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EFFECT OF ORAL ADMINISTRATION OF *LACTOBACILLUS PARACASEI* L9 ON MOUSE SYSTEMIC IMMUNITY AND THE IMMUNE RESPONSE IN THE INTESTINE

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Abstract: A probiotic strain *Lactobacillus paracasei* L9, which was isolated from human intestine, was investigated for its immunomodulatory activity *in vivo*. Results showed that L9 improved systemic immunity by enhancing the phagocytic activity of peritoneal macrophages, the proliferation ratio of splenocytes, the IgG level in the serum and the level of IgA in the mucosa. Further, L9 induced the Th1-polarized immune response by elevating the IFN-γ/IL-4 ratio in the mucosa. This effect was confirmed by the enhanced IL-12-inducing activity of macrophages after in vitro stimulation of L9. Also detected was increased expression of TLR-2 mRNA in the mucosa. We predict that L9 could enhance innate immunity by activating TLR-2 in the mucosa, and enhance acquired immunity by promoting Th1 polarization through induced production of IL-12 by macrophages.

Key words: *Lactobacillus paracasei* L9; immunomodulatory activity; systemic immunity; Th1 immunity; TLR-2

INTRODUCTION

Immunomodulatory activity is an important criterion for selecting and evaluating probiotics as it is thought to be responsible for many probiotic effects, such as their anti-inflammatory, anti-infection and antitumor activities [1]. The immunomodulatory capacities of probiotic lactobacilli are strain-specific [2]. Therefore, it has been suggested that the immunomodulatory activity of a specific strain must be evaluated before development and application [3].

Systemic immunity, consisting of innate immunity (macrophage phagocytosis, etc.) and acquired immunity, have been widely used to evaluate the immunomodulatory effect of probiotics [1,4-6]. Gut mucosa is the first line of immune defense [7], where probiotics act to induce innate immunity and acquired immunity [8]. Activation of innate immunity is reported to be mediated by Toll-like receptors (TLRs) and is a critical step for the development of acquired immunity. T helper cells are key components of acquired immunity responses [9], which can be polarized into T helper type-1 (Th1), T helper type-2 (Th2) or regular T (Treg) cells by specific lactic acid bacteria (LAB) strains [10]. Some LAB strains have been demonstrated to induce interleukin (IL)-12 production from dendritic cells (DCs) and macrophages, converting a Th2 response into a Th1-dominated response and suppressing antigen-specific IgE production in mice [11,12], whereas some strains are capable of controlling inflammatory diseases by inducing the development of $CD4^+$ Forkhead box P3 $(Foxp3)^+$ Tregs cells [13].

Lactobacillus paracasei L9 (L9) was initially isolated from a human intestine. Previous studies indicated that L9 was efficient in preventing ulcerative colitis [14] and capable of relieving constipation [15], suggesting that L9 is a potential probiotic strain. The objective of this study was to evaluate the effect of

oral administration of L9 on the systemic immunity and the innate and acquired immunity in the mucosa.

MATERIALS AND METHODS

Bacterial strains and bacteria preparation

The *Lactobacillus paracasei* L9 (CGMCC NO. 9800) used in this study was isolated from human intestine and stored in our lab. L9 was inoculated in Man, Rogosa, and Sharpe (MRS) broth under aerobic condition at 37°C for 12 h. Centrifugation (4000 rpm, 15 min) was used to separate the bacterial strain. The bacterial culture was washed twice with saline, and resuspended in saline to prepare the required concentration.

Animal groups used in *in vivo* experiments

For the *in vivo* experiments, six-week-old male BALB/c mice were kept in a temperature-controlled environment (22±2°C) with a 12 h light-dark cycle and with free access to water and standard rodent chow throughout the experiment. All animals were treated in accordance with the guidelines on the care and use of animals and with the approval of the Animal Ethics Committee of the China Agricultural University.

After a 5 day acclimation period, the mice were randomly divided into 4 groups, with each group containg 8 mice. The L9(L), L9(M), and L9(H) groups were orally administered a low dose (10^6 CFU/mL), a medium dose (10^8 CFU/mL), and a high dose (10^{10} CFU/mL) of L9, respectively, at a volume of 0.2 mL once a day. The control group received the same volume of sterile saline at the same time. After feeding for 7 weeks, the mice were euthanized by cervical dislocation.

Measurement of serum IgG

Blood was collected from the orbital cavity before euthanasia. Samples were allowed to clot at room temperature for 3 h and then at 4°C overnight. The samples were centrifuged at 1000 rpm for 10 min to isolate the supernatants. The supernatants were then stored at -20°C until use. IgG concentrations were determined by enzyme-linked immunosorbent assay (ELISA) kits (USCN Life Science, Wuhan, China) according to the manufacturer's instructions.

Phagocytosis of peritoneal macrophages

Macrophage phagocytosis of chicken red blood cells (CRBC) was measured as previously described [16]. After the mice were killed, the macrophage suspension was prepared and mixed with equal volumes of 1% CRBC solution. Five hundred μL of the mixture was spread onto glass slides to make a smear for each mouse. The smears were incubated at 37°C for 30 min in a wet box, fixed with methanol for 1 min and then stained by 12.5% (v/v) Giemsa-phosphoric acid dye (Sigma-Aldrich, Shanghai, China) for 15 min. The number of macrophage ingesting CRBC out of a total of at least 100 cells was calculated by direct visual enumeration using a light microscope. The phagocytosis ratio of macrophages was defined as in Eq.1.

$$\text{Phagocytosis ratio} = \frac{\text{number of macrophages ingesting CRBC}}{\text{number of macrophages}} \times 100\%$$

Splenic lymphocyte proliferation assay

Spleens were removed aseptically from killed mice, placed in a Petri dish, and then minced in sterile Hank's solution through a sterilized mesh (200 mesh) to obtain the single cell suspension. After the red blood cells were depleted by distilled water, the remaining cells were washed twice and resuspended to a final concentration of 2×10^6 cell/mL by RPMI-1640 medium. To determine cell viability, trypan blue (BD Biosciences) was used. Splenic lymphocyte proliferation was implemented according to the method described previously [17], with modifications. Splenic lymphocytes (2×10^5) that were either exposed or were not exposed to 1.5 μg/mL concanavalin A (ConA, Sigma-Aldrich, Shanghai, China) were seeded in 96-well plates and incubated at 37°C for 72 h. At 4 h before the end of the incubation period, 10 μL of CCK-8 (Cell counting kit-8, Beyotime, Beijing, China) was added per well. After incubation, optical density (OD) at 490 nm was measured on an ELISA reader (Bio-Rad Model 680). The proliferation of splenic lymphocytes was calculated by the proliferation ratio as follows: Proliferation ratio

$$= (OD_{ConA}\text{-}OD_{control}) / (OD_{ConA}^{-}\text{-}OD_{control}) \times 100\%,$$

where OD_{ConA}, $OD_{control}$ and OD_{ConA}^{-} represent the OD value of wells containing cells, CCK-8 and ConA, wells containing CCK-8 and wells containing cells and CCK-8, respectively.

Secretory IgA (sIgA) concentration in the intestinal mucus

The concentration of SIgA in the intestinal mucus was measured as described in a previous report, with modifications [18]. A 5-cm length of intestine tissue of the ileum was obtained from each animal, dissected and washed with saline. Intestinal mucus was collected and centrifuged at 700 *g* for 10 min after being dissolved in 1 mL of phosphate-buffered saline (PBS). The supernatant was removed and centrifuged at 12000 *g* for 15 min. The supernatant was taken for measurement of sIgA by commercial ELISA kit (USCN Life Science, Wuhan, China) following the manufacturer's instruction. The total protein content of intestinal mucus was assayed by bicinchoninic acid (BCA) kit (Takara, Beijing, China) against a bovine serum albumin (BSA) standard curve, simultaneously. The concentration of sIgA was expressed as microgram per milligram of protein.

Gene expression in mucosa

Real-time reverse transcription polymerase chain reaction (RT-PCR) assays were used to evaluate mRNA expression of TLR-2, interferon (IFN)-γ, IL-4, Foxp3 and transforming growth factor (TGF)-β. After collecting mucus, the intestinal segment was scraped with a coverslip to obtain the mucosa [19]. The collected mucosa was stored by freezing at -80°C. Total RNA was extracted from the mucosa using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and reverse transcribed using MMLV reverse transcriptase (Promega, Madison, WI, USA) to generate cDNA for use in RT-PCR. Each cDNA sample was stored at -20°C until further real-time PCR analysis. The specific oligonucleotide primers were synthesized by Invitrogen. The primer sequences for each gene are shown in Table 1. Real-time PCR reactions were performed using the Roche Light Cycler Instrument 1.5 using Light Cycler Fast Start DNA Master PLUSSYBR Green I kit (Roche, Castle Hill, Australia). Briefly, the 20-μL reaction mixtures contained 10 μL of Master Mix, 0.4 μL of 0.75 μM forward primer and reverse primer, 2 μL of the cDNA sample and 7.6 μL of Milli Q water. Each sample was run in duplicate. The RT-PCR program was at 95°C for 30 s, 40 cycles at 95°C for 15 s, 60°C for 30 s and 72°C for 30 s. At the end of the program, melt curve analysis was performed. The fold expression or repression of the target gene relative to the internal control gene (GAPDH) in each sample was calculated by the formula:

Table 1. Primer sequences used for RT-PCR.

Gene	Primer sequence	Reference
IL-4	Forward:5'-GGTCTCAACCCCCAGCTAGT-3' Reverse:5'-GCCGATGATCTCTCTCAAGTGAT-3'	[26]
IFN-γ	Forward:5'-AGCGGCTGACTGAACTCAGATTGTAG-3' Reverse:5'-GTCACAGTTTTCAGCTGTATAGGG-3'	[27]
TGF-β	Forward:5'-GGCGGTGCTCGCTTTGTA-3' Reverse:5'-GTTGTTGCGGTCCACCATTAG-3'	[28]
Foxp3	Forward:5'-CTCATGATAGTGCCTGTGTCCTCAA-3' Reverse:5'-AGGGCCAGCATAGGTGCAAG-3'	[29]
TLR-2	Forward:5'-TCTAAAGTCGATCCGCGACAT-3' Reverse:5'-CTACGGGCAGTGGTGAAAACT-3'	This study
GAPDH	Forward:5'-GTGTTCCTACCCCCAATGTGT-3' Reverse:5'-ATTGTCATACCAGGAAATGAGCTT-3'	[30]

$$2^{-\Delta\Delta\text{crossing point (CP)}},$$

where $\Delta Cp = Cp_{\text{target gene}} - Cp_{\text{internal control}}$ and $\Delta\Delta Cp = \Delta Cp_{\text{test sample}} - \Delta Cp_{\text{control sample}}$.

Assay for IL-12-inducing activity *in vitro*

Mouse peritoneal macrophages were obtained from male BALB/c mice as reported before [16]. Mouse macrophages (2×10^5 cells) were stimulated with L9 at three doses (2×10^4 CFU, 2×10^5 CFU and 2×10^6 CFU) for 24 h. The highest value of IL-12 production was extrapolated as the IL-12-inducing activity. Culture supernatants were collected and IL-12 levels measured by ELISA kits (USCN Life Science, Wuhan, China) according to the manufacturer's instructions.

Statistical analysis

The results were expressed as means±standard deviation (SD). Data analysis was carried out using SPSS software, version 20.0. Differences among groups were compared using one-way ANOVA tests followed by Duncan's post hoc test. Values of $P<0.05$ were considered significant.

RESULTS

Effects of oral administration of L9 on systemic immunity in mice

The phagocytosis ratio of peritoneal macrophage to CRBC was determined to evaluate the innate immune response. As shown in Fig. 1, the phagocytosis ratio of mice fed with L9(M) and L9(H) were significantly higher than the control group ($P<0.05$). The L9(M) group had a slightly but not significantly higher phagocytosis ratio than L9(H) ($P>0.05$).

As an important index of cellular immunity in the acquired immune response, the proliferation ratio of splenic lymphocytes was detected. Viability of splenic lymphocytes exceeded 95% of all cell populations in all cases. No significant difference in splenocyte proliferation ratio was found between the L9(L) group and the control group ($P>0.05$; Fig. 2). The proliferation ratio of mice fed with L9(M) and L9(H) was significantly higher than that of the control mice ($P<0.05$).

B-cell function, a symbol of humoral immunity in the acquired immune response, was investigated by measuring the concentration of IgG in serum. Results showed that IgG production was significantly increased in mice fed with high doses of L9 ($P<0.05$; Fig. 3), while no significant changes were observed in mice fed with the medium and low doses of L9 ($P>0.05$).

The concentration of sIgA in the intestinal mucus was measured by ELISA (Fig. 4). In comparison to the control mice, sIgA concentrations were significantly increased in mice fed with the high dose of L9 ($P<0.05$), but not in mice fed with a medium or a low doses of L9 ($P>0.05$).

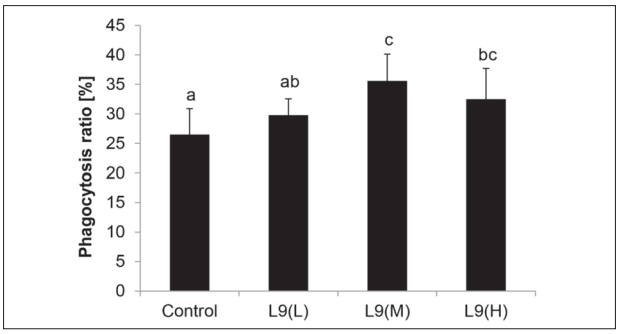

Fig. 1. Effect of administration of L9(L), L9(M), L9(H) and saline on peritoneal macrophage phagocytosis. The activity of these cells was determined by a phagocytosis assay of chicken red blood cells (CRBC). Values are means±SD. The phagocytosis ratio was expressed as the percentage of phagocyting macrophages in 100 calculated macrophages. ^{a,b,c} – data marked with different letters are significantly different at $P<0.05$. L9(L), low dose (10^6 CFU·mL^{-1}), L9, L9(M), middle dose (10^8 CFU·mL^{-1}) L9, L9(H), and high dose (10^{10} CFU·mL^{-1}) of *Lactobacillus paracasei* L9.

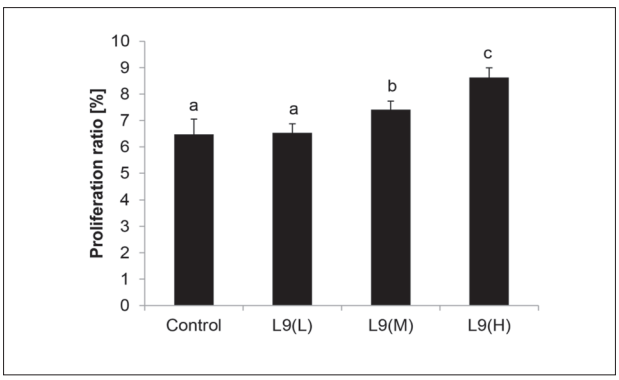

Fig. 2. Proliferation of splenic lymphocytes from untreated controls and mice treated with L9(L), L9(M) and L9(H) in response to ConA. Splenocytes were prepared and cultured with or without ConA for 72 h. Splenic lymphocyte proliferation was measured by the modified MTT method as described in the text, and is shown as a proliferation ratio. The values are presented as means±SD. ^{a,b,c} – data marked with different letters are significantly different at $P<0.05$

Effects of oral administration of L9 on the expression level of receptor TLR-2 mRNA in the mucosa

The expression of TLR-2 mRNA in the mucosa was measured to investigate whether the LAB strain could initiate the immune response through this receptor. As shown in Table 2, significant increases in the mRNA

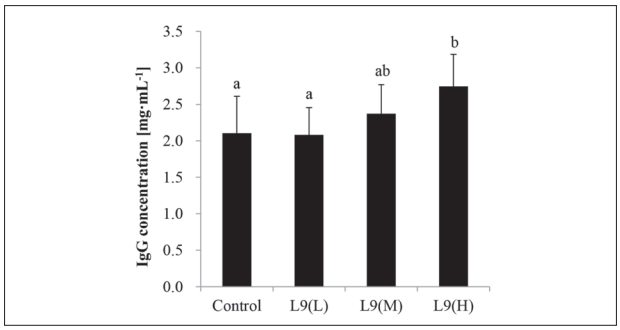

Fig. 3. Production of IgG antibodies from mice fed with L9(L), L9(M), L9(H) and saline. The concentration of IgG was determined using ELISA. All data are presented as means±SD. [a,b] – data marked with different letters are significantly different at $P<0.05$

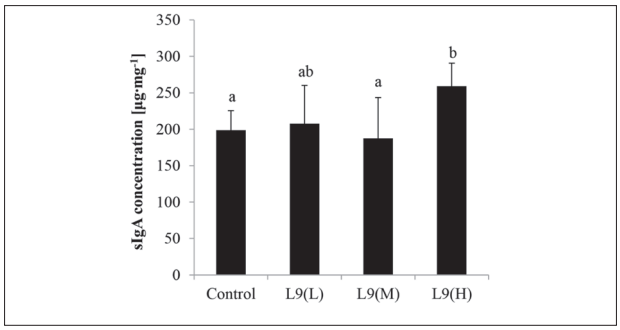

Fig. 4. Effect of oral administration of LGG, L9(L), L9(M), L9(H) and saline on sIgA production in the intestines. sIgA concentration in the intestinal mucus was detected by ELISA. The values are presented as means±SD. [a,b] – data marked with different letters are significantly different at $P<0.05$

expression of TLR-2 were found in L9-treated mice ($P<0.05$). Moreover, the L9(H) group had a significantly higher level of TLR-2 mRNA expression than the L9(L) and L9(M) groups ($P<0.05$).

Effects of oral administration of L9 on T helper cell polarization in the mucosa

To determine the influence of the tested LAB strain, we examined the expression of mRNAs for IFN-γ, IL-4, and TGF-β and Foxp3, representative proteins of Th1-, Th2- and Treg-type immune responses, respectively. As shown in Table 2, the L9(M) and L9(H) groups exhibited a relatively high ability of enhancing IFN-γ mRNA expression ($P<0.05$), unlike the L9(L) group ($P>0.05$), as compared to the control group. The L9(H) and L9(M) groups had equivalent IFN-γ mRNA levels ($P>0.05$). The expression of IL-4 mRNA exhibited an opposite tendency to that of IFN-γ. Specifically, the L9(L), L9(M) and L9(H) groups displayed a slightly but not significantly lower level of IL-4 mRNA than the control group ($P>0.05$). The IFN-γ/IL-4 ratios in the L9(M) and L9(H) groups were significantly higher than the control group ($P<0.05$). The mRNA expression of TGF-β and Foxp3 were not significantly different ($P>0.05$) in all of the tested groups (Table 2).

Effect of L9 on the IL-12-induced activity of macrophages *in vitro*

Judging by the elevated IFN-γ/IL-4 ratio, the L9 strain could induce a Th1-polarized immune response. To confirm the induction of Th1-type immunity by the L9 strain, the IL-12-inducing activity of macrophages was examined *in vitro*. The results in Fig. 5 show that the concentration of IL-12 was significantly increased after the macrophages were stimulated with the L9

Table 2. Effects of LAB on the expression of mRNAs for TLR-2, Foxp3, TGF-β, IFN-γ, and IL-4, and on the IFN-γ/IL-4 ratio in the mucosa.

	mRNA expression					
	TLR-2	**Foxp3**	**TGF-β**	**IFN-γ**	**IL-4**	**IFN-γ/IL-4**
Control	1±0.24[a]	1.00±0.32	1.00±0.42	1±0.43[a]	1±0.22	1±0.11[a]
L9(L)	1.49±0.23[b]	0.87±0.28	0.87±0.35	1.03±0.24[a]	0.91±0.28	1.02±0.13[a]
L9(M)	1.43±0.30[b]	0.93±0.24	0.87±0.28	2.31±0.46[b]	0.76±0.16	2.17±0.35[b]
L9(H)	1.87±0.27[c]	0.85±0.26	0.94±0.41	1.70±0.57[b]	0.75±0.16	2.15±0.58[b]

Real-time PCR analysis of the expression of TLR-2, Foxp3, TGF-β, IFN-γ and IL-4 mRNAs, and the value of IFN-γ/IL-4 in the mucosa of mice treated with L9(L), L9(M), L9(H) and saline. Data are mean values±SD obtained from three independent assays of the relative mRNA expression of transcripts. [a,b,c] – data marked with different letters are significantly different at $P<0.05$.

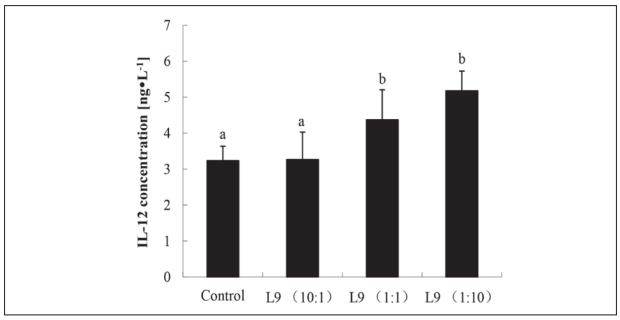

Fig. 5. Effect of different doses of L9 strain on IL-12-producing activity of macrophages *in vitro*. The bracketed number behind L9 represents the ratio of macrophage cell to L9 strain. The concentration of IL-12 was determined by ELISA kits. Data are presented as means±SD. Significant differences compared to model control group are designated as $^{*}P<0.05$

strain, at 1:1 and 1:10 macrophage cell : L9 strain ratios ($P<0.05$). Meanwhile, the IL-12 concentration rose with the increment in L9 dose. As described in the Materials and Methods, the highest value of IL-12 production was extrapolated as the IL-12-inducing activity. Therefore, the IL-12-inducing activity of the macrophages was significantly increased after they were stimulated with the L9 strain ($P<0.05$).

DISCUSSION

To evaluate the immunomodulatory activity of L9, we administered different doses of L9 to mice and detected the effect of L9 on the systemic immune response *in vivo*. Our results show that L9 enhanced innate immunity by elevating phagocytosis of macrophages, strengthening the acquired immunity by improving splenic lymphocyte proliferation and increasing the serum IgG concentration and the concentration of sIgA in the mucus. It was suggested that strains of LAB that are capable of affecting a wider array of immune functions are likely to be more beneficial to human health [6]. L9 was shown to be a beneficial strain, capable of enhancing many aspects of systemic immunity.

Probiotics are known to be not only involved in activating the systemic immune response, but also the mucosal immune response. Gut epithelial and immune cells are continually sampling gut microbes, and bacterial strains can signal through pattern-recognition receptors, resulting in the modulation of various intracellular signaling pathways [20]. Our results showed L9-treated mice to have a higher IFN-γ/IL-4 ratio than the control group and the mRNA expression of TGF-β and Foxp3 did not significantly changed in the mucosa, which suggested that L9 tends to shift towards a Th1 cytokine profile, namely the Th1 polarized strain. It is speculated that probiotics induce the IL-12 production of gut macrophages; thereafter, IL-12 promotes the differentiation of naive $CD4^{+}$ T cell into Th1 cells. We demonstrated that the IL-12-producing activity of macrophages was enhanced by L9 *in vitro*. According to the above results, we arrived at the conclusion that L9 can promote Th1 polarization by inducing IL-12 production by macrophages in mucosa.

Th1 cells and cytokines, including IL-12 and IFN-γ, play crucial roles in host health. Enhancement of their activities could protect against viral infection, alleviate diseases and suppress tumors [21]. We also suggest that enhanced systemic immune effects, which include lymphocyte proliferation and phagocytosis of macrophages can be attributed to the enhancement of Th1-type immune activity, since the Th1-type cytokines, IL-12 and IFN-γ, could promote lymphocyte proliferation and macrophage activity, respectively. In addition to the beneficial effects of a Th1-polarized immune response, constitutive Th1 activation is associated with autoimmune diseases. However, previous reports revealed that oral administration of probiotic LAB did not induce pathological inflammation in murine models [22,23]. While the high dose of L9 (10^{10}cfu/mL) used in this study was much higher than the dose recommended for human consumption (10^{6}cfu/mL), it did not cause overregulation of the Th1-type immune response. More importantly, the safety assessment of L9 done by CTC supports its safety in oral administration.

TLR-2s are known to be a family of receptors that play crucial roles in the innate immune system, where some LAB can lead to the activation of the Th1 immune response [24]. Our results showed that L9 could enhance TLR-2 mRNA expression, suggesting that L9 could initiate the immune response of the host

through TLR-2. Furthermore, it was shown that *Lactobacillus delbrueckii* TUA4408L and its extracellular polysaccharides exerted immunomodulatory effect via TLR-2 and 4 [25]. We observed that L9 could produce a large amount exopolysaccharides (EPS) when compared with other LABs; thus, L9 could be recognized by TLR-2 through EPS, initiating the subsequent immune response.

In summary, we demonstrated that L9 can enhance systemic immunity, as characterized by increased phagocytosis and splenocyte proliferation ratios, and increased concentrations of IgG in the serum and sIgA in the mucus. L9 induced Th1 polarization, which led us to conclude that L9 might promote Th1 polarization by inducing IL-12 production by macrophages via TLR-2 activation in the mucosa. The presented data demonstrate that L9 is an immunomodulating strain that can enhance systemic immunity, and it is very effective in activating the Th1 immune response in the mucosa.

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Authors' contributions: Fazheng Ren conceived the experiment. Yunabo Zhu designed and performed the experiment, analyzed data and wrote paper together with Jun Zhu. Liang Zhao and Ming Zhang modified and polished the manuscript. Huiyuan Guo provided essential materials and was involved in experiment discussion

Conflict of interest disclosure: The authors have declared that no conflict of interests exists.

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