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In Vitro Screening of Lactic Acid Bacteria with RAW264.7 Macrophages and the Immunoregulatory Mechanism

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Abstract: Lactic acid bacteria (LAB) are commonly consumed as probiotics to improve gut barrier function and boost the immune system. This study aimed to screen LAB with high immunomodulatory activity using RAW264.7 macrophages. According to the results, *Limosilactobacillus reuteri* AUc2301 was selected from 84 screened strains that can stimulate RAW264.7 cell proliferation. *Limosilactobacillus reuteri* AUc2301 significantly enhanced the phagocytosis activity of RAW264.7 cells. In the ELISA test, *Limosilactobacillus reuteri* AUc2301 significantly promoted the release of interleukin-6, IL-1β, the tumor necrosis factor, and nitric oxide in RAW264.7 macrophages. In addition, *Limosilactobacillus reuteri* AUc2301 significantly inhibited the excessive release of IL-6, IL-1β, TNF-α, prostaglandin E2 as well as NO and the high expression of cyclooxygenase-2 in RAW264.7 macrophages induced by lipopolysaccharide. In further mechanism studies, *Limosilactobacillus reuteri* AUc2301 could regulate the nuclear factor-κB signaling pathway in RAW264.7 macrophages. Collectively, the screened *Limosilactobacillus reuteri* AUc2301 showed good immunomodulatory activity in vitro, and it has the potential to be developed as a novel probiotic.

Keywords: RAW264.7macrophages; immunomodulatory activity; LAB; NF-κB; in vitro

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1. Introduction

The immune system serves as the body's defense system for immune response, immune function, and self-defense, and is composed of immune organs, immune cells, and immune molecules [1]. Macrophages are an essential component in the creation of immune cells in the body's immune system and they play a significant role in immune defense and stability maintenance [2]. They are among the first cell types to react during the immune response. Lipopolysaccharide (LPS) effectively induces macrophages, which are key mediators of the innate immune response. Various inflammatory mediators and cytokines are released by macrophages to protect the body against foreign infections. The key proinflammatory mediators generated when exposed to LPS stimulation encompass tumor necrosis factor (TNF)-α, interleukin (IL-1β), reactive oxygen species (ROS), reactive nitrogen species (RNS) such as nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS), and prostaglandin E2 (PGE2) produced by cyclooxygenase-2 (COX-2) [3–5]. When diverse external stimuli attach to pattern-recognition receptors on the surface of macrophages, these stimuli can activate defense systems by initiating several different signaling pathways [6]. Nuclear factor kappa-B (NF-κB), a transcription factor sensitive to oxidative stress, governs the expression of genes that activate the immune system, including cytokines such as IL-1β, TNF-α, interferon-γ (IFN-γ) and IL-6 [7]. Dysregulation of NF-κB may occur in chronic inflammatory diseases, and substances that modulate NF-κB have the potential to be innovative therapeutic agents [8]. Moreover, NF-E2-related factor-2 (Nrf2) plays a pivotal

role in adjusting cellular oxidative stress, which serves as a trigger for cellular immune responses [9]. Nrf2 is responsible for regulating the expression of antioxidant enzymes by interacting with the antioxidant response element (ARE), subsequently facilitating the activation of gene transcription involved in the antioxidant response [10–12].

It is known that immune homeostasis plays a pivotal role in maintaining host health [13]. In general, the immune system is a tightly regulated network for maintaining immunological homeostasis [14]. However, inadequate nutrition, chemotherapeutic application, various stressors, may influence or disrupt the balance, resulting in immunological disorders [15]. In recent decades, studies have increasingly emphasized the investigation of specific signaling pathways involved in macrophage immunoregulation [3,16]. A large number of studies have focused on the maintenance of immune homeostasis, which is linked to various human diseases including inflammatory bowel disease (IBD), obesity, diabetes, allergic disorders, and neurodegenerative diseases [17]. Traditional fluoroquinolones, rifaximin and other antibiotics have shown effectiveness in preventing certain immune disorders. Antibiotics can inhibit the growth of harmful microorganisms and reduce the number of bacteria. When the body is infected, the body can be effectively protected, by inhibiting the immune system's inflammatory response to bacterial infections, thus improving the body's energy metabolism and enhancing the function of the body's immune system [18,19], although they can also have side effects. Moreover, long-term use of these traditional drugs pose challenges such as high economic burden and drug resistance. Hence, there is an immediate need to investigate therapies that are effective, safe, and non-toxic to regulate immunological homeostasis.

In recent years, probiotics have been attracting a lot of attention as potential immunomodulatory agents and their mode of function has been increasingly studied. Regulating host immunity is one of the most common roles of probiotics [20]. In addition, previous research has demonstrated that some probiotics have strain-specific immunomodulatory effects on the host and immune cells by engaging Toll-like receptors (TLRs) that stimulate IFN pathways [21]. For instance, *Lactiplantibacillus plantarum* F1 was shown to enhance the immune response by activating NK cells and macrophages, and *Lactobacillus sakei* can enhance the phagocytosis of macrophages through the TLR2-mediated NF-κB signaling pathway, and increase the expression of nitric oxide (NO) and various cytokines [22–24]. *Lacticaseibacillus rhamnosus* IMC501 regulates immunity by inhibiting the invasion of pathogens; *Lacticaseibacillus rhamnosus* GG regulates the innate immune response [25–27]. Furthermore, *Lactobacillus* strains, including *Lacticaseibacillus rhamnosus* JLAU103, *Lacticaseibacillus rhamnosus* VL8, and *Lacticaseibacillus rhamnosus* WXD030, have been found to enhance the immunocompetence of RAW264.7 macrophages to varying extents [28–30], suggesting that LAB could serve as a potentially valuable source of natural immunomodulators. LAB play a crucial role in influencing the immunomodulatory activity [31]. Probiotics have the advantages of possible preventive and therapeutic effects, relatively low cost and availability, and safety with low side effects; however, the beneficial effects of probiotics vary depending on the type and number of strains. Therefore, it is still necessary to screen new potential probiotic strains.

This study aimed to screen probiotics with high immunomodulatory activity from 84 LAB strains by analyzing their growth and their influence on RAW264.7 macrophages. To the best of our knowledge, our study provides evidence of the ability of probiotics to modulate immune responses in macrophages and the theoretical foundation for the development of functional food.

2. Materials and Methods

2.1. LAB Strains and Cultivation Conditions

The LAB strains utilized in this study were provided by Ausnutria Dairy (China) Co., Ltd. Strains (Changsha, China) were inoculated in MRS liquid medium (HKM, Guangzhou, China).

2.2. Cell Culture

RAW264.7 macrophages were obtained from the Shanghai National Collection of Authenticated Cell Cultures of the Chinese Academy. RAW264.7 macrophages were cultured with DMEM medium (HyClone, LU, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (Gibco, NY, USA) and 1% mixture of penicillin and streptomycin (Gibco, NY, USA). All cells were cultured at 37 °C in an atmosphere containing 5% carbon dioxide. When the cells were about 80% confluent, after discarding the medium, the cells were digested with 0.25% trypsin (Gibco, NY, USA) solution for 3 min and centrifuged, cell culture medium was added to terminate the digestion, and the digested cells were collected into a centrifuge tube. The cells were centrifuged at 2000× g for 5 min, and the supernatant was discarded. Cell concentration was adjusted according to experimental requirements.

2.3. Growth Characteristics

Growth characteristics were explored according to a previous study [32]. Strains were maintained in a mixture of MRS broth (200 µL) and glycerol (200 µL) at −80 °C. A measure of 2% v/v bacterial suspension was inoculated into MRS medium and cultured for 3 generations. The strains' suspensions were cultured in MRS medium broth under anaerobic conditions at 37 °C and their optical density (OD) values were measured by an automatic growth curve instrument (BIO, Shanghai, China) at 600 nm wavelength each hour for 24 h.

2.4. Cell Viability Assay

Cell viability assay was performed as previously reported with some modifications [33]. RAW264.7 cells were inoculated into 96-well plates at a density of 5.0×10^4 cells/mL. The tested strains were adjusted to an OD_{600nm} value of 0.8, and incubated with adherent RAW264.7 cells for 12 h. After the supernatant of each well was removed and the cells were washed three times with warmed PBS, 100 µL complete medium was added to each well and 10 µL CCK-8 reagent (Beyotime, Beijing, China) was added to each well. After incubation for 2 h, three wells were taken from each group for measurements. The OD values were measured at 450 nm by a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). The blank control group represents cells without any treatment. The *Lacticaseibacillus rhamnosus* GG was used as a positive control. Cell viability was calculated using Equation (1) provided below:

$$\text{Cell Viability}(100\%) = \frac{\text{ODexperimental cell} - \text{ODblank}}{\text{ODcontrol} - \text{ODblank}} \times 100\% \quad (1)$$

ODexperimental cell represents the OD of cells treated with experimental strains, ODblank represents the OD of the cultured medium, and ODcontrol represents the OD of cells together with the cultured medium.

2.5. NO Determination

RAW264.7 cells were adjusted to a concentration of 2.5×10^5 cells/mL and uniformly introduced into 24-well plates, following the experiment performed as previously reported with slight modifications [34]. Cells were incubated with or without LPS (2.5 mg/mL) for 24 h. RAW264.7 cells were cultured with various strains (OD_{600nm} = 0.8) for 12 h. Afterward, the supernatant was collected, and the concentration of NO was determined using the NO kit (Promega, MW, USA), following the provided instructions. *Lacticaseibacillus rhamnosus* GG served as the positive control group.

2.6. TNF-α, IL-6, IL-1β and PGE2 Production Detection

RAW264.7 cells were treated according to the method described in Section 2.5. The effects of strains on the production of TNF-α, IL-6, IL-1β, and PGE2 were determined using ELISA kits (LunChangShuoBIotech, Xiamen, China), according to the operating instructions.

2.7. Phagocytosis Assay

The effects of LAB strains on the phagocytic activity of RAW264.7 cells were examined using a neutral red uptake assay [33]. Phagocytosis was evaluated utilizing the Phagocytosis Assay Kit (Beyotime, China). RAW264.7 cells were inoculated into 96-well plates at a concentration of 5×10^4 cells/mL. Then, RAW264.7 cells were cultured with various strains (OD_{600nm} = 0.8) for 12 h. Subsequently, the culture medium was removed and 100 μL neutral red solution was added and incubated for 2 h. The supernatant was discarded and the cells were washed with PBS thrice to remove the neutral red that was not phagocytized by RAW264.7 cells. Neutral red was extracted by RAW264.7 cells using lysis buffer (1% acetic acid:50% ethanol = 1:1). The absorbance was measured at 540 nm with a full-wavelength enzyme-linked immunoassay reader. Phagocytosis activity was calculated according to Equation (2)

$$\text{Phagocytic Activity } (100\%) = \frac{As}{Ac} \times 100\% \quad (2)$$

where *As* is the absorbance of the test sample at 540 nm, and *Ac* is the absorbance of the control at 540 nm.

2.8. Western Blotting

Samples of 2×10^4 cells were implanted into a 6-well plate, cultured overnight, stimulated with LPS (2.5 μg/mL) for 24 h, and then treated with bacterial cultures at an OD_{600nm} = 0.8 for 12 h. The cells were lysed on ice with RIPA cleavage buffer containing protease inhibitors. The protein concentration of the lysate was determined with the Bradford method. The same amount of protein was then loaded, dissolved on a 10% SDS-PAGE, and transferred to the polyvinylidene fluoride membrane (PVDF). The membrane was blocked by 5% skim milk buffer dissolved in Tris-HCl buffered salt solution + Tween wash buffer (TBST) for 1 h, incubated with the corresponding primary antibody (Abcam, Cambridge, UK) at 4 °C overnight, and then matched with the secondary antibody for 1 h. The antibody was stored at −20 °C and centrifuged at 12000 rpm for 5 min prior to the use of the antibody (all the antibodies attached to the tube wall or cap were centrifuged). Protein bands were shown using a chemiluminescence kit (Thermo Fisher Scientific, 32109).

2.9. Statistical Analysis

Version 21 of SPSS software (IBM Inc., Armonk, NY, USA) was used to conduct the statistical analysis. One-way analysis of variance (ANOVA) was used to determine whether differences between groups were statistically significant before Tukey's post hoc test was applied at $p < 0.05$. The average and standard deviation (SD) from three separate studies were used to represent all results.

3. Results

3.1. Growth Characteristics

As shown in Supplementary Figures S1–S5, the strain growth curves exhibit a typical 'S' shape, indicating the characteristic four growth stages: latency, logarithmic growth period, plateau, and aging phase. Based on the inferred growth curves, all strains were categorized into five groups according to their growth stage characteristics (Table 1). Additionally, the growth curves provided insights into the optimal culture duration for performing cell experiments with different groups of strains. Specifically, the first group of strains required a culture time of 8 h, the second group required 11 h, the third group required 12 h, the fourth group required 24 h, and the fifth group required 20 h.

Table 1. Growth characteristics of LAB strains.

Group	Strains	Logarithmic Stage	Culture Time for Cell Experiments
Group 1	AU04, AU05, AU06, AU07, AU21, AU78, AU80, AU22, AU26, AU28, AU33, AU51, AU58, AU53, AU68, AU77, AU38, LGG	2–8 h	8 h
Group 2	AU01, AU02, AUc2301, AU10, AU11, AU12, AU13, AU14, AUY2301, AU16, AU17, AU18, AU19, AU20, AU37, AU41, AU42, AU44, AU48, AU49, AU50, AU55, AU62, AU71, AU73	1–11 h	11 h
Group 3	AU08, AU09, AU27, AU30, AU40, AU47, AU54, AU67, AU74, AU75, AU24, AU72, AU81, AU82, AU83, AU84, AU35	6–12 h	12 h
Group 4	AU23, AU31, AU32, AU36, AU60, AU61, AU66, AU76, AU29, AU34, AU45, AU56, AU25	16–24 h	24 h
Group 5	AU39, AU43, AU46, AU52, AU53, AU57, AU59, AU63, AU64, AU65, AU79, AU69	8–20 h	20 h

Cell experimental culture time refers to the time when all strains are cultured to the stable stage before cell experiment.

3.2. Effect of LAB on Proliferation Activity of RAW264.7 Macrophages—Preliminary Screening

As shown in Table 2, different LAB had varying effects on RAW264.7 macrophages. In contrast, the viability of RAW264.7 macrophages was significantly enhanced by the following 34 strains: AUc2301, AU07, AUY2301, AU17, AU41, AU49, AU50, AU26, AU55, AU31, AU73, AU71, AU58, AU52, AU79, AU59, AU62, AU57, AU63, AU64, AU61, AU68, AU39, AU04, AU23, AU30, AU51, AU37, AU38, AU70, AU74, AU76, AU83, AU84, when compared to the control group ($p < 0.05$). Furthermore, these LAB strains showed no cytotoxic effects on RAW264.7 macrophages at the tested optical density of 0.8. The macrophage survival rate of *Lacticaseibacillus rhamnosus* GG group in the positive control group reached (128.96 ± 1.91%). The LAB that promoted macrophage proliferation and exhibited a survival rate exceeding the positive control group were selected as the secondary screening of LAB, including strains AUc2301, AU07, AUY2301, AU17, AU41, AU49, AU50, and AU64. The basic information for the tested strains is shown in Table 3. The viable cell count of the LAB during the secondary screening was approximately 1×10^8 CFU/mL at an optical density of OD_{600nm} = 0.8. The ratio of bacteria to cells was 100:1. Information regarding the viable cell count of the screened strains and the ratio to cell quantity can be found in Supplementary Table S1.

Table 2. Effects of different strains on macrophage activity of RAW264.7.

Strain	Cell Viability (%)	Strain	Cell Viability (%)	Strain	Cell Viability (%)	Strain	Cell Viability (%)
AU04	109.36 ± 6.33	AU12	91.07 ± 6.38	AU09	98.05 ± 2.15	AU34	87.36 ± 0.82
AU05	84.81 ± 11.31	AU13	99.50 ± 15.99	AU27	86.10 ± 2.81	AU36	93.06 ± 3.47
AU06	97.91 ± 14.58	AU14	88.20 ± 5.81	AU30	105.72 ± 1.09	AU56	88.57 ± 1.42
AU07	131.10 ± 10.79	AUY2301	132.76 ± 17.11	AU40	89.25 ± 1.13	AU60	97.31 ± 2.95
AU21	83.23 ± 10.89	AU16	97.05 ± 3.44	AU47	97.26 ± 0.24	AU61	108.82 ± 0.42
AU22	73.75 ± 2.28	AU17	146.58 ± 6.45	AU54	83.21 ± 1.47	AU66	74.97 ± 0.98
AU26	102.38 ± 1.73	AU18	90.21 ± 7.61	AU67	73.83 ± 2.57	AU76	108.84 ± 1.47
AU28	75.45 ± 1.26	AU19	90.35 ± 4.63	AU74	102.47 ± 2.64	AU45	84.60 ± 5.43
AU33	81.11 ± 0.38	AU20	95.32 ± 2.07	AU75	99.28 ± 0.44	AU39	120.34 ± 2.09
AU38	104.75 ± 1.64	AU37	104.75 ± 3.84	AU24	82.01 ± 0.95	AU43	89.05 ± 3.76
AU51	128.96 ± 1.91	AU41	152.99 ± 5.29	AU72	77.86 ± 1.63	AU46	73.47 ± 1.63
AU53	91.18 ± 2.12	AU42	98.92 ± 1.76	AU81	92.30 ± 1.51	AU52	115.29 ± 0.42

Table 2. *Cont.*

Strain	Cell Viability (%)	Strain	Cell Viability (%)	Strain	Cell Viability (%)	Strain	Cell Viability (%)
AU58	109.63 ± 1.63	AU44	89.90 ± 2.38	AU82	104.99 ± 1.43	AU57	117.17 ± 0.76
AU68	111.04 ± 1.96	AU48	103.60 ± 5.17	AU83	105.60 ± 0.68	AU59	127.54 ± 0.50
AU77	101.04 ± 1.96	AU49	133.60 ± 5.17	AU84	98.26 ± 1.55	AU63	119.33 ± 3.76
AU80	77.86 ± 1.85	AU50	142.19 ± 1.68	AU35	93.86 ± 1.33	AU64	145.32 ± 0.97
AU01	88.48 ± 8.84	AU55	111.59 ± 5.86	AU23	102.29 ± 2.19	AU65	71.66 ± 3.35
AU02	96.26 ± 9.93	AU62	96.51 ± 3.52	AU25	96.51 ± 2.87	AU79	113.47 ± 3.03
AUc2301	132.32 ± 8.61	AU71	111.31 ± 1.65	AU29	97.34 ± 2.85	AU69	79.54 ± 24.43
AU10	87.98 ± 5.95	AU73	128.06 ± 1.91	AU31	127.81 ± 0.99	AU78	77.86 ± 1.85
AU11	91.94 ± 6.38	AU08	92.08 ± 2.74	AU32	92.79 ± 0.25	LGG	128.96 ± 1.91
AU53	91.18 ± 1.53	con	100%				

Table 3. Basic information for tested strains.

Strain	Species	Source
AUc2301	*Limosilactobacillus reuteri*	Cat feces
AU07	*Pediococcus acidilactici*	Cat feces
AUY2301	*Lactiplantibacillus plantarum*	Southern milk source
AU17	*Lactobacillus brevis*	Southern milk source
AU41	*Lacticaseibacillus rhamnosus*	Southern milk source
AU49	*Pediococcus acidilactici*	Dog manure
AU50	*Pediococcus acidilactici*	Dog manure
AU64	*Lactiplantibacillus plantarum*	Breast milk

3.3. Effect of LAB on the Release of IL-6, IL-1β, TNF-α and NO in RAW264.7 Macrophages

As shown in Figure 1A–D, in contrast to the control group, the intervention of different LAB significantly elevated the levels of IL-6, IL-1β, and TNF-α. When the cells received intervention from AUY2301, the levels of inflammatory cytokines in the medium were 2.5 and 2 times higher compared to the control for TNF-α, and IL-6, respectively. For details, the concentration of IL-1β and NO for the AUc2301 treatment group was four-fold and five-fold higher than the control group, respectively ($p < 0.05$).

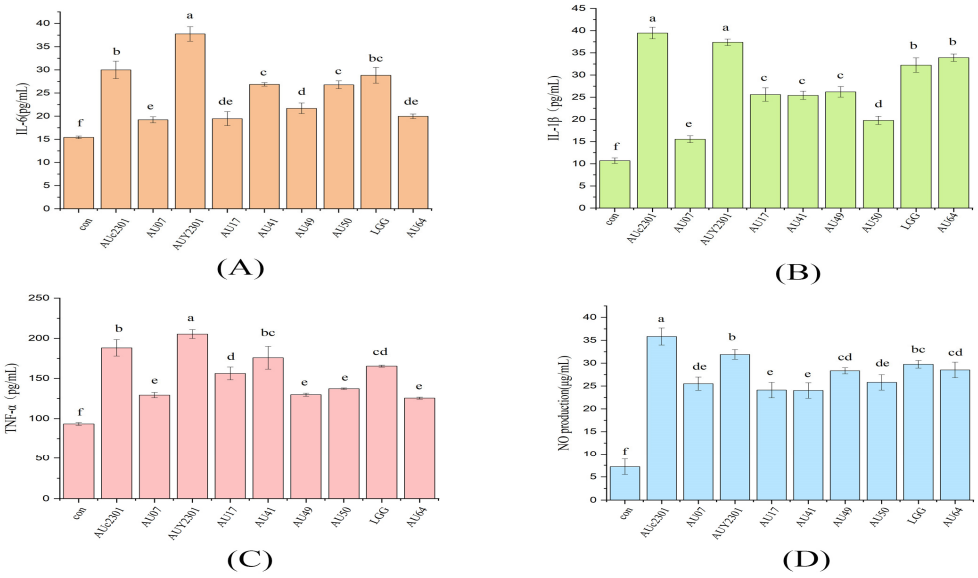

Figure 1. Effect of different strains on the release of IL-6 (**A**), IL-1β (**B**), TNF-α (**C**), and NO (**D**) of RAW264.7 macrophages. (Data are presented as mean ± SD of three independent experiments (n = 3)). Different letters on the bars indicate significant differences between means at $p < 0.05$ based on Tukey's post hoc test.

3.4. Effect of LAB on the Release of IL-6, IL-1β, TNF-α and NO in RAW264.7 Macrophages Stimulated by LPS

In modulating inflammatory and immunological responses, cytokines are crucial [34]. Cytokines such as TNF-α, IL-6, and IL-1β are also potent immunomodulators for stimulating macrophages [35]. As depicted in Figure 2A–D, the formation of IL-6, IL-1β, TNF-α, and NO was relatively low in the control group but was significantly increased by the LPS group ($p < 0.05$). The LPS-induced IL-6, IL-1β, TNF-α, NO production of RAW264.7 cells pretreated with the different LAB was significantly lower than that in the LPS group. Furthermore, the levels of IL-1β and TNF-α were lower in AU64 pretreated RAW264.7 cells than those in other LAB pretreated LPS-stimulated RAW264.7 cells. In comparison to other LAB-pretreated LPS-stimulated RAW264.7 cells, AUc2301-pretreated RAW264.7 cells showed lower NO levels. AU41-prepped RAW264.7 cells had lower levels of IL-6 than other pretreated LAB and pretreated LPS groups ($p < 0.05$).

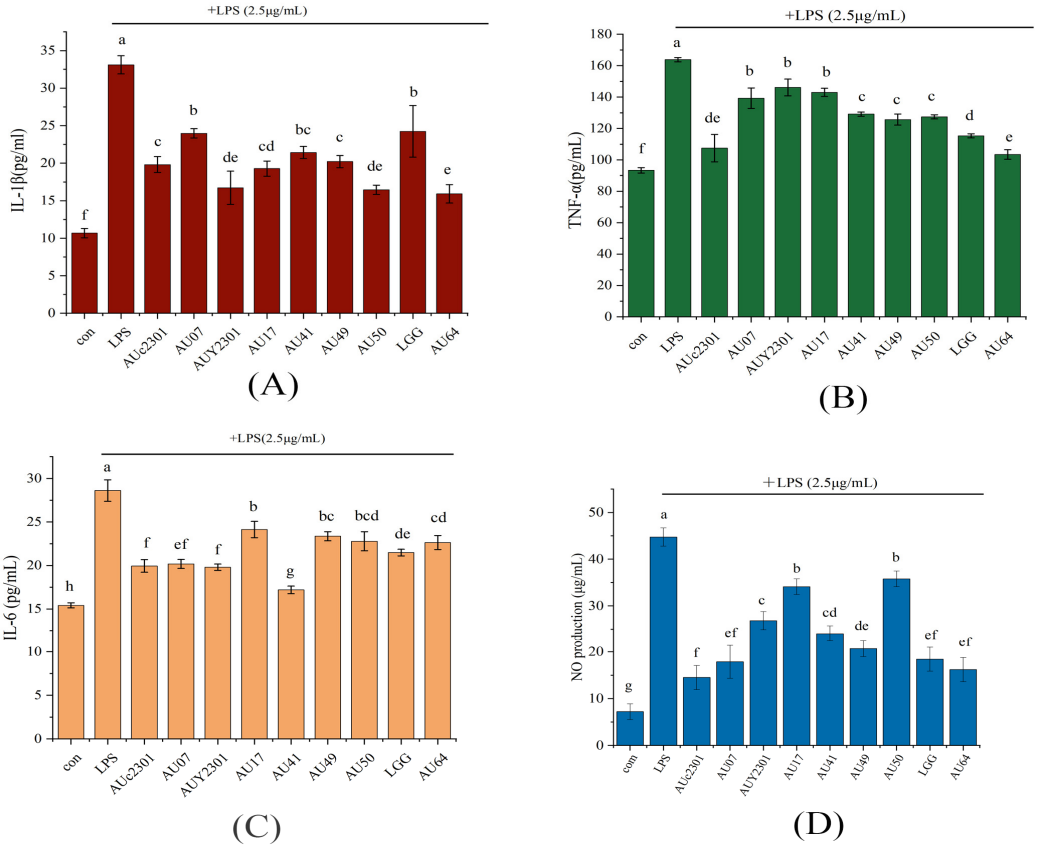

Figure 2. Effect of different strains on the release of IL-1β (**A**), TNF-α (**B**), IL-6 (**C**), and NO (**D**) of RAW264.7 macrophages activated by LPS. (Data are presented as mean ± SD of three independent experiments (n = 3)). Different letters on the bars indicate significant differences between means at $p < 0.05$ based on Tukey's post hoc test.

3.5. Effect of LAB on Phagocytosis Activity of RAW264.7 Macrophages

A neutral red uptake assay was used to assess how the chosen strains affected macrophage phagocytosis. As shown in Figure 3, compared to the control group, all the tested LAB, except for AU07, enhanced the phagocytosis activity of RAW264.7 macrophages ($p < 0.05$). The phagocytic activity of the AUc2301 group achieved 122.45 ± 13.57%.

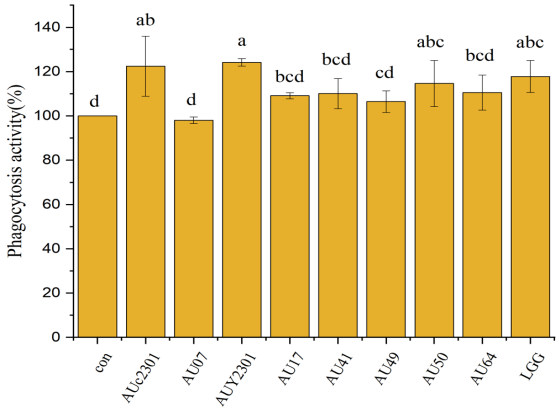

Figure 3. Effect of the selected strains on the phagocytosis of RAW264.7 macrophages. (Data are presented as mean ± SD of three independent experiments (n = 3)). Different letters on the column indicate significant differences between means at $p < 0.05$ based on Tukey's post hoc test.

3.6. Effects of LAB on Release of PGE2 and Expression of COX-2 of RAW264.7 Macrophages by LPS

In this study, PGE2 production in RAW264.7 macrophages activated by LPS was detected by ELISA. As shown in Figure 4C, the level of PGE2 for the LPS group was higher than that of the control group ($p < 0.05$). Compared with the LPS group, different LAB could significantly reduce PGE2 content in RAW264.7 macrophages induced by LPS ($p < 0.05$). The inhibition of the production of PGE2 varied between different LAB, within a range of 102.12–211.80 ρg/mL. Among them, strain AUc2301 and strain AUY2301 showed a higher inhibitory effect than other LAB, and the level of PGE2 was reduced to 168.84 ± 6.41 ρg/mL and 130.59 ± 3.64 ρg/mL, respectively. To investigate whether the effects of strain AUc2301 involved the regulation of COX-2 proteins associated with PGE2 production, we examined their expression using Western blot analysis. As shown in Figure 4A,B, the expression level of COX-2 protein after LPS stimulation was significantly higher than that in the control group ($p < 0.05$). After pretreatment with AUc2301, the expression of COX-2 protein decreased gradually ($p < 0.05$). These findings suggest that AUc2301 inhibits PGE2 production by downregulating the expression of COX-2 protein.

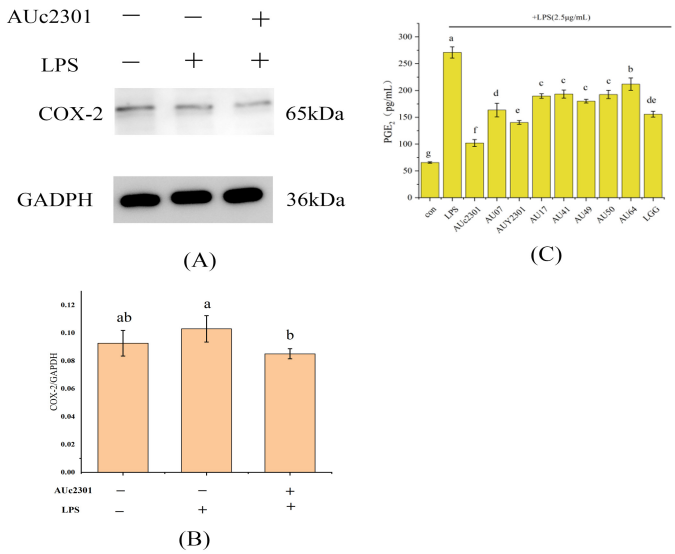

Figure 4. Effect of AUc2301 on expression of COX-2 of RAW264.7 macrophages activated by LPS (**A**,**B**). Effect of screened strains on PGE2 release from RAW264.7 macrophages (**C**). (Data are presented as mean ± SD of three independent experiments (n = 3)). Different letters on the bars indicate significant differences between means at $p < 0.05$ based on Tukey's post hoc test.

3.7. Effect of LAB on NF-κB Activation of RAW264.7 Macrophages Stimulated by LPS

To investigate whether the NF-κB pathway mediated the cytokine production in AUc2301-treated LPS-activated RAW264.7 macrophages, in Figure 5A,C, the expression of phosphorylated p65 was significantly downregulated by AUc2301, which reduced the proportion of p-p65/p65 ($p < 0.05$). The results suggested that AUc2301 could promote the expression of NF-κB from cytoplasm to nucleus. It demonstrated that AUc2301 could activate the NF-κB pathway and promote p65 nuclear transfer. Moreover, as shown in Figure 5B–D, AUc2301 also inhibited the LPS-induced degradation of IκB in RAW264.7 macrophages ($p < 0.05$). This clearly indicated that AUc2301 played an immunomodulatory role by activating the pathway of NF-κB.

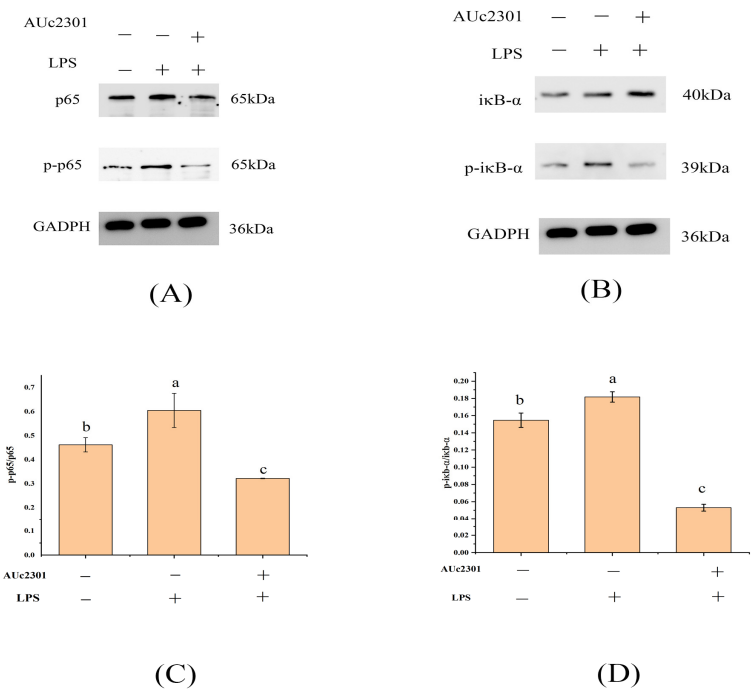

Figure 5. Effect of AUc2301 on the NF-κB activation in RAW264.7 cells activated by LPS. (Data are presented as mean ± SD of three independent experiments (n = 3)). Different letters on the bars indicate significant differences between means at $p < 0.05$ based on Tukey's post hoc test). (**A**–**C**) Effect of AUc2301 on the expression of p65 non-phosphorylated protein in RAW264.7 cells activated by LPS. (**B**–**D**) Effect of AUc2301 on the expression of IκB non-phosphorylated protein in RAW264.7 cells activated by LPS.

4. Discussions

The human innate immune system serves as the initial barrier against pathogens, providing the first line of defense. Beneficial bacteria known as probiotics have a significant impact on the development and maturation of the human immune system within the intestinal tract [24,36]. Probiotics can induce an immune response in the host by binding to immune cells [37]. It is of great significance to investigate the complex interactions between *Lactobacillus* and the host [38]. The effects of probiotics on immune responses have been demonstrated by in vitro and in vivo experiments [39,40]. However, their effects on immunity are still very limited [41,42]. Therefore, the present study was carried out to screen for more immunologically active strains of 84 LAB isolated from different sources.

Because of their lengthy durations and high costs, animal studies are not appropriate for the extensive screening of probiotics. Instead, in vitro macrophage RAW264.7 cells were employed to assess the immunologically active effects of probiotics. Macrophages are one of the most important innate immune cells in the organism and play a dominant role in directing immune processes such as host inflammatory responses [43]. Macrophage activa-

tion is considered a major and necessary step in the stimulation of the immune system [44]. Therefore, the proliferation activity could also be used for preliminary screening of probiotics with better immunity effects. In this study, the selected *Lactobacillus* strains had no toxic effect on RAW264.7 cells and promoted cell proliferation. Therefore, the optical density of 0.8 used for the experimental strains in the investigation that followed was based on these results. The same findings were reported by a study that found the *Lactiplantibacillus plantarum* ML2018 [45] promoted the growth of RAW264.7 macrophages at experimental concentrations of 0.07 to 1.2 μg/mL. In addition, *Lactiplantibacillus plantarum* JLK0142 had no toxic effect on RAW264.7 cells and enhanced RAW264.7 macrophage proliferation [46].

A common index for assessing the activity of macrophage function regulators is phagocytic activity [45]. Interestingly, in this study, the AUc2301 increased the phagocytic activity much more than the other strains of the tested LAB, indicating that the AUc2301 had a better immunomodulatory effect (Figure 3), similar to the results of experiments reported in the literature in which the probiotic *Escherichia coli Nissle* 1917 increased the phagocytosis of RAW264.7 macrophages [47]. Enhanced macrophage phagocytic activity was also reported in *Bifidobacterium* RH from animals [48]. Previous research demonstrated that probiotics increased phagocytic activity by recognizing several pattern-recognition receptors on the surface of macrophages, including the Fc receptor (FcR), Toll-like receptors (TLR4), and complement receptor type 3 (CR3), and regulated the expression of these receptors afterward [47]. Consequently, in the current study, we hypothesized that the secretion of the strain may enhance the phagocytosis of RAW264.7 macrophages by recognizing one or more receptors, but further studies are required before a conclusion can be drawn.

Cytokines, which are small proteins secreted by cells, play a vital role in mediating and regulating immunity, inflammation and hematopoiesis. Activated macrophages release key mediators involved in the host defense response, such as IL-6, IL-1β, TNF-α which stimulate the immune system against pathogenic infections [34]. The immunomodulatory effects of probiotics have been extensively studied. Research has revealed that LAB can induce the production of proinflammatory cytokines such as TNF-α, IL-6, and IL-1β from a variety of cell types [48–50]. In the current research, the release of IL-6, IL-1β, TNF-α were enhanced by AUc2301, which may have been due to the presence of bacterial-secreted soluble substance receptors on the surface of RAW264.7 macrophages. These receptors could interact with the secretions of LAB, activating intracellular signaling pathways such as NF-κB, adenosine monophosphate-activated protein kinase (AMPK), and phosphatidylinositide 3-kinases (PI3K-Aκt). Consequently, the mRNA expression of IL-6, IL-1β, and TNF-α was upregulated, leading to the release of these cytokines (Figure 6). Our findings align with previous studies where *Lactiplantibacillus plantarum* KFCC11389P was reported to upregulate the release of IL-6 and IL-1β from RAW264.7 macrophages after 24 h of co-culture [51]. In addition, NO is a non-specific effector gas molecule produced by iNOS that is vital in controlling the death of tumor cells and preventing the proliferation of many dangerous bacteria [52].

Macrophages can exhibit overactivation in response to pathogenic substances, such as LPS, leading to the excessive production of several proinflammatory cytokines, including IL-6, TNF-α, and IL-1β [19]. This cascade of responses can result in inflammatory diseases and pathological conditions through both cellular and humoral effector systems. Consequently, targeting this process could be a valuable approach for developing anti-inflammatory agents by preventing the excessive release of cytokines that trigger inflammation [51]. AUc2301 significantly inhibited the release of IL-6, IL-1β, TNF-α, in stimulated-LPS RAW264.7 cells. Consistent with these results, a study demonstrated that EPS derived from *Trichoderma* spp. strain B2 significantly diminished the release of proinflammatory mediators (such as TNF-α and IL-6) from RAW264.7 macrophages activated by LPS [53]. Production of TNF-α and IL-6 in cells was previously shown to be significantly increased from different probiotic LAB [54].

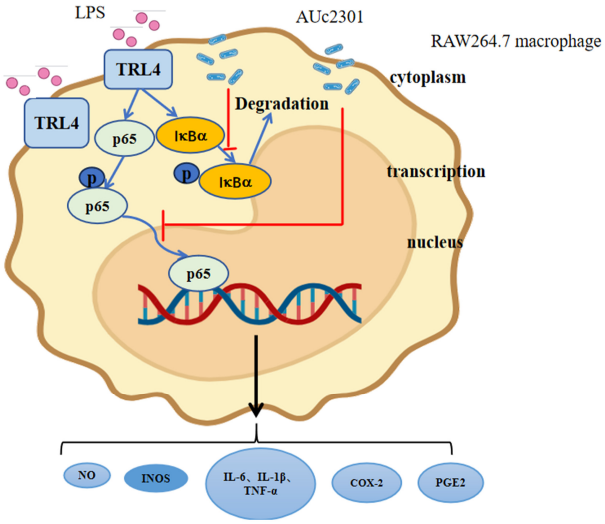

Figure 6. Effect of AUc2301 on the NF-κB activation in RAW264.7 cells stimulated by LPS.

The overproduction of PGE2 leads to inflammatory and pathological states. A crucial enzyme, COX-2, transforms arachidonic acid into prostaglandin H_2 to produce PGE2 [53]. LPS is known to increase COX-2 expression in macrophages, which subsequently significantly increases PGE2 production [51]. Thus, reducing COX-2 synthesis can significantly inhibit cytokine production in LPS-activated RAW264.7 macrophages. In the present study, AUc2301 could significantly reduce PGE2 release and the expression of COX-2 protein in LPS-induced RAW264.7 macrophages ($p < 0.05$). The results showed that AUc2301 exhibited immunomodulatory effects not only by suppressing the activity of proinflammatory cytokines (IL-6, IL-1β, and TNF-α), but also by inhibiting the secretion of PGE2 through the downregulation of COX-2 expression levels in LPS-activated RAW264.7 macrophages. According to previous studies, the *Lactiplantibacillus casei* 3260 significantly reduced COX-2 protein expression in LPS-stimulated macrophages [55].

In response to specific stimuli, such as LPS, NF-κB undergoes phosphorylation and translocation into the nucleus to regulate the expression of inflammatory cytokines, including iNOS, NO, COX-2, IL-6, and TNF-α, thus activating the NF-κB signaling pathway. Activation of this pathway involves the phosphorylation and subsequent degradation of IκB, which is a crucial step for NF-κB activation in LPS-activated RAW264.7 cells. In the cytoplasm, the p65/p50 heterodimer, the most common NF-κB dimer, remains bound to an inhibitor of NF-κB (IκBα) and remains inactive. However, LPS stimulation leads to the phosphorylation of both p65 and IκBα, which enhances NF-κB activation during inflammatory processes. NF-κB, located downstream of the TLR4-mediated signaling pathway, is a significant transcription factor that plays an essential role in regulating the immune response [56]. Due to their high molecular mass, the soluble compounds produced by LAB are unable to penetrate cells. Consequently, the initial step in immunomodulation involves binding to TLR4 receptors. This binding activates downstream signaling pathways, such as NF-κB, leading to the secretion and expression of cytokines, including NO, TNF-α, IL-6, IL-10, and IL-1β. These cytokines play a crucial role in regulating the immune system and contribute to the immunomodulatory activity exerted by LAB [57]. The NF-κB signaling pathway acts upstream of cytokine production, including TNF-α and IL-6, and plays a crucial role in regulating the secretion of cytokines. The activation of NF-κB is pivotal in controlling the expression of proinflammatory cytokines. A large number of studies have demonstrated that various pathways can induce NF-κB activation in macrophages, leading to phosphorylation of NF-κB proteins. This phosphorylation triggers their translocation from the cytoplasm to the nucleus, initiating gene transcription [57]. In Figure 6, AUc2301 attenuated the LPS-induced expression of p-p65 protein in RAW264.7 cells, promoted nuclear translocation of p65 and inhibited the degradation of IκB-α. These results indicated

that AUc2301 could restrain the production of proinflammatory cytokines, NO, and COX-2 by activating NF-κB signaling pathway (Figure 6).

5. Conclusions

Taken together, *Limosilactobacillus reuteri* AUc2301 was selected from 84 strains of LAB with strong immune activity in our results. It can enhance the proliferative activity and phagocytic activity of RAW264.7 macrophages, and promote the secretion of IL-6, IL-1β, TNF-α and NO from RAW264.7 macrophages. In addition, pretreatment with the *Limosilactobacillus reuteri* AUc2301 could inhibit the excessive release of IL-6, IL-1b, TNF-a, PGE2, and NO in LPS-stimulated RAW264.7cells. The results above demonstrated that *Limosilactobacillus reuteri* AUc2301 has dual immunostimulatory activities in vitro. Moreover, it was found that the *Limosilactobacillus reuteri* AUc2301 could downregulate the expression of COX-2 protein and mediate the NF-κB signaling pathway in cells. *Limosilactobacillus reuteri* AUc2301 could have the potential to be used for designing functional foods or clinical immunomodulators, but their beneficial effects and action mechanism need to be further confirmed in vivo.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pr12050903/s1, Figure S1: Growth curves of strains in group 1; Figure S2: Growth curves of strains in group 2; Figure S3: Growth curves of strains in group 3; Figure S4: Growth curves of strains in group 4; Figure S5: Growth curves of strains in group 5; Table S1: The Counts of Viable Bacteria of the Tested Strains.

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Conflicts of Interest: Authors Lina Pan, Jiaqi Wang, Rongxue Tang and Wenli Kang were employed by the company Ausnutria Dairy (China) Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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