Oral delivery of *Lactobacillus paracasei* via microcapsule modulates gut health and intestinal microbiota

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Abstract

Background:

The beneficial impact of probiotics on host health is impaired due to the significant loss of survivability during gastric transit, small intestinal enzymes, and bile acids. Encapsulation helps to preserve the probiotics species from severe environmental factors. This study investigated the effects of oral delivery of probiotics via microcapsule on different parameters of gut health.

Methods:

Lactobacillus paracasei, highly sensitive probiotic species to gastric acid, was encapsulated with novel encapsulating material, polyacrylate resin, to get a microcapsule. C57BL/6 male mice were equally divided into three groups; supplementing basal feed, a mixture of encapsulating material and *Lactobacillus paracasei*, and encapsulated *Lactobacillus paracasei* (microcapsule) for four weeks. Fecal moisture percentage was measured regularly, which was elevated in the encapsulated group, but not in the mixed group. Based on this data, mice from control and encapsulated groups were sacrificed to study the different parameters of gut health.

Results:

Compared to control, encapsulated probiotics increased villi height, the ratio of villi height to crypt depth, and decreased crypt depth. Simultaneously, the tight junction proteins were upregulated on encapsulated group showing enhancement of intestinal barrier functions. The level of SigA and mucin increased along with gene expression of MUC-2 but, albumin level was decreased. In addition, we found a rise in the relative gene expression of anti-inflammatory factor (IL-10) and decreased pro-inflammatory factors (IL-1 β , IL-6, IL-8, and TNF- α). Meanwhile, microbiota metabolites, fecal LPS and TMAO were downregulated while SCFA and lactate were upraised compared to control. Furthermore, GSH-Px and TAOC level were increased and MDA was decreased. Microbiota analysis revealed the proportion of firmicutes was higher at the phylum level on an encapsulated group, while *Lactobacillus* was elevated at the genus level. We also found a remarkable increase in the population of *Lactobacillus murinus* at the species level. In summary, the oral delivery of probiotics via microcapsule effectively improves the animals' gut health by improving morphology, barrier function, anti-inflammatory action, antioxidant ability and modulating gut microbiota.

Background

Intestine helps in food digestion and absorption of food-derived nutrients in the host [1]. The three components of the intestine named, the single cell layer epithelium, microbiome, and immune system together have a vital role in nourishing homeostasis in host health [2]. Simple epithelium contributes for

the maintenance of host-microbe symbiosis by controlling the nutrient uptake and protecting against stress [2, 3]. The microbial environment of the gut is complex due to the presence of an abundant number and species of microbes, which is dominated by gram-positive *Firmicutes* and gram-negative *Bacteroidetes*. The diversity of microbiome is closely associated with host intestinal health [4]. The enteric microbiome, inhabiting the gastrointestinal tract, has a significant contribution to nutrient and drug metabolism, detoxification, prevention of the pathogens' colonization along with the induction and regulation of essential components of the host innate and adaptive immune system [5, 6]. Similarly, they also modulate intestinal barrier function, which defends the host against exterior environment such as pathogenic bacteria and helps in the absorption of nutrients, by regulating tight junction of epithelial cells [7]. Moreover, the immune system organizes the principal aspect of the symbiotic relationship between the host and highly diversified microorganisms. But, the changes in the composition of the gut microbiome or disruption of the interaction between host microbes and immune system affect intestinal health and can lead to the development of autoimmune diseases or disorders [8]. Thus, maintenance of the gut microbiota is very important to regulate immune homeostasis and impart other health benefits to the host.

The use of antibiotics, shifts in diet, age, or infection can disturb the gut microbiota leading to pathogenic, inflammatory, and metabolic conditions [9]. Various approaches have been suggested to regulate the composition of the microbial community, which include inoculation of probiotics [10], oligosaccharides, dietary fibre [11], traditional chinese medicine [12], fecal microbiota transplantation (FMT) [13], and so on. Probiotics play a very important role in intestinal health, by restoring gut microbiome composition and providing favorable environment for the gut microbes that results in the treatment of many infections [10, 14]. The effects of using different strains of probiotics, administration methods, and application doses vary greatly on livestock and poultry. This might be due to environmental effects on probiotics or the physiological situation of the host itself. Thus, the health potential of probiotics cannot be fully utilized. Higher temperature, oxygen level, and Relative humidity (RH) are harmful to many probiotic microorganisms [15]. Similarly, when probiotic species are ingested, it needs to encounter harsh environmental complexity in the GI tract. Generally, they can survive at the pH range of about 6–7 [16] but, gastric fluids are highly acidic (pH around 1–3) that can be deleterious to probiotic species [17]. In addition, High ionic strength and enzyme (pepsin) activity in the stomach [15, 16], bile acid, and digestive enzymes (lipases, proteases, and amylases) in the small intestine also affect the viability of probiotics [18]. As a result, there is a reduction in the number of bacteria reaching the hindgut. Thus, several techniques are reported to overcome these barriers, which enhance mucoadhesion properties and improve viability in the GI tract to increase colonization [19, 20]. Microencapsulation has been recommended as a propitious solution to solve the issue regarding its viability and preservation [21–23].

Microencapsulation protects the probiotics from environmental stress, GI tract insult and holds its structure in the upper GI tract before releasing it in the intestinal area, enhancing its efficacy [16, 24]. This technology has been widely used in clinical medicine for the controlled release of encapsulated drugs [25]. However, this is not common in the livestock and poultry industry. But, the material used for

microencapsulation should be selected wisely. Some encapsulating material may not break down on the targeted site, while others can dissolve partially, preventing the complete release of probiotic species into the hindgut and excrete out without utilization [26]. Considering these facts, we have developed a microcapsule to deliver the probiotics in the intestine for its efficient utilization. *Lactobacillus paracasei* was taken as a probiotic species, encapsulated with Polyacrylate resin III, to use as a feed additive. *Lactobacillus paracasei* is an extensively used probiotic strain, but it is highly sensitive to low pH [27]. Polyacrylate resin III is pH sensitive material that can dissolve in the intestinal juice of $\text{pH} \geq 7$; thus, it can protect *L. paracasei* from gastric acid, release on lower GI tract and enhances its potential as a probiotic. Nevertheless, there is no research using this matter as encapsulating material for probiotics in livestock or poultry for intestinal site-specific release.

Thus, the objective of our study was to improve the *L. paracasei*'s potential as a probiotic when used as a feed additive as a microcapsule. For this, we have observed, measured, or quantified several indicators of gut health. This technique can be beneficial for livestock and poultry to improve the acceptance of feed or additives having a foul odor, prevent the loss of beneficial bacteria number after administration, and release drugs or medicine at the specific area to improve the overall performance of the host.

Materials And Methods

Animals, Experimental Design, and Dietary Treatments

A total of 30 C57BL/6 male mice (Five weeks) were obtained from the Animal Experiment Center of Guangdong Province (Guangzhou, Guangdong, China) and were caged individually, in a sterile and controlled environment with a temperature of $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$, relative humidity of $65\% \pm 10\%$ and 12 hr light/12 hr/dark cycle. For two weeks, mice were provided with standard mouse feed and water ad libitum. After two weeks of adaptation, mice were assigned into three groups ($n = 10$) according to their weight: one group was control (Control) which received a standard diet, the second group was un-encapsulated *Lactobacillus paracasei* (0.1% MELP), which received 1g/Kg of a mixture of *Lactobacillus paracasei* (0.25g/Kg) and coating material (0.75g/Kg), and the last group was 0.1% encapsulated *Lactobacillus paracasei* (0.1% ECLP) which received 1g/Kg of encapsulated *Lactobacillus paracasei*. Here, encapsulated *Lactobacillus* was prepared by encapsulating *L. paracasei* with Polyacrylate resin III, by Hefei Ansheng Pharmaceutical Technology Co., Ltd, Hefei, China. Feed was prepared with water by maintaining the pH of 3.5 to protect encapsulating material from disintegration in the presence of water. The animals were individually housed in cages. Each animal's body weight and feed intake were recorded weekly. The Nuclear magnetic resonance system (Body Composition Analyzer MiniQMR23-060H-I, Niumag, China) was used to measure the body composition.

Fecal sample collection

For moisture content, fresh feces were collected every day for a week and then once a week for three weeks. Feces were collected instantly after ejection and placed on airtight tubes to prevent evaporation. All the fecal samples were also collected from the litter to measure dry fecal weight and further studies.

For Fecal moisture content, fresh feces were weighed in a tube, dried for 24 hours at a 60°C dry oven, and reweighed to get dry weight. The moisture content was determined as: (weight of feces before drying – weight of feces after drying) / weight of feces before drying × 100%. Other feces collected in sealed tubes (10ml) were dried for 48 hours at 37°C to protect them from the loss of volatile nutrients that can be evaporated and lost at a higher temperature. Dried feces were weighed to obtain dry fecal weight and stored at -20°C for later use.

Sample collection and processing

After feeding for 28 days, group showing better fecal quality was sacrificed along with control. Blood was harvested from an eyeball. Blood samples were collected in a 1.5ml centrifuge tube, placed for 1 h at room temperature for clotting, and centrifuged (3500 rpm, 15 min, and 4°C). The serum of each sample was separated and stored at -20°C for subsequent detection and analysis. From the dissected mice, tissues and internal organs were observed and weighted. Intestinal tissues and their contents were stored at -80°C for future use. The parts of separated specimens of the intestinal section (duodenum, jejunum, and ileum) were fixed in a 4% paraformaldehyde solution for morphological study.

Morphological Study

The specimens placed in paraformaldehyde were embedded in paraffin wax and slices were sectioned at 5 µm. The obtained sections were stained with hematoxylin and eosin (HE) by mounting in a glass slide. Afterwards, the slides were observed under the microscope (Olympus, Tokyo, Japan) for villi length (V) and crypt depth (C). The measurement of V and C was done using Image (Image-Pro Plus 6.1 Media Cybernetics, Rockville, MD, USA). Finally, the ratio of V and C (V/C) was determined.

Biochemical indices

The quantification of serum malondialdehyde (MDA), glutathione peroxidase (GSH-PX), fecal mucin, Secretory immunoglobulin A (sIgA), albumin, Lipopolysaccharide (LPS), and Trimethylamine N-oxide (TMAO) were carried out by enzyme-linked immunosorbent assay (ELISA) kits from Shanghai Ruifan Biotechnology Co., Ltd., China. Similarly, blood and fecal urea nitrogen (BUN), ammonia, and hydrogen peroxide (H₂O₂) were computed using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) as per the instructions of company. Furthermore, Total antioxidant capacity (TAOC) in blood serum was detected by a commercial product of Solrabio Life Science Beijing, China. For fecal/fecal digesta contents, the feces and fecal digesta were diluted with double distilled water at the ratio of 1:5 (weight/volume), homogenized, and centrifuged (12000 rpm, 5 minutes, 4°C). The obtained supernatant was used to detect different contents by following the kit instructions. Digesta pH was measured by inserting the sterile glass electrode of a pH meter (Thermoscientific™ Eutech Elite pH Spear) to a tube containing digesta and the values were noted down.

Determination of mRNA Expression

The expressions of mRNA were determined by quantitative real-time PCR (qRT-PCR). For RNA extraction, intestinal tissues stored at -80°C were taken. Total RNA was extracted from Intestinal sections

(duodenum, jejunum, and ileum) using RNA extraction kit (Guangzhou Magen Biotechnology Co., Ltd, China) as per the manufacturer’s instructions, and then determined using a Nanodrop spectrophotometer. Complementary DNA (cDNA) was synthesized by using 2 µg of total RNA by treating with DNase I (Takara Bio Inc., Shiga, Japan) to obtain a final volume of 20 µL by using Random Primer 9 (Takara Bio Inc., Shiga, Japan) and M-MLV reverse Transcriptase (Promega, Madison, WI, USA) by following the protocol of company. Then, total cDNA was mixed with antisense primers, SYBR green Real-Time PCR master Mix and Nucleic acid-free water. The qPCR reaction had a final volume of 20 µL. The qPCR was carried out with the Applied Biosystems QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific, USA). GAPDH was used as control and the relative gene expression of mRNA was calculated by $2^{-\Delta\Delta Ct}$. The primers sequences used for PCR are provided in Table 1.

Table 1

Gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
GAPDH	GGAGCGAGACCCCACTAACA	CGGAGATGATGACCCTTTTG
MUC-2	ATGCCACCTCCTCAAAGAC	GTAGTTTCCGTTGGAACAGTGAA
Claudin- 1	TGGATGGCTGTCATTGGG	GTGTTGGGTAAGAGGTTGTTTTTC
Occludin	CAGCCTTCTGCTTCATCG	GTCGGGTTCACTCCCATTA
ZO-1	GGGAAAACCCGAAACTGAT	CGCCCTTGGAATGTATGTG
IL-1β	TGTGCTCTGCTTGTGAGGTGCTG	CCCTGCAGCTGGAGAGTGTGGA
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-8	CACCCTCTGTCACCTGCTCAA	ATGGCGCTGAGAAGACTTGGT
IL-10	GCTCTCTGAAGAAAGCTGCAC	CACTTTCCCATCTTCATCATCA
TNF-α	CCGCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG

Detection of concentration of Lactate and SCFAs in digesta

The determination of SCFA and lactic acid concentrations were done via HPLC (High Performance Liquid Chromatography). In brief, the fecal and jejunal contents were taken out from a refrigerator, thawed at 4°C and mixed thoroughly. Around 0.2 g of the contents from each sample were diluted with double distilled water at the ratio of 1:5 (w/v) and mixed using a vortex for around 20 minutes to break all the feces. The mixture was centrifuged for 15 minutes, 12000× g at 4°C. Around 400 µL of the supernatant was extracted via pipette after centrifugation. By the use of a disposable syringe, the supernatant was filtered via a 0.22 µm filter, placed in a glass sample bottle, and injected into a glass column of 4.6 mm × 250 mm dimension. As per the protocol of the company and the parameters of the machine, the test was carried out.

16S rRNA Gene Sequencing Analysis

After 21 days of feeding, fresh fecal samples from 4 mice of control and encapsulated groups were collected on a well sterilized 1.5 ml tube twice a day for five days, placed on liquid nitrogen, and stored at -80°C until DNA extraction. 16S rRNA sequencing was performed at Beijing Novogene Co., Ltd. The steps and procedure of experiment are described in the previous article [28]. Briefly, CTAB/SDS method was used to extract genome DNA. The purity and concentration of obtained DNA were examined by using 1% Agarose gel and diluted to 1ng/μL using sterile water. The V3-V4 region of the 16S rRNA gene was amplified by using universal primer 338F and 806R with a barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Then the PCR products were detected on 2% agarose gel electrophoresis. The obtained mixture was purified with Qiagen Gel Extraction Kit (Qiagen, Germany). The sequencing library was generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA), and quality was assessed using a Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The library was sequenced on an Illumina NovaSeq platform, and 250 bp paired-end reads were generated.

Data analysis and statistics

Firstly, raw tags were obtained by using the software flash (version 1.2.7, <http://ccb.jhu.edu/software/FLASH/>) [29], and high quality clean tags were obtained by using QIIME(version 1.9.1, http://qiime.org/scripts/split_libraries_fastq.html) [30]. Chimera sequencing were detected using UCHIME Algorithm, (http://www.drive5.com/usearch/manual/uchime_algo.html) and effective tags were obtained by the removal of chimera sequences [31]. Sequences analysis was performed by using Uparse software (Uparse version 7.0.10,<http://drive5.com/uparse/>). Sequences with $\geq 97\%$ similarity were assigned to the same OTUs [32]. As per the Mothur method and SILVA (<http://www.arb-silva.de/>) database [33], representative sequences for each OTU were annotated with taxonomic information. OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences. The alpha and beta diversities of the gut microbiota were analyzed by QIIME software (version 1.7.0). Graphpad Prism 8.0.1 (Chicago, IL, USA) was used for statistical analysis. All the experimental results are expressed as means $\pm$ standard error of the mean (SEM). Methods of statistical analyses were chosen as per the design of each experiment and are mentioned in the figure legends, p values of < 0.05 were considered statistically significant.

Results

Encapsulated probiotics increased fecal weight and moisture content

The effect of encapsulated and un-encapsulated *Lactobacillus* was studied on growth performance and fecal parameters. There were no significant differences ($P > 0.05$) in a body weight gain or feed intake throughout the experiment (Fig. 1A-B). The administration of encapsulated *Lactobacillus* improved the fecal qualities compared to un-encapsulated *Lactobacillus* and control feed groups. Dry fecal weight was significantly increased on the 6th and 14th days of the experiment in encapsulated *Lactobacillus* group

(Fig. 1C). The fecal moisture percentage was also significantly increased in the encapsulated group ($P < 0.05$) compared to the control (Fig. 1D) from the 2nd day of the experiment. Similarly, on the 28th day, QMR was carried out, but no difference was found in the lean and fat mass among the groups (Fig. 1E). Blood urea nitrogen (BUN) and Blood glucose also showed no significant difference (Fig. 1F-G) between the three groups.

Encapsulated probiotics improved overall histomorphometric parameter and barrier function of the intestine

Control and encapsulated groups of mice were sacrificed to investigate the change in the tissue and organ structure. Figure 2A and 2B show the organ index for the liver, muscle and adipose tissue. Liver and part of skeletal muscle; gastrocnemius (GAS), extensor digitorum longus (EDL), and Tibialis anterior (TA) didn't show any change in their weight in both groups. Interestingly, the Soleus (SOL) mass was increased significantly ($P < 0.05$) in encapsulated *Lactobacillus* group. Additionally, the weight of white adipose tissue (WAT), i.e., inguinal (iWAT) and gonadal (gWAT), as well as the weight of brown adipose tissue (BAT) in both groups were similar. To find out the morphological change and structure, the length of the small intestine and colon were measured. In the encapsulated group, a noticeable difference was found in the intestinal length and colon length (Fig. 2C-E). The value was found to be numerically higher but had no significant difference ($P > 0.05$). Using H&E staining, the histomorphological study was made on different sections of the intestine (Fig. 2F). All the intestinal section showed normal tissue. Villus height, crypt depth and the ratio of villus height to crypt depth (V/C) in the three evaluated intestinal segments (duodenum, jejunum and ileum) are shown in Fig. 2G-I. The addition of encapsulated *Lactobacillus* increased V/C in the duodenum, jejunum and ileum compared to control group ($P < 0.05$). In addition, the dietary treatment increased the villus height significantly ($P < 0.05$) and decreased the crypt depth numerically in duodenum and jejunum. Furthermore, the ileal villus height was increased numerically ($P = 0.08$), and crypt depth was decreased significantly ($P < 0.05$) in the encapsulated group. Additionally, qRT-PCR was performed to determine the effects of encapsulated *Lactobacillus* on tight junction proteins in intestinal tissues. We observed an increase in the mRNA expression levels of claudin-1, occludin, and zonula occludens-1 (ZO-1) in the duodenum, jejunum and ileum as shown in Fig. 2J-L.

Encapsulated probiotics improved immunity and antioxidant capacity

Fecal Secretory immunoglobulin A (SIgA) and albumin contents were quantified as an indicator of the intestinal barrier. Encapsulated *Lactobacillus* raised the quantity of SIgA ($P < 0.05$) and lowered the level of albumin ($P = 0.054$) (Fig. 3A-B). Mucin level in feces was also analyzed to investigate the effect on a gut barrier function which was significantly upraised in our encapsulated group (Fig. 3C). An increase in mucin contents denotes that encapsulated *Lactobacillus* enhanced the mucin-related gene expression in the intestinal region. So, analysis of mucin gene; mucin-2 was quantified by qRT-PCR on the different

sections of the intestine. There was an increase in Muc-2 mRNA expression in all the sections of the intestine and a significant increase was found in the ileum section (Fig. 3D). To examine the inflammatory cytokines, the mRNA expression of interleukin 1 β (IL-1 β), interleukin 6 (IL-6), interleukin 8 (IL-8), Tumor necrosis factor alpha (TNF- α), and interleukin 10 (IL-10) in the different tissue of the intestine were detected by qRT-PCR. We observed the level of IL-1 β , IL-6, IL-8 and TNF- α were significantly or numerically lower in the duodenum, jejunum and ileum (Fig. 3E-I). IL-1 β level was significantly decreased in the duodenum segment and was also reduced in the jejunum ($P = 0.06$) and Ileum ($P = 0.06$). In addition, the expression of IL-6 was reduced in the duodenum ($P = 0.07$), jejunum ($P = 0.05$) and ileum ($P < 0.05$). We also found that IL-8 expression was downregulated in the duodenum, jejunum ($P = 0.05$) and ileum ($p = 0.07$). A significant decrease ($P < 0.05$) of TNF- α mRNA expression was observed in the ileum section, but no significant difference was found in the duodenum and jejunum ($P = 0.05$). Additionally, the consumption of encapsulated *Lactobacillus* increased the level of IL-10 compared to those in the control group, suggesting the improvement of inflammation. As shown in Fig. 3J-K, treatment of 0.1% encapsulated *Lactobacillus* elevated the level of Total Antioxidant Capacity (TAOC) and Glutathione peroxidase (GSH) in blood serum. In addition, we observed the reduction of maleic dialdehyde (MDA) level ($P < 0.05$) in the encapsulated group (Fig. 3L).

Encapsulated probiotics regulated and promoted microbial metabolites

The feces stored in the refrigerator were used to determine effect on the different fecal indices for microbiota study. The fecal lipopolysaccharide (LPS) and trimethylamine-N-oxide concentration (TMAO) were decreased (Fig. 4A-B) in encapsulated *Lactobacillus* group compared to control with a significant difference. In addition, the pH of digesta was decreased in the jejunum, colon ($P = 0.08$) and cecum ($P < 0.05$) (Fig. 4C). The value of urea nitrogen in feces was determined for three weeks period. We found the values were decreased for the whole period. For the first week, no significant difference was seen, but for the second and third week, there was a significant reduction ($P < 0.05$) in its level (Fig. 4D). Similarly, fecal ammonia didn't show any considerable variation for three weeks (Fig. 4E). The effect of encapsulated *Lactobacillus* on peroxide production revealed an elevation in H₂O₂ production for the same period (Fig. 4F). To show the additional potential of encapsulated *Lactobacillus* to influence the gut microorganisms, SCFA level was examined. Figure 4G-I shows lactic acid and SCFAs concentration in digesta of jejunum, colon and cecum along with feces. Encapsulated *Lactobacillus* increased the levels of lactate and acetate in colon digesta, cecum digesta and feces significantly. But, in the jejunum, the rise in lactate and acetate was seen but was not significant. Furthermore, the levels of propionic acid were close in both groups but were slightly higher in the treatment group.

Encapsulated probiotics altered fecal microbiota and their predicted function

To study the effects of encapsulated *Lactobacillus* on fecal microbiota, feces were analyzed by 16S rRNA sequencing. We investigated that the consumption of encapsulated *Lactobacillus* decreased ($P < 0.05$) the observed species (Fig. 5A) in encapsulated group. Similarly, the microbial diversity within the samples was analyzed by using the Shannon index, which determines the richness and evenness of microbial communities. We observed a significant reduction in Shannon index ($P < 0.05$) in the treatment group (Fig. 5B). We used non-metric multidimensional scaling (NMDS) plots to find the relationship between samples and species (beta diversity) and observed a distinct clustering of the control and treatment group samples (Fig. 5C).

To evaluate the effect of encapsulated *Lactobacillus* on microbial composition, bacteria on different levels were analyzed. A Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was used to express the enrichment of microbiome on a different level (Fig. 5D-H). On the phylum level, no significant differences were observed between the control and treatment group, but it was observed that Firmicutes and Bacteroidota followed by Campilobacterota were dominant over other phyla. The result also revealed that consumption of encapsulated *Lactobacillus* elevates the relative abundance of Firmicutes (0.3757 vs 0.4975, $P = 0.18$) and reduces the Bacteroidota (0.3761 vs 0.3469, $P = 0.72$) (Fig. 5D). In addition, we analyzed the dominant bacteria in order and family level. The proportion of Lactobacillales order and Lactobacillaceae family were dominant and higher in the encapsulated group than in the control. We also revealed the significant increase in Lachnospirales order and Lachnospiraceae family in encapsulated *Lactobacillus* group (Fig. 5F).

Lactobacillus and *Bacteroides* were dominant bacteria at the genus level. The relative abundance of *Lactobacillus*, *Turicibacter*, and *Lachnoclostridium* were elevated in the treatment group, and *Bacteroides* was slightly reduced. There was a statistically significant reduction of *Romboutsia* in the treatment group (Fig. 5G). Additionally, we also observed reduction of harmful bacteria like *Streptococcus*, *Helicobacter*, *Corynebacterium*, etc. without statistical significance. At the species level, *Lactobacillus* sp. was dominant over others. *Lactobacillus murinus* was the dominant species in both groups, but it was significantly higher in the encapsulated group. Similarly, *Lactobacillus reuteri*, and *Bacteroides thetaiotaomicron* were markedly elevated in the treatment group, while *Lactobacillus acidophilus*, *Bacteroides fragilis* and *Romboutsia ilealis* were significantly reduced (Fig. 5H).

To further analyze the relative difference between the control and encapsulated *Lactobacillus* in terms of function prediction of microbiota in feces, a histogram with FC and P value was used to analyze their KEGG pathway based on 16s rRNA sequencing. The feces of mice had shown that encapsulated *Lactobacillus* group has a higher relative abundance of microbiota involved in fructose, mannose and lipoic acid metabolism. We found those microbiomes related to antimicrobial resistance genes and biosynthesis of ansamycins were enriched. However, the pathways in cancer, salmonella infections, bacterial chemotoxins and biofilm formation in *Escherichia coli* were reduced in the treatment group (Fig. 5I).

Discussion

The purposes of microencapsulation are to mitigate the poor viability of probiotics due to the harsh environment on the upper part of the GI tract and to release probiotics at a controlled rate on the lower part for its beneficial action to the host. Previously, several microencapsulation methods and materials have been studied on different strains of probiotics to analyze their viability. Alginate-pectin microgel [34], chitosan-alginate [35], cellulose sulphate [36], etc., as encapsulating material protected different bacterial and fungal strains of probiotics from unfavorable gastrointestinal environment and improved the survival of bacterial cell, intestinal delivery and release resulting to several health benefits. However, pectin encapsulated probiotics did not enhance the effect of probiotics supplementation [26]. In our study, Polyacrylate resin was used as encapsulating material that can hold its structure at lower pH and release probiotic strain on the hindgut for the complete release of probiotic strain. Moreover, microcapsule formed improved overall gut performance.

Our encapsulated probiotics induced no comparable difference in body weight and food intake for four weeks. Similar results were recorded after the supplementation of different probiotic species [37, 38]. But, some strains of probiotics have shown notable improvement in growth performance [37, 39, 40]. The moisture content in feces, which determines the softness or firmness, studies on probiotic species [41, 42]. The production of lactic acid [41] may be the reason for a higher moisture content that helps to alleviate constipation and induce bowel movement. Interestingly, we observed a significant increase in soleus weight that could be due to the variation in the gut microbiota and metabolites produced by them that influence the skeletal muscle mass [43]. But, for an accurate conclusion, further research is necessary.

The present study revealed that probiotic *Lactobacillus* species could improve intestinal morphology as we observed an increase in the villus height, decrease in crypt depth and V/C ratio on different intestinal tissues. They are the standard index for intestinal health and indicate enhancement in digestion and absorption by increasing the epithelium surface layer [44]. Similarly, intestinal epithelium layer represents the most important barrier against pathogenic molecules and bacteria. The integrity of the intestinal epithelial cell layer is maintained by adherens junctions, tight junctions, and desmosomes. Claudin, occludin and Zonula occludens (ZO-1) are majorly studied tight junction proteins [45]. The expression of claudin-1, occludin and ZO-1 were increased in the different intestinal tissue. Our result is consistent with other studies on a different strain of probiotics [46, 47].

LPS, also called endotoxins and TMAO, are intestinal microbiome-derived toxins correlated with inflammation, cardiovascular disease (CVD), and other diseases on the host [48]. The levels of these toxins were significantly reduced in the encapsulated group, suggesting *Lactobacillus paracasei* reduce the toxins production and the expression of markers of inflammation [49]. Short Chain Fatty Acids (SCFAs) are other key metabolites of microbiota in the colon. Lactate, short-chain hydroxyl-fatty acid, is produced by several bacterial species and converted to SCFA by lactate fermenting bacteria [50, 51]. The three monocarboxylic short-chain organic acids, lactate, acetate and propionate, can maintain immune and intestinal homeostasis by downregulating the pro-inflammatory response in intestinal epithelial cells, showing antimicrobial and anti-inflammatory effects [52, 53]. Our study showed a rise in the SCFA and

lactate value in colonic, cecal and fecal content. Our study is consistent with several other probiotic species of *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, that produce Lactate and SCFA [54]. The modulation of microbiome metabolites is associated with a change in gut microbiota. Probiotics have a vital role in maintaining the gut microbiota by increasing the population of beneficial bacteria and reducing the pathogens by competing with them [55]. Previously, several studies were conducted about the impact of different strains of probiotics including *Lactobacillus* and it is reported that the probiotic modulates the microbiome on the different intestinal segments and control over microbes ecosystem [56]. In our study, we also found that the supplementation of encapsulated *Lactobacillus paracasei* changed the microbiome population.

The study on jejunal microbiota of chicken showed that probiotics could reduce the Shannon number on day 42 [39]. However, Zhang et al., 2015 investigated an increase in Shannon index in pro and pre encapsulated probiotics groups [20]. While another study on probiotics, *Bacillus sp.*, increased Shannon index number in the colon but decreased in cecum against enterohemorrhagic *Escherichia coli* on mice [47]. Interestingly, we found a decrease in observed species and Shannon index. The treatment of encapsulated probiotic limited microbiome to predominantly *Lactobacillus* genus (0.4000 vs. 0.1952) and *Lactobacillus murinus* species (0.3194 vs. 0.1314), which may be the reason for the decrease in richness and diversity. We observed a clear difference between the control and encapsulated probiotic treated group, i.e., Beta diversity, which illustrates *L. paracasei* affects the microbiota in mice.

In our study, phylum Firmicutes was elevated and Bacteroidetes was slightly reduced in the treatment group, consistent with a previous study on probiotic species [39]. An increase in firmicutes and a decrease in Bacteroidetes phyla are correlated with increased absorption of nutrients [57], which indicated that supplementation of encapsulated *Lactobacillus* improves digestion by improving absorption. We also found upraised value in firmicutes to Bacteroidetes ratio (0.99 and 1.43) after supplementing the encapsulated probiotic, which is believed to be the marker for obese animals. However, we didn't find any difference in weight gain in mice between those groups. A recent study has presented that this biomarker is still difficult to associate with the weight and health of an individual [6]. Lachnospiraceae family can be beneficial to the host as its members are chiefly responsible for producing short-chain fatty acids [58], which were upraised in our treatment group.

Genus *Lactobacillus*, belonging to firmicutes phylum, was enriched in the treatment group ($P = 0.05$) as in the previous study on several probiotic species [39, 59]. They exert a positive effect on the host health by maintaining immune homeostasis, improving gastrointestinal barrier function, and suppressing pro-inflammatory cytokines [56]. Some species under *Lactobacillus* genus also can produce several inhibitory substances, including H_2O_2 , that may limit the growth of pathogens to protect intestinal mucosa by strengthening toxic oxidation [60]. In our study, the production of H_2O_2 was increased for three weeks. This can be another reason for the decrease in species richness in the treatment group. Similarly, *Lactobacillus* also can reduce the production of endotoxins, LPS [61]. *Turicibacter* was found to be higher in the encapsulated group. *Turicibacter* metabolism and its association with the host animal are not well studied, but it has a remarkable action for reducing metabolic stress and has an anti-inflammatory and

anti-obesity effect [62]. Furthermore, we observed a significant reduction in genus *romboustia*, which is considered to be present in a healthy body and maintain the health status of the host. But, it is also reported as a possible marker of gut dysbiosis [63].

Lactobacillus murinus relatively and significantly increased in the treatment group. Lebovitz and Theus, 2019 has reported the applications of *L.murinus* from the various studies [64] and outlined that it has a beneficial effect on the host, including antimicrobial production, antagonist against pathogens, intestinal barrier, and can be developed as a potential probiotic. *Bacteroides thetaiotaomicron* helps in Carbohydrate metabolism, lipid metabolism, and enervates the production of pro-inflammatory cytokines and finally helps to strengthen the host-microbiome ecosystem [5]. This species was enriched in our study.

As per the function prediction, the pathway associated with the antimicrobial resistance gene was increased. Biosynthesis of antibiotics may be the reason for the enrichment of the antimicrobial resistance gene. Here, the pathway related to the synthesis of ansamycins (antibiotic) was also enriched. So, further study is necessary to find its validity on commercial production of livestock or poultry for the long run with the different doses as this may develop a negative influence on gut health. Pathway associated with lipoic acid metabolism may elevate host antioxidant properties and anti-inflammation [65].

Epithelial cell of the intestine establishes a physical and chemical barrier for preventing antagonism between immune cells of host and gut microbes to protect the mucosa from inflammation [1]. SIgA is an abundant antibody class found in the intestinal lumen, illustrated as the first line of defense to protect intestinal epithelium from pathogens and enterotoxins and has a key role in immune protection [66]. The result in this study showed that the SIgA quantity was upraised in feces of mice. Similarly, we observed a rise in mucin content and MUC-2 expression after the administration of encapsulated *L. paracasei*. Mucin fundamentally helps in epithelium lubrication and involved in immune functions. Furthermore, MUC2 covers the intestinal tract and safeguard the GI tract from pathogens attacks [1, 67, 68]. Fecal albumin is another index for measuring intestinal permeability, which estimates the flow from the blood to the lumen. A healthy intestinal barrier checks the overflow of albumin in the intestinal space. A higher value of albumin indicates the disruption of the intestinal barrier [69]. As expected, fecal albumin quantity was reduced in our study, which is a good sign for a healthy intestinal epithelium layer. Cytokines have the function to balance the intestinal immunity in the host. Intestinal Infections lead to inflammation that raises several pro-inflammatory factors [70]. Li et al., 2016 had described that supplementation of *Lactobacillus acidophilus* against *E.coli* decreased the secretion of pro-inflammatory cytokines and increased the secretion of anti-inflammatory cytokines compared to control, indicating probiotics can act as an anti-inflammatory supplement [71]. In our current study, the supplementation of encapsulated *L. paracasei* decreased the expression of TNF- α , IL-1 β , IL-6 and IL-8 and increased the expression of IL-10 in different intestinal tissues. Our results are in line with the earlier studies with other probiotics [37, 58, 72]. Thus, we can suggest that encapsulated *L. paracasei* can show anti-inflammatory effects and protect the

gut. But, interestingly, there was a difference in the mRNA expression within a tissue. So, further studies are needed to draw a specific conclusion from this.

Higher production of reactive oxygen species (ROS) than antioxidants leads to oxygen stress in host health. Due to such imbalance, there is a disturbance in a cell leading to damage of DNA, lipids, and proteins. During ROS production, several antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GPx) defend against oxidative stress for balancing the system. However, MDA activity is increased during oxidative damage [73]. In the earlier studies, different strain of probiotics including, *Lactobacillus plantarum* KFY02 [58], *Bacillus velezensis*, and *Bacillus subtilis* [37], improved the antioxidant level of animals by increasing antioxidant enzymes. In this study, the quantity of TAOC and GSH-Px were increased while MDA level was decreased, which concludes our encapsulated probiotics can enhance the antioxidant capacity of animals.

Conclusion

In this study, we developed a novel microcapsule that keeps its structure in gastrointestinal transit and releases the probiotic species completely in the hindgut to improve the overall health performance of the host. We demonstrated that the encapsulation of probiotics with Polyacrylate resin can upregulate the anti-inflammatory cytokines and downregulate pro-inflammatory cytokines. Additionally, it enhances the intestinal barrier function, antioxidant ability, improves intestinal histomorphometry, and promotes microbial metabolites. All these activities are associated with the change in the composition of gut microbiota. Our study suggests that the microcapsule developed can be applied to the commercial production of livestock and poultry. Moreover, more research may be required to improve the efficacy of microcapsule.

Declarations

Ethics approval and consent to participate

Animal care and procedures were performed as per the guidelines and were approved by the Animal Subjects Committee of South China Agricultural University.

Consent for publication: Not applicable

Availability of data and materials

The datasets generated and/or analyzed during this study are available from the corresponding author on a reasonable request.

Conflict of Interest: The authors declare no conflict of interest.

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Authors' contributions:

IG, GZ, GS, CZ: Conceived and designed the experiments.

IG, GX, YZ, JL, JZ: Collection of samples, execute and conduct experiment, data collection and analysis.

IG: Drafted the manuscript.

GZ, QJ, GS, CZ: Revised the manuscript, execute the experiment and finance, and laboratory analysis.

All the authors have read and approved the final version of manuscript.

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Figures

Figure 1

Effect of Encapsulated probiotic on growth performance, fecal output, and metabolism: Body weight gain (A), Cumulative feed intake (B), Dry fecal weight (C), Fecal moisture %, * represents significant difference of 0.1% ECLP with control and * with 0.1% MELP (D), Body composition analysis by QMR (E), Blood Urea Nitrogen (BUN) (F), Glucose (G). The data are presented as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, ($n=8-10$ per group). Control group supplemented with basal feed without additives, 0.1% MELP group supplemented with 0.1% of a mixture of encapsulating material and *L. paracasei* and 0.1% ECLP group supplemented with 0.1% of encapsulated *L. paracasei*.

Figure 2

Effect of encapsulated probiotic on histomorphometry, specific organ and intestinal barrier function: Liver, Muscle (GAS, TA, EDL, SOL) mass (A), mass of adipose tissue (B) (n=8-10 per group), Representative image of the intestine (C), supplemented with control feed (i) supplemented with dietary encapsulated *L. paracasei* (ii), Intestine length (D), Colon length (E), Representative histology of intestinal section, stained with hematoxylin and eosin (H and E), Bar 100µm (F), The average: Villus height (G), Crypt depth (H), Ratio of Villus height to Crypt depth (I) mRNA expression of Claudin-1 (J), Occludin (K), ZO-1 (L) quantified against housekeeping gene GAPDH on intestinal tissues (n=6-8 per group). The data are presented as the mean $\pm$ SEM. *P < 0.05, **P < 0.01. Control group supplemented with basal feed without additives and 0.1% ECLP group supplemented with 0.1% of encapsulated *L. paracasei*.

Figure 3

Effect of encapsulated probiotics on immunity and antioxidant capacity Fecal sIgA (A), Fecal albumin (B), Fecal mucin (C), mRNA expression of MUC-2 (D), IL-1β (E), IL-6(F), IL-8(G), IL-10(H) and TNF-α (I) quantified against GAPDH, TAOC (J), GSH-Px (K), MDA (L). Here, single bar for feces represents the feces collected on third week. The data are presented as the mean ± SEM. *P < 0.05, **P < 0.01. (n=6-8 per group). Control group supplemented with basal feed without additives and 0.1% ECLP group supplemented with 0.1% of encapsulated *L. paracasei*.

Figure 4

Effect of encapsulated probiotic on microbiome metabolites: Fecal LPS (A), Fecal TMAO (B), Digesta pH (C), Fecal Urea Nitrogen (D), Fecal Ammonia (E), Fecal H₂O₂ (F), Lactic acid (G), Acetic acid (H), Propionic acid (I). Here, single bar for feces represents the feces collected on third week. The data are presented as the mean $\pm$ SEM. *P < 0.05, **P < 0.01, ***P < 0.001 (n=6-8 per group). Control group supplemented with basal feed without additives and 0.1% ECLP group supplemented with 0.1% of encapsulated *L. paracasei*.

Figure 5

Effect of encapsulated probiotic on modulation of microbiota: Observed species (A), Shannon index (B), Nonmetric multidimensional scaling (NMDS) (C), Composition of gut microbiota by using KEGG enrichment analysis: Phylum (D), Order (E), Family (F), Genus (G), Species (H) where the size of the bubble represents the relative abundance of bacteria, the x-axis label shows log2 (FC) that represents fold change, which was calculated as firstly, the relative content of bacteria in the control group was divided by our treatment group to get first value (FC). By using excel, $\log(\text{FC}, 2)$ was calculated to get Log2 (Fold change). Bacteria that are up-regulated relative to the control has a positive log2FC value, and

downregulated relative to the control has negative log2 FC value, left y-axis label represents the bacteria at a different level and a different color on the right side shows significance (P value), Prediction of metabolic function at KEGG (level 3) (l), left Y-axis shows different predicted function, X-axis shows the log2 (FC) that represents fold change. Control group supplemented with basal feed without additives and 0.1% ECLP group supplemented with 0.1% of encapsulated *L. paracasei*.

Supplementary Files

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- [GraphicalAbstract.png](#)