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Improvement of the Gut Environment of Sandwich-Coated Lactic Acid Bacteria with Aloe Polysaccharides

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ABSTRACT: Recent gut microbiome research has emphasized the important role that lactic acid bacteria (LAB) play in promoting digestive and overall health. This study aims to solve the problem of lactic acid bacteria reaching the gut and improve their efficacy by using a sandwich coating method containing aloe polysaccharides. The robust encapsulation method developed is designed to protect the LAB from degradation from the acidic and basic environment during digestion, ensuring their stability and efficacy. The sandwich coating utilizes LbL technology and consists of three layers: protein, cellulose, and aloe polysaccharide. The results of the study showed that the sandwich coating significantly improved the survival rate of lactic acid bacteria under conditions such as pH 2.0, pH 2.5, and bile salt concentrations of 0.3% and 2.0%. The coated lactic acid bacteria also inhibited the growth of harmful bacteria such as E. coli, Staphylococcus aureus, and Salmonella, while effectively stimulating the production of immune markers such as nitric oxide(NO), IL-1β, TNF-α, and NF-κB. These findings suggest that aloe polysaccharide-containing sandwich coating LAB can improve gut health and immune function by ensuring the stability and activity of lactic acid bacteria during digestion.

KEYWORDS: Lactic acid bacteria, Aloe Polysaccharides, Sandwich Coating, Encapsulation, Immune Function

I. INTRODUCTION

Lactic Acid Bacteria (LAB) play an important role in the gut by breaking down carbohydrates such as glucose and lactose to produce short-chain fatty acids, which inhibit the growth of harmful bacteria and the production of toxins in the body and support the growth of beneficial bacteria. [1-3] So far, studies have reported that LAB have a variety of health functions, such as promoting serotonin and dopamine production, relieving depression [4-6], enhancing gut-brain coordination [7,8], regulating TNF-α and IgA [9-11], activating apoptosis and autophagy, promoting colon cancer cell death [12,13], and regulating TH1/TH2 cytokine balance through gut immune regulation. [14,15] In addition, probiotics, defined as 'live microorganisms that can benefit the host when consumed in appropriate amounts,' are known to exert bioactive effects once they enter the body and settle in the intestines, helping to normalize the beneficial bacteria. [16] This has led to their growing importance as a health food for a variety of conditions related to gut health and immunity.

In order for lactic acid bacteria to work in the human body, they must possess three characteristics: acid and bile resistance, intestinal reachability, and intestinal fixability. This means that lactic acid bacteria must survive digestive enzymes such as stomach acid (pH 1.8-2.0) during the digestive process, reach the intestine as live bacteria without passing out in the feces, and colonize and live in the intestine. [17]

Therefore, based on these characteristics, methods for coating lactic acid bacteria have been developed, and the existing coating technology is encapsulation. Encapsulation is 'wrapping a useful substance in a particulate state with a wall material' and is divided into macro, micro, and nano capsules according to size. [18] Encapsulation methods can be divided into extrusion and emulsion. In the case of extrusion, alginate is mainly used as a coating material, and in the case of emulsion, carrageenan, cellulose acetate

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phthalate, chitosan, gelatin, etc. are generally safe ingredients that can be applied to food. However, they are less stable in the presence of chelating substances, phosphates, lactate, and citrates after encapsulation, so researchers have been working to overcome this limitation by multi-coating processes, cross-linking with cationic polymers, or starch blending. However, these additional treatments complicate the process and are difficult to commercialize due to factors such as long manufacturing time, low productivity, and limitations of large-scale production. [19-21]

Meanwhile, Aloe is a perennial succulent herbaceous plant in the lily family, and its transparent fleshy part is composed of about 99% water and 1% polysaccharides. There are only three or four species available for food or medicinal use, including Aloe vera L. and Aloe arborescens M. Among them, Aloe vera has been medically proven to have anti-inflammatory and antitumor effects on human and animal intestinal health and immune activity. [22,23] In addition, it is known that the gel component of Aloe vera contains polymeric heteropolysaccharides, which are beneficial for maintaining internal structural and functional properties.

In this study, we aimed to prepare lactic acid bacteria with increased coating stability, freezing stability, storage stability, and environmental stability to artificial gastric juice and artificial interest juice by sandwich coating with first protein, second cellulose, and third aloe polysaccharide to check the effect of improving the intestinal environment

II. EXPERIMENTATION

Preparation of Aloe Polysaccharide

The raw leaves of Aloe vera, which are 3 to 5 years old, were collected from KimJeongMoon Aloe Jeju Farm, washed three times with purified water in a KGMP facility and peeled to obtain a viscous polysaccharide by U-tech's patented process. (Item notification and manufacturing number: 1992063113512).

Lactobacillus cultivation and Strain preparation

Lactobacillus planatrum (KCCM11322, ATCC8014) was inoculated into MRS medium (Difco, USA) and cultured for 24 hours at 30~37℃ by fed-batch culture method. The lactic acid bacteria count was 2.58×10^{9}/mL on average, and the bacteria were then recovered from the lactic acid bacteria culture by centrifugation at 6500 rpm for 2 min using a high-speed centrifuge at 3-5℃.

Custom coating and cryoprotectant formulation for stable sandwich-coated lactic acid bacteria

Protein coating agent was prepared by mixing protein (isolated soy protein 100%, Harbin Hi-Tech Sobean Food Ltd, China. Cellulose(hydroxyethylcellulose 100%, Hebei Crovell Biotech Ltd) coating agent, and aloe polysaccharide(Aleo vera gel 100%, Kim Jung Moon Aloe Co., Korea) coating agent were prepared in their respective mixing ratios and coated on lactic acid bacteria, and then palm oil (100% refined palm oil, Sun Bio Naturals Private Ltd, India) was mixed as a cryoprotectant to prepare samples 0 through 5, respectively. The mixing ratios for Samples 0 through 5 are shown in Table 1. At this time, the protein, cellulose, aloe polysaccharide, and palm oil were filled with lactic acid bacteria culture to make a total of 100%. Then, the sandwich-coated LAB were frozen in a -80°C ultra-low freezer for 12 h and then lyophilized using a freeze-dryer (Ilshin Bio, FD-8508, Korea) for 120 h at a condensing temperature of -80°C and a pressure of 5 mm Torr or less, ground, and stored in a refrigerator at 4°C for use. The sandwich-coated LAB powder samples were diluted 10-fold with sterile water and applied to the medium (Difco, USA), incubated at 37°C for 48 hours, and the colonies were counted and expressed as CFU/g. [24]

Manufacturing of Sandwich-Coated Lactic acid bacteria

Table 1. Mix ratio of sample0~5 depending on the coating and cryoprotectant

Sample	Protein(%)	Cellulose(%)	Aloe Polysaccharides(%)	Palm Oil(%)
Sample0	0	0	0	0
Sample1	5	15	5	5
Sample2	5	10	5	10
Sample3	10	10	5	5
Sample4	10	5	5	10
Sample5	15	4	4	4

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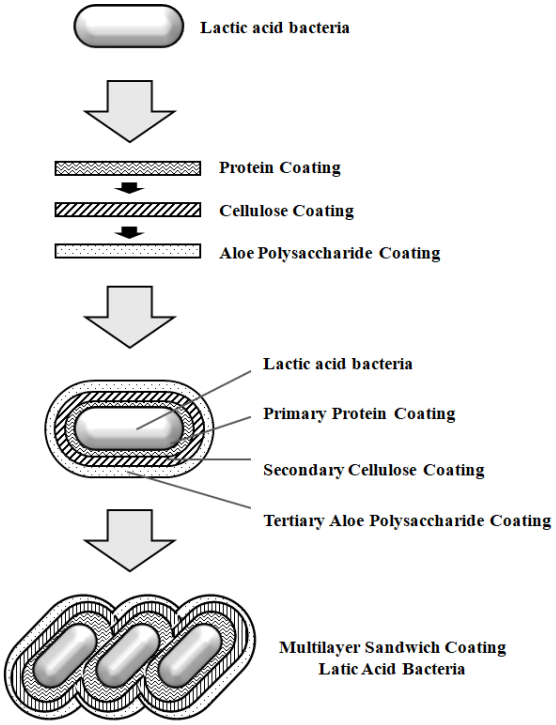

Fig. 1. Manufacturing Process of the Aloe Polysaccharide-containing Sandwich-Coated Lactic Acid Bacteria

The mixture was homogenized by stirring for more than 30 minutes according to the mixing amount of each sample 0 to 5, and then the protein coating agent and LAB were first mixed and centrifuged to recover the first coated lactic acid bacteria. Then, as shown in Figure 1 the cellulose coating and aloe polysaccharide coating were sequentially stacked using the self-assembly method to complete the sandwich coating.

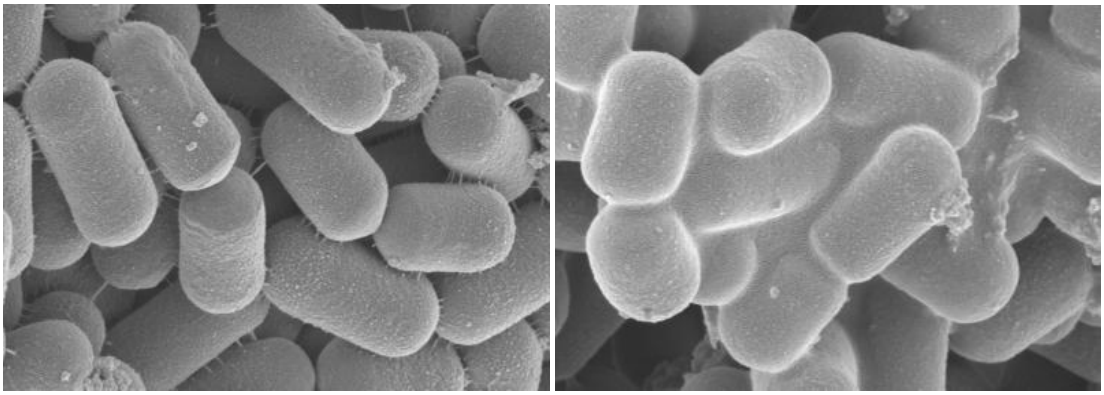

Fig. 2. Photographed by Electron Microscopy (A) Lactic Acid bacteria Powder before Coating; (B) Sandwich-Coated Lactic Acid Bacteria in three layer

Storage reliability

100 g of sandwich-coated LAB powder (named sample 6) prepared under the conditions of the most stable sample in the freeze-drying stability experiment in 2.3 was stored in a 4℃, 25℃, 37℃ thermostat for 6 months, and then the total bacterial count was determined.

Digestive enzyme stability

Preparing artificial gastric juice with 1000 U/mL pepsin and 1 N-HCl, pH adjusted to 2.0 and 2.5, and artificial bile juice with 1000 U/mL lipase, 0.3% bile salt, and 2.0% bile salt, pH adjusted to 8.0 with 1 N-NaOH. 1 g of sandwich-coated LAB powder of the most stable sample was added to

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each of the artificial gastric juice and artificial bile juice, and the total bacterial count was determined at 37°C at 0.5, 1, and 2 hours.

Intestinal adhesion and stability

In vitro experiments were performed using intestinal endothelial cells (Caco-2 cells, Korea Biotechnology Institute, Korea). The cells were cultured at 37°C using 20% inactivated fetal calf serum and 0.2% penicillin/streptomycin. The cultured cells were treated with trypsin for 10 minutes at 37°C, then counted with a hemocytometer and incubated until the cell concentration reached 1.0×10^5 cells/mL to form a complete monolayer. Caco-2 cells were then inoculated with 0.5 mL of DMEM and the most stable sample (0.5 mg; 2.58×10^9 CFU/mL in PBS) and incubated at 37°C for 3 hours. After incubation, the cells were washed five times with PBS and fixed with methanol for 5 min. The cells were subjected to Gram stain and observed under a light microscope (×1,000), and the number of LAB adhering to 50 Caco-2 cells was counted in 10 fields of view, and the mean value of the number of LAB adhering per 5 Caco-2 cells was calculated.

In in vivo experiments, the small intestines of 8-week-old albino rats were harvested after 1 week of oral administration, and the tissues were fixed in 4% glutaraldehyde, 1% paraformaldehyde for 2 hours, and washed with PBS. [25] After tissue fixation, the tissues were dehydrated in 50%, 60%, 70%, 80%, 90%, and 100% alcohol for 30 min each, and dehydrated in alcohol:isoamyl acetate at 2:1, 1:1, 1: 2, 100% isoamyl acetate for 30 min each, then dried in a critical point dryer (CPD, HCP-2, Hitachi, japan) at 45℃ for 20 min and 20℃ for 5 min, coated with ion supper (H-1010, Hitachi, japan) for 30 s, and observed using an analytical high resolution scanning electron microscope (Supra55VP, Carl Zeiss, germany).

Inhibit harmful gut bacteria

The sandwich-coated LAB were sprinkled on 2 μL of the medium and incubated anaerobically at 30°C for 24 hours to form spots. Then, the above pathogens mixed with 7 mL of brain heart infusion agar and 0.7% of the above pathogens were added to the medium in which the sandwich-coated lactic acid bacteria were cultured and incubated anaerobically at 30℃ for 24 hours, and the inhibition degree was evaluated by measuring the diameter of the inhibition ring in mm.

Gut immune function activity

The most stable sandwich-coated LAB were cultured using MRS medium (Difco, USA) (named as Example 7), and macrophage RAW 264. 7 Cells (Korea Cell Line Bank, Korea) were cultured at 37°C, 5% CO2 using Dulbecco's modified Eagle's medium (DMEM; PAA, Austria) medium supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories) and 100 units/mL of streptomycin and penicillin (Gibco Laboratories). The inflammatory response model was treated with 1 μg/mL lipo polysaccharide (LPS; Sigma, USA) per well, while the control was treated with PBS (pH7.2).

For nitric oxide (NO) measurement, RAW 264.7 cells were inoculated at 5.0×10^5 cells/mL, and PBS and LPS cultures were harvested, and NO was measured with the Griess reagent system (Promega, USA), and NO concentrations were calculated using a sodium nitrite standard curve (0-100 μM).

For the measurement of IL-1β and TNF-α activity, RAW 264.7 cells were inoculated at 5.0×10^5 cells/mL and treated with LAB and LPS, cultures were harvested 48 hours later and measured in triplicate using IL-1β, TNF-α ELISA kit (eBioscience, San Diego,USA).

For the measurement of NF-κB activity, RAW BLUE cells were cultured using Dulbecco's modified Eagle's medium (DMEM; PAA, Austria) supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories, USA) and 100 units/mL of streptomycin and penicillin (Gibco Laboratories, USA), while LPS were treated with 100 ng/mL of lipopolysaccharide (LPS; Sigma, USA) per well.

III. RESULTS AND DISCUSSION

Observing Sandwich-coated Lactic Acid Bacteria through an electron microscope

The protein, cellulose, and aloe polysaccharide coatings were coated by self-assembly method, and the surface of the LAB was coated in a layered structure to form a multilayer thin film on the lactic acid bacteria. Fig. 2.(A) is an electron microscopy image of LAB before coating, and (B) is an electron microscopy image of aloe polysaccharide-containing sandwich-coated LAB coated in three steps. Comparing the two figures, in A, the LAB are individually separated, while in B, the coated LAB are densely packed together. This is due to the formation of intercellular bonds due to the tertiary coating. These bonds can protect the LAB from the external environment and contribute to their survival rate.

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Evaluate lyophilization stability between coating and cryoprotectant ratios

Commercially, LAB are available in powdered form. Therefore, the survival rate of lactic acid bacteria was measured after freeze-drying each sample to determine the ratio of the most stable protein, cellulose, aloe polysaccharide coating and palm oil as a cryoprotectant. As shown in Figure 3, the survival rate of sample 3 is the highest. Therefore, it was concluded that the sandwich coating of 10% protein, 10% cellulose, 10% aloe polysaccharide, and 5% palm oil could maximize the freezing stability of lactic acid bacteria. This is possible because each material acts complementary to the other, minimizing physical damage to the lactic acid bacteria, maintaining their physicochemical internal structure and functional properties, and as a protective agent with a high degree of polymerization.

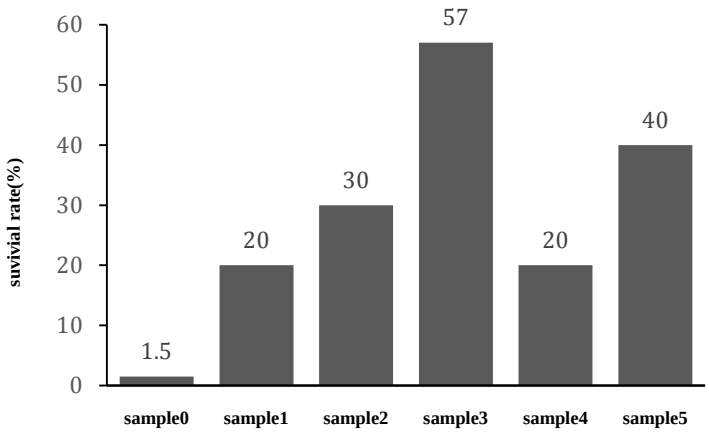

Fig. 3. Lactic Acid Bacteria Survival Rate of Coated LAB Samples

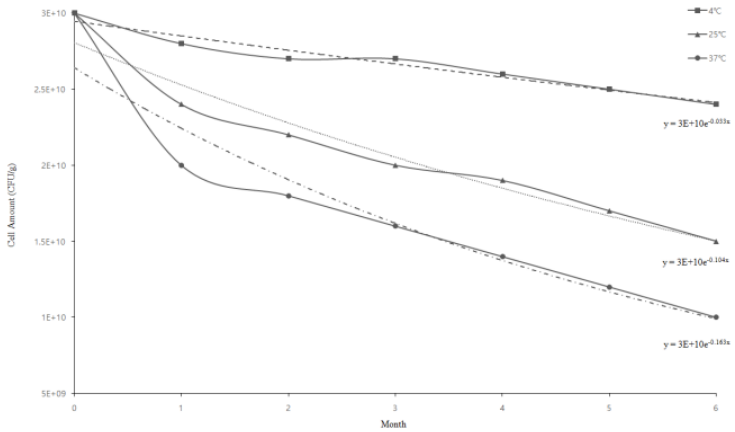

Fig.4. Change in lactic acid bacteria count over months at 4, 25, and 37°C plotted as an exponential decay graph

Evaluate lactobacillus survival under different temperature conditions

In order for LAB to be marketed, it must be well preserved without dying when left at room temperature. Therefore, the storage stability was evaluated by checking the change in total LAB count over time under different temperature conditions. As shown in Figure4, the sandwich-coated LAB powder showed more than 80% survival rate for 6 months at 4℃. When regression analysis was applied with an exponential decay curve [26], survival rates of over 67% for 12 months, over 55% for 18 months, and over 45% for 24 months were obtained. The survival rate was 50% after 6 months at room temperature (25℃) and 33% at body temperature (37℃). Therefore, the

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sandwich-coated lactic acid bacteria powder of sample 6 showed excellent long-term preservation at low temperature (4℃). However, since the survival rate was measured to be lower at room temperature and body temperature than at low temperature, it was determined that if the sandwich-coated lactic acid bacteria is sold in the market, it should be stated that it should be refrigerated or stored at low temperature.

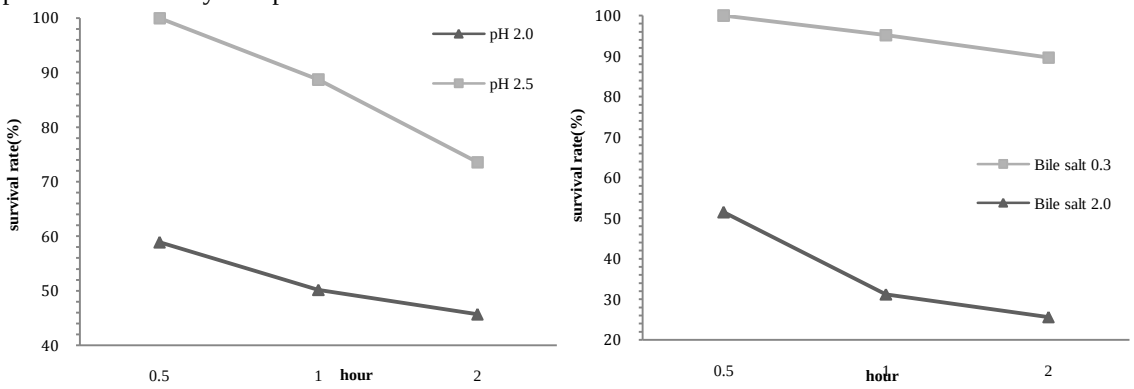

Fig.5. Lactic acid bacteria viability at 0.5, 1, and 2 hours (A) in pH2.0, 2.5 (B) in bile salt 0.3%, 2.0%

Evaluate digestive enzyme stability

In order for the sandwich-coated LAB powder to be beneficial to the human body, it must be able to withstand stomach acid and digestive enzymes and reach the small/ large intestine reliably. To investigate this, we prepared artificial gastric juice and artificial ear fluid, and the survival rate of LAB over time for samples with pH 2.0 and 2.5 for bile salt 0.3% and 2.0% in figure 5. A and B. As a result, the sandwich-coated LAB showed 45.7% survival rate after 2 hours of treatment at pH 2.0, maintained more than 100% survival rate after 0.5 hours of treatment at pH 2.5, and secured 73.6% survival rate after 2 hours of treatment. Therefore, the sandwich-coated LAB powder has a very good effect on acid resistance and gastric juice resistance. At 0.3% bile salt, the viability of LAB remained above 100% at 0.5 hours of treatment, and 89.7% at 2 hours of treatment. However, at 2.0% bile salt, the viability of LAB at 0.5 hours of treatment was 51.5%, indicating that the stability of LAB at 2.0% bile salt was somewhat lower. At 0.3% bile salt, the survival rate can be secured, so the bile tolerance and artificial salt tolerance of the sandwich-coated LAB powder are excellent in the range of 0.3% bile salt. Therefore, it means that the sandwich-coated LAB containing aloe polysaccharide prepared by us can reach the intestine without being degraded in the digestive process and can work well in the human body.

Assessing Lactic acid bacteria adhesion and stability in the gut

It is important for LAB to reach the human intestine in a live state and colonize it. Therefore, in vitro experiments were conducted to investigate the intestinal adhesion by inoculating sandwich-coated lactic acid bacteria powder on a monolayer of medium cultured with intestinal endothelial cells. The results showed that the sandwich-coated LAB powder of sample 6 had a stable adhesion property to Caco-2 cells compared to the control group, as shown in Table 2. In the in vivo experiment, the small intestine was removed from rats after oral administration for 8 weeks, and the results were observed using an analytical high resolution scanning electron microscope, and it was confirmed that the sandwich-coated lactic acid bacteria of sample 6 was deposited in the intestine as shown in figure 6.

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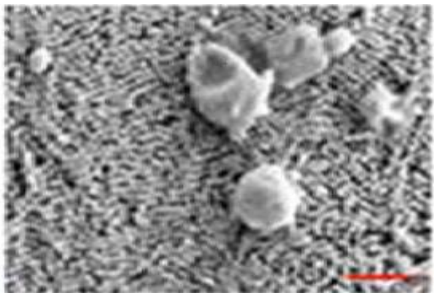

Fig.6. Small intestine of a mouse fed sandwich-coated LAB as seen through an electron microscope

Evaluate the inhibition of harmful bacteria in the gut

A robust coating of LAB is of little use if the lactic acid bacteria do not provide a health benefit in the gut. Therefore, since the stability of the LAB sandwich coating was demonstrated above, we evaluated whether it would be effective in the gut. Pathogenic bacteria such as E coli, Salmonella enteritidis, and Staphylococcus aureus, which are known to be harmful in the gut, were used to test the pathogen inhibition of the sandwich-coated LAB. Table 3 shows that the control group had no inhibitory activity against any bacteria, while the sandwich-coated LAB of sample 6 had excellent inhibitory activity against all bacteria. These results suggest that sandwich-coated lactic acid bacteria can maintain and improve intestinal health by inhibiting the proliferation of harmful bacteria in the intestine, rather than simply maintaining stability in the stomach.

Assess gut immune activity

To evaluate whether the sandwich-coated LAB were beneficial to gut immune function, cytokine activities such as nitric oxide (NO), IL-1β, and TNF-α were measured.

First, the nitric oxide (NO) activity showed that the sandwich-coated lactic acid bacteria of sample 7 produced more than 20 times more NO compared to the negative control PBS, as shown in Figure 7.(A), confirming the superiority of NO activity. This indicated that sandwich-coated lactic acid bacteria can activate immunity by inhibiting cell proliferation or modulating immune responses in the intestine, inducing immune and allergic responses by modulating TH1 and TH2 cytokine responses, and regulating the expression of various proinflammatory cytokines in inflammatory responses. [27]

The results of IL-1β and TNF-α measurements showed that compared with the control group, the sandwich-coated LAB of sample 7 was more active, with more than 8 and 3 times higher concentrations of IL-1β and TNF-α, respectively, suggesting that sandwich-coated LAB may be an effective host defense against fungal, bacterial, and parasitic infections. [28]

Finally, the ability to modulate the activity of NF-κB, a transcription factor that plays an important role in the immune response, was confirmed by the superior NF-κB activity of the sandwich-coated lactic acid bacteria in sample 7 compared to the control. [29,30].

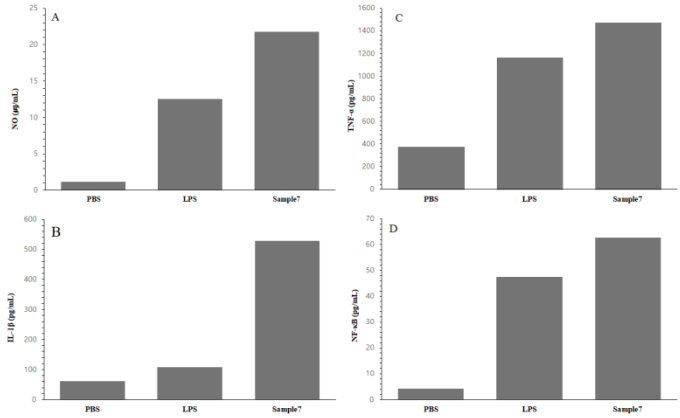

Fig.7. Amounts(pg/mL) of (A) NO (B) IL-1β (C) TNF-α (D) NF-κB after PBS, LSP, and Smaple7 administration.

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IV. CONCLUSION

This study confirms that sandwich coating with aloe polysaccharides is effective in improving the survival and functionality of lactic acid bacteria. The sandwich coating of LAB by self-assembly method (layer-by-laver (LBL)) by combining firstly protein, secondly cellulose, and thirdly aloe polysaccharide in appropriate proportions was used to maximize the survival rate of lactic acid bacteria after freeze-drying. The lactic acid bacteria at 4℃ and body temperature showed high survival rate, which can be used for long-term preservation of lactic acid bacteria and extend the shelf life of lactic acid bacteria products in commercial distribution. After entering the body, the lactic acid bacteria showed a high survival rate against digestive enzymes, gastric acid and bile during the digestive process, and its intestinal adhesion was also high, which maximized intestinal health and internal function. However, the reduced survival rate at 2.0% bile salts suggests that further research into the mechanisms of protection in high concentrations of bile salts is warranted. Once effectively colonized in the intestine, lactic acid bacteria can inhibit the growth of harmful bacteria and promote the production of NO, IL-1β, TNF-α, and NF-κB, which can contribute not only to improving intestinal health, but also to regulating immune response and inhibiting inflammatory responses, thereby improving immune function and preventing inflammation-related diseases. Therefore, the aloe polysaccharide-containing sandwich-coated lactic acid bacteria have a high potential for long-term preservation and commercial distribution and can be expected to be useful in related industries such as lactic acid bacteria-based functional foods, general foods, fermented foods, fermented milk, cosmetics, and pharmaceuticals.

Table 2. Relative amount of adherent lactic acid bacteria per Caco-2 cell in the control group and sample 6 as observed by light microscopy

(+): existing

Group	Caco-2 cells Adhesive
Control Group	+
Sample 6	+++

Table 3. Relative inhibition by comparing the size of the inhibitory ring formed around the spot after incubation with harmful bacteria

(+): existing (-): removing

Group	Inhibits harmful bacteria		
	E. coli	*Salmonellosis*	*Staphylococcus*
Control Group	+	+	+
Sample 6	– –	– – –	– – –

V. ACKNOWLEDGEMENTS

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