with viable bacteria count of about 10^9 cfu/mL in the curdled milk is obtained.

The strain of *Lactobacillus plantarum* BDLP0001 is inoculated into MRS liquid medium and cultured for activation at the temperature of 37°C for 12-16h, and the activated culture is continued for two generations, then the activated culture obtained is inoculated into new MRS liquid medium with a ratio of 2-4% (v/v) to culture for 16-18h, and then the cell sediment is obtained via centrifugation at the temperature of 4°C for 15min with the speed of 4000r/min, and the cell sediment is suspended in a certain amount of sterilized skimmed milk, then said bulk starter culture (2) is obtained.

Applied Embodiment 2: *Lactobacillus plantarum* BDLP0001-containing beverage

Raw milk is cooled to 40°C after pasteurization at the temperature of 95°C for 20min or high temperature sterilization at the temperature of 140°C for 2s and then said bulk starter culture (1) or (2) of *Lactobacillus plantarum* BDLP0001 in applied Embodiment 1 is added to make the concentration of *Lactobacillus plantarum* BDLP0001 up to 10^6 cfu/mL or more and then stored at 4°C, i.e., said *Lactobacillus plantarum* BDLP0001-containing beverage is obtained.

Applied Example 3: Preparing *Lactobacillus plantarum* BDLP0001-containing

fermented milk

Raw milk is sterilized at the temperature of 95°C for 20min and cooled to 37°C, and then a ratio of 3-5% (v/v) of bulk starter culture (1) or (2) of *Lactobacillus plantarum* BDLP0001 in applied Embodiment 1 as well as mutualistic commercial starter cultures, i.e., *Lactobacillus Bulgarica* is inoculated into the sterilized milk, and then after being mixed uniformly, the inoculated milk is fermented at 37°C till the titration acidity reaches to 0.6 calculated by lactic acid and then stored at 4°C, i.e., said *Lactobacillus plantarum* BDLP0001-containing fermented milk is obtained.

It should be understood that, after reading the contents of the present invention described above, the person skilled in the art can make various modifications of the invention, these equivalent forms also fall within the scope defined by the appended claims of the present application.

What is claimed is:

1. A strain of *Lactobacillus plantarum* that produces exopolysaccharides, wherein it is deposited in China General Microbiological Culture Collection Center with an accession number of CGMCC No.5222.

2. A bulk starter culture of said strain of *Lactobacillus plantarum* according to claim 1, wherein it is prepared according to a method comprising the following step (a) or (b):

(a) inoculating said strain of *Lactobacillus plantarum* according to claim 1 into sterilized milk added with whey protein and culturing till the milk begin to curd, continuing the activated culture for two generations to obtain a mother starter culture; inoculating said mother starter culture with a ratio of 3-5% (v/v) into new sterilized milk added with whey protein and culturing till the new milk begins to curd, and then obtaining said bulk starter;

(b) inoculating said strain of *Lactobacillus plantarum* according to claim 1 into liquid medium and culturing for activation, continuing the activated culture for two generations, inoculating the activated culture obtained with a ratio of 2-4% (v/v) into new liquid medium and culturing for 16-18 h, obtaining the cell sediment through solid-liquid separation, and suspending the cell sediment in sterilized milk, and then obtaining said bulk starter culture.

3. The bulk starter culture according to claim 2, wherein a viable count of bacteria in said bulk starter culture is no less than 10^9 cfu/mL.

4. Use of strain of *Lactobacillus plantarum* CGMCC No.5222 in fermented foods.

5. The use according to claim 4, wherein said fermented foods refer to *Lactic acid bacteria*-containing milk beverage or fermented milk.

6. The use according to claim 5, wherein said *Lactic acid bacteria*-containing milk beverage is prepared according to a method comprising the following steps: cooling raw milk after sterilization, and mixing with said bulk starter culture of *Lactobacillus plantarum* according to claim 2 uniformly to make the concentration of *Lactobacillus plantarum* up to 10^6 cfu/mL or more, and then obtaining said *Lactic acid bacteria*-containing milk beverage.

7. The use according to claim 5, wherein said fermented milk is prepared according to a method comprising the following steps: cooling raw milk after sterilization, inoculating a ratio of 3-5% (v/v) of bulk starter culture of *Lactobacillus plantarum* according to claim 2 as well as a ratio of 3-5% (v/v) of mutualistic

commercial starter cultures into the sterilized milk, mixing uniformly and fermenting till the titration acidity reaches to 0.6-0.7 calculated by lactic acid, and then obtaining the fermented milk containing said *Lactobacillus plantarum*.

8. Exopolysaccharides extracted from said strain of *Lactobacillus plantarum* according to claim 1.

9. The exopolysaccharides according to claim 8, wherein a method of extraction comprises the following steps: centrifuging the fermentation liquor of said strain of *Lactobacillus plantarum* according to claim 1 after boiling in order to remove thalli and condensed protein, precipitating protein from supernatant via trichloroacetic acid method to remove the protein, obtaining a precipitate by adding alcohol, dissolving the precipitate in water and then dialyzing with water.

10. Use of said exopolysaccharides according to claim 8 or 9 in immune-enhancing medicament, healthcare product or food product.

Sequence Listing

<110> Bright Dairy & Food Co., Ltd

<120> Strain of exopolysaccharide-secreting *Lactobacillus plantarum* and application thereof

<130> P4-124006C

<160> 1

<170> PatentIn version 3.4

<210> 1

<211> 1446

<212> DNA

<213> *Lactobacillus plantarum*

<400> 1

cttaggcggc tggttcctaa aaggttacct caccgacttt ggggtgttaca aactctcatg	60
gtgtgacggg cgggtgttac aaggcccggg aacgtattca ccgcggcatg ctgatccgcg	120
attactagcg attccgactt catgtaggcg agttgcagcc tacaatccga actgagaatg	180
gctttaagag attagcttac tctcgcgagt tcgcaactcg ttgtaccatc cattgtagca	240
cgtgtgtagc ccaggtcata aggggcatga tgatttgacg tcatccccac ctctctccgg	300
tttgtcaccg gcagttctac cagagtgcgc aacttaatgc tggcaactga taataagggt	360
tgcgctcggt gcgggactta acccaacatc tcacgacacg agctgacgac aacctatcac	420
cacctgtatc catgtccccc aagggaacgt ctaatctctt agatttgcat agtatgtcaa	480
gacctggtaa ggttcttcgc gtagcttcga attaaaccac atgctccacc gcttgtgcgg	540
gccccgtca attcctttga gtttcagcct tgcggccgta ctccccaggc ggaatgctta	600
atgcgttagc tgcagcactg aagggcggaa accctccaac acttagcatt catcgtttac	660
ggtatggact accagggtat ctaatcctgt ttgctaccca tactttcgag cctcagcgtc	720
agttacagac cagacagccg ccttcgccac tgggtgttctt ccatatatct acgcatttca	780

ccgtacaca tggagttcca ctgtcctctt ctgcactcaa gtttcccagt ttccgatgca	840
cttcttcggt tgagccgaag gctttcacat cagacttaaa aaaccgcctg cgctcgcttt	900
acgcccaata aatccggaca acgttgcca cctacgtatt accgcggctg ctggcacgta	960
gtagccgtg gctttctggt taaataccgt caatacctga acagttactc tcagatatgt	1020
tcttctttaa caacagagtt ttacgagccg aaacccttct tctctcacgc ggcgttgctc	1080
catcagactt tcgtccattg tggaagattc cctactgctg cctcccgtag gagtttgggc	1140
cgtgtctcag tcccaatgtg gccgattacc ctctcaggtc ggctacgtat cattgccatg	1200
gtgagccgtt accccaccat ctagctaata cgccgcggga ccatccaaaa gtgatagccg	1260
aagccatctt tcaaactcgg accatgcggt ccaagttggt atgcggtatt agcatctggt	1320
tccaggtggt atcccccgct tctgggcagg ttcccacgt gttactcacc agttcgccac	1380
tcactcaaat gtaaatcatg atgcaagcac caatcaatac cagagttcgt tcgactgcat	1440
gtatag	1446