Effects of *Ilisha elongata* proteins on proliferation and adhesion of *Lactobacillus plantarum*

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ABSTRACT

The effects of aquatic proteins on the proliferation and adhesion of intestinal probiotic bacteria were investigated by in vitro fermentation and mouse in vitrointestinal tissue models. Compared with the control group, the *Illisha elongata* protein reduced the growth time of Lactobacillus plantarum (LP45) by 34.25% and increased the total number of colonies by 6.61%. The *Ilisha elongata* salt-solubale protein performed better than water-soluble protein in vitro proliferation of LP45. *Ilisha elongata* salt-soluble protein significantly increased the number of viable bacteria adhering to intestinal, and caused changes in the amount of polysaccharides, proteins and biofilms in the intestinal tissue model. These results indicate that the *Ilisha elongata* protein is beneficial to the proliferation and adhesion of probiotics in the intestinal, and can be used as an active protein beneficial to intestinal health.

Introduction

Observational findings achieved during the past two decades suggest that the gut microbiota may contribute to the metabolic health of the human host. (Fan, & Pedersen, 2020) Intestinal probiotics are active microorganisms that colonize the human body and alter the composition of the flora in a particular part of the body. Probiotics colonize the intestinal tract and can effectively improve the intestinal microenvironment and regulate intestinal homeostasis. (Phimister, Lynch, & Pedersen, 2016) Diet strongly impacts the relationship between humans and their microbial hosts (Gentile, & Weir, 2018). Based on studies in animals and humans, dietary intake appears to be a major regulator of gut microbiota structure and function for both short- and long-term (Schroeder et al., 2017, Shoaie, et al.,2015, David, et al.,2014, Desai, Seekatz, Koropatkin, Kamada, & Martens, 2016). Dietary nutrients are not only essential for human health but also for the health and survival of the trillions of microbes that reside within the human intestines. (Gentile, & Weir, 2018)

Protein is one of the essential nutrients for human life activities and can provide the amino acids required by human protein synthesis. Three primary sources of dietary protein are animal protein, vegetable protein and dairy protein, and aquatic products are one of the main sources of animal protein in the human diet. A diet rich in aquatic proteins is beneficial to intestinal health. One study found that the addition of fish meal to the diet of mice caused alterations in the intestinal microbiota, affecting the fermentation and production of spoilage compounds in the intestinal microbiota (An, 2014). Martone, et al. found that fish by-products could be used as an alternative substrate for microbial cultures (Martone, Olinda, & Jorge, 2005).

An important prerequisite for probiotics to colonize the intestinal tract is that probiotics can adhere and proliferate on the intestinal tract. The proliferation of probiotics requires the supply of nutrients, and proteins and polysaccharides can be used as growth-promoting factors for probiotics. The adhesion of probiotics is closely related to the mucin secreted by probiotics, which includes surface epithelial protein, mucus binding protein, bacterial hair protein, extracellular polysaccharide, lipopolysaccharide, peptidoglycan and other components (Hynonen, Ulla & Palva, 2013). The rich proteins in aquatic products can be used as growth promoting factors for probiotics, and aquatic proteins provide essential amino acids for probiotics through the proteolytic system of probiotics.

However, the functional interactions between aquatic product proteins and intestinal probiotics are still superficial to the best of our knowledge. Therefore, the aim of this study was to investigate the effect of aquatic product proteins on the proliferation and adhesion of intestinal probiotics.

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2. Materials

2.1. Materials and reagents

The aquatic products were purchased from Guangzhou Huangsha Seafood Market, and *Bifidobacterium lactis* (M8), LP45, and *Bifidobacterium bifidum* (BBi32) were preserved by our laboratory. Beef extract, yeast extract, peptone, Tween-80, triammonium citrate, manganese sulfate monohydrate, magnesium sulfate heptahydrate, agar and glucose were purchased from Bioengineering (Shanghai) Co. Pentobarbital and glutaraldehyde were purchased from Shanghai Maclean Biochemical Technology Co. Crystalline violet was purchased from Shanghai Yuanye Biotechnology Co. All other chemical reagents and solvents used in the experiments were of analytical reagent grade.

2.2. Preparation of aquatic protein

The aquatic proteins were prepared according to the method described by Sathivel et al (Sathivel, Yin, Bechtel, & King, 2009), the fresh raw aquatic products were first pretreated, fish was skinned, gutted and deboned and shellfish were shelled and gutted. The pretreated samples were homogenized for 3 min using a homogenizer (Jiuyang) and the mixture was continuously stirred for 60 min at room temperature. During the heating process, the fat cells ruptured and released the oil into the liquid phase. The heated suspension was centrifuged at 4000 r/min for 15 min to obtain three separated layer segments with insoluble material at the bottom; the middle heavy liquid phase contained soluble protein and the top phase contained crude lipid, and the middle layer was separated and collected to obtain water-soluble protein solution. The insoluble material was collected and mixed with 5 times the volume of 1.1 mol/L KCL solution, homogenized for 3 min using a homogenizer, and the homogenate was centrifuged at 4000 r/min for 15 min at 4 °C. The insoluble precipitate was separated and collected, the above extraction steps were repeated, and the supernatant of the two salt extracts was collected to obtain the salt-soluble protein solution, which was dialyzed at 4 °C for 48 h. The water-soluble protein solution and the dialyzed salt-soluble protein solution were mixed for 48 h. After the salt-soluble protein solution was mixed to obtain the aquatic product protein solution, the aquatic product protein solution was freeze-dried to obtain the crude aquatic product protein. The water-soluble protein and salt-soluble protein of *Ilisha elongata* were obtained by respectively freeze-drying the water-soluble protein solution and the dialyzed salt-soluble protein solution.

2.3. Measurement of probiotic growth curve and calculation of growth generation time

MRS medium: peptone 10 g/L, beef extract 5 g/L, yeast extract 5 g/L, glucose 20 g/L, Tween-80 1 g/mL, sodium dihydrogen phosphate 2 g/L, anhydrous sodium acetate 5 g/L, trimonium citrate 2 g/L, manganese sulfate 0.02 g/L, magnesium sulfate 0.1 g/L, distilled water 1000 mL, agar 15 g/L.

Protein-deficient MRS(Pd-MRS) medium: beef extract 5 g/L, glucose 20 g/L, Tween-80 1 g/mL, sodium dihydrogen phosphate 2 g/L, anhydrous sodium acetate 5 g/L, trimonium citrate 2 g/L, manganese sulfate 0.02 g/L, magnesium sulfate 0.1 g/L, distilled water 1000 mL, agar 15 g/L.

The probiotic lyophilized powder was activated continuously for 3 generations, inoculated into MRS medium, and incubated at 37 °C, 200 rpm overnight. Then inoculate 1% into MRS-protein medium. After setting up the grouping, 200 μL of bacterial solution was added to each well in a 96-well plate, which was placed in an enzyme marker and the 24-h growth curve was measured at OD600 nm. The doubling time was calculated by linearly fitting the slope of the logarithmic phase of probiotic growth with the formula (1).

$$g = \frac{\ln 2}{k} \tag{1}$$

Where, g is the doubling time; k is the slope.

2.4. Colony counting of probiotic bacteria

The activated probiotic bacteria were inoculated in polysaccharide-MRS medium and incubated at 37℃, 200 rpm incubator. After 24 h of fermentation, the bacterial solution was diluted to 10^{-6} dilution, 100 μL of the bacterial solution was inoculated in solid MRS medium and coated evenly with a coating stick, and they were incubated in an anaerobic incubator at 37℃ for 24 h. The bacteria were counted by ImageJ. The calculation formula (2) was as follows.

$$N = \frac{C \times 10}{10^{-D}} \tag{2}$$

Where, N = CFU/mL; C = number of bacterial monoclonal; D = dilution multiple

2.5. The establishment of the mouse in vitro intestinal tissue adhesion model

The mouse adhesion model of in vitro intestinal tissue was established based on the method described by Leila-ouassila et al. (Leila-ouassila, Farzin, Luc, & Jean, 1995). Briefly, mouse intestinal tissues were taken from the intestine of healthy adult mice, and the mice were anesthetized using pentobarbital and dissected in an ultra-clean table after they had entered deep anesthesia; the mouse intestinal segments were removed and divided into duodenum, jejunum, ileum, cecum, and colon according to their physiological structure. Each intestinal segment was placed in a sterile flat dish and washed with 0.01 M PBS buffer at 4℃; the membranes, blood vessels and fat on the outside of the intestine were removed using sterile surgical instruments; PBS was injected from one end of the small intestine with a syringe to rinse, and the intestine was cut longitudinally using a scalpel with the intestinal lumen opened upwards; the contents of the intestine were gently scraped out with the back of a sterile blade, and the intestinal tissue was rinsed with PBS; each intestinal segment was divided into 5-mm-long intestinal segments using a sterile blade.

LP45 was cultured overnight to obtain fresh seed solution, and the bacterial solution was centrifuged and resuspended with 0.01 M PBS, and the concentration of each group was diluted to 108 CFU/mL to obtain LP45 bacterial solution. Two intestinal segments (one for counting and related index determination, one for electron microscopy observation) were placed in a sterile 24-well plate, and 1 mL of LP45 bacterial solution was added to each well. The intestinal tissues were then incubated in a shaker incubator (ZHP-160, Shanghai Jingqi) at 37 °C for 30 min. The intestinal tissues were then washed three times in 1 mL of sterile PBS to eliminate all non-adherent bacteria. One piece of intestinal tissue was soaked in 1 mL PBS, cut into homogenate with surgical scissors, and each diluted homogenate was applied on MRS agar medium and incubated in an anaerobic incubator (HYQX-III, Shanghai Xinuo) for 24 h and then colonies were counted. The count was expressed as the number of viable bacteria per mm of the tissue surface. Another piece of intestinal tissue was placed in a sterile well plate, fixed with 1 mL of 2.5% glutaraldehyde for 12 h; washed three times with 0.1 M phosphate buffer (pH = 7.3); then washed three times with distilled water at 4 ℃; finally freeze-dried for 18 h. The specimens were examined by scanning electron microscopy (Phenom ProX, Pure G6) after drying.

2.6. Determination of adhesion-related indicators

The changes in polysaccharide content in the intestinal tissue model of adherent probiotics were measured using the phenol sulfate method

(Dubois, Gilles, Hamilton, Rebers, & Smith.1956). The change in protein content in the intestinal tissue model of adherent probiotics was determined by the KOMAS Brilliant Blue G-250 method (Bradford, 1976). The determination of biofilm biomass was improved on the basis of the crystalline violet staining method described by Rajamani et al. (Rajamani, et al., 2019). 200 μL of 1% crystalline violet was added in 200 μL of intestinal tissue homogenate from the intestinal tissue model of adherent probiotic bacteria. After a 15-min incubation, the samples

were washed three times using 0.01 M PBS. Then,1 mL of 95% ethanol was added, and 200 μL of the final solution was transferred to a 96-well plate and measured the absorbance at 570 nm. Determination of molecular weight of proteins by SDS-PAGE.

2.7. Statistical analysis

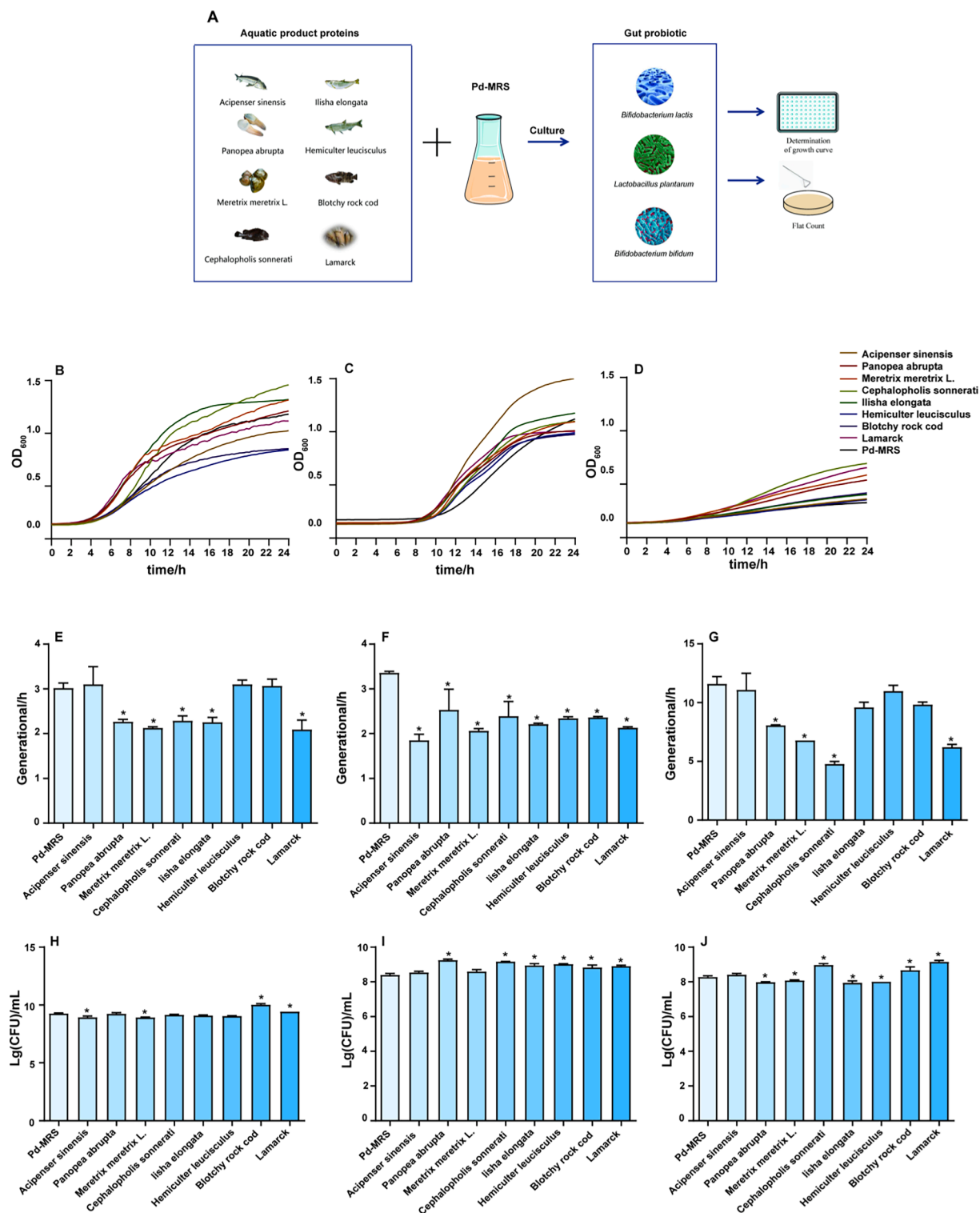All values were represented as mean $\pm$ SEM. Statistical analysis was

Fig. 1. Schematic diagram of cultivating 3 kinds of probiotics with 8 kinds of aquatic proteins(A);Growth curves of M8, LP45, BBi32 (B, C,D); Growth generation times of M8, LP45, BBi32 (E, F,G); The total number of colonies of M8, LP45, BBi32 (H, I,J). *$p < 0.05$, compared with the Pd-MRS group.

carried out using SPSS 22.0 software. One-way analysis of variance (ANOVA) was adopted to compare the significant differences among all of the groups using Tukey's analysis. Differences were considered to be significant at $p < 0.05$.

Results

3.1. Effect of aquatic proteins on the growth of 3 probiotic species

In addition to oligosaccharides, proteins, hydrolysates, and peptides have also been shown to have prebiotic functions that promote the growth of probiotic bacteria (Zhang, Zhang, Li, & Liu, 2020). The growth of probiotic bacteria requires the supply of nutrients such as carbon and nitrogen sources. For MRS medium, which is a commonly used culture for probiotics, the carbon source of it is glucose, and the nitrogen source is provided by yeast extract, beef extract, and peptone. In this study, aquatic proteins (*Acipenser sinensis, Panopea abrupta, Meretrix meretrix L, Cephalopholis sonnerati, Ilisha elongata, Hemiculter leucisculus, Blotchy rock cod, Lamarck*) were used as the research object. add the same amount of aquatic protein to the nitrogen-deficient MRS medium to replace the missing protein, explore its effect on the growth of probiotics (*Bifidobacterium lactis* M8, *Lactobacillus plantarum* LP45, *Bifidobacterium bifidum* BBi32).

Fig. 1A shows a schematic diagram of aquatic protein culturing probiotics. As shown in Fig. 1B, 1C and 1D, the growth curves of probiotics reflected the different proliferation of probiotics by each aquatic protein experimental group, Among them, M8 andLP45 significantly used aquatic protein for its own growth and proliferation, where Bifidobacterium bifidum BBi32 could only utilize aquatic protein for growth and proliferation to a lesser extent. This is due to the different protease species activities of different probiotics, so they behave differently in terms of their ability to utilize aquatic proteins. The protein hydrolysis system of lactic acid bacteria consists of three main groups: the first group is the extracellular proteases that hydrolyze macromolecular proteins into peptides; the second group is one of several transport systems that translocate peptides into cells; and the third group contains multiple peptidases that further hydrolyze translocated peptides into cells to form free amino acids that are ultimately metabolized or synthesized into proteins(Pritchard, & Tim, 1993, Zhang, Zhang, Li, & Liu, 2020).Fig. 1E, 1F and 1G show the effect of different aquatic protein experimental group on the growth generation time of probiotic bacteria, the most obvious of which is that each aquatic protein experimental group significantly reduced the growth generation time of LP45. From the perspective of microbial fermentation, the logarithmic growth time of microorganisms reflects the growth rate of microorganisms, the growth rate of probiotics was significantly increased by significantly reducing the growth time of probiotics. As shown in Fig. 1E,the experimental groups of *Panopea abrupta, Meretrix meretrix L, Cephalopholis sonnerati, Ilisha elongata*, and *Lamarck* decreased the growth generation time of M8 and were significant, while the experimental groups of *Acipenser sinensis, Hemiculter leucisculus, Blotchy rock cod* and significantly increased the growth generation time of M8. As shown in Fig. 1F, each aquatic protein experimental group significantly reduced the growth generation time of LP45. As shown in Fig. 1G, only the experimental groups of *Panopea abrupta, Meretrix meretrix L, Cephalopholis sonnerati,* and *Lamarck* significantly decreased the growth generation time of BBi32. Fig. 1H, 1I, 1 J shows the effect of each aquatic protein experimental group on the viable bacteria count of probiotics after 24 h' fermentation. When M8 was fermented for 24 h, only the *Blotchy rock cod* experimental group significantly increased the total number of probiotic colonies compared with the control group, and when LP45 was fermented for 24 h, all the other six aquatic protein experimental groups significantly increased the total number of probiotic colonies compared with the control group, except for the *Acipenser sinensis* and *Meretrix meretrix L.* experimental groups. When BBi32 was fermented for 24 h, only the experimental groups of *Cephalopholis sonnerati, Blotchy rock cod*

and *Meretrix meretrix L.* significantly increased the total number of probiotic colonies compared with the control group. The results show that the influence of aquatic protein on the growth of probiotics is determined by the types of protein and probiotics.

To screen the probiotic bacteria with dominance protein utilization ability, we compared the change of colony count of standard protein (soy protein) and control group during the fermentation. As shown in Fig. 2A, LP45 utilized standard protein significantly increased the total number of colonies after 24 h of fermentation, and Fig. 1I also reflected that LP45 significantly utilized aquatic protein. Bifidobacteria are strictly anaerobic in nature, thus reducing their effectiveness in adults, hence the increased interest in Lactobacillus as a potential probiotic (Ravinder, Ashwani, Manoj, Be Hare, Shalini, & Hariom, 2012). To screen for aquaproteins that significantly proliferated LP45 growth, we compared the effect of each experimental group of aquaproteins on LP45 growth generation time and the total number of colonies. As shown in Fig. 2B, the experimental groups of *Panopea abrupta, Cephalopholis sonnerati, Hemiculter leucisculus,* and *Ilisha elongata* improved the total number of colonies of probiotic bacteria most significantly, compared to the control group (10.27%, 9.18%, 7.49%, and 6.61%, respectively). The experimental groups of *Acipenser sinensis, Meretrix meretrix L., Lamarck.* and *Ilisha elongata* were the most significant in reducing the growing generation of probiotic bacteria, and the total number of colonies was reduced by (44.82%, 38.74%, 36.58% and 34.25%) compared with the control group, respectively. Considering the two representative indicators of probiotic growth and proliferation, it was found that the *Ilisha elongata* protein had a significant effect on the proliferation speed and number of LP45.

3.2. Screening of Ilisha elongata protein types and addition amounts

The protein of *Ilisha elongata* mainly includes sarcoplasmic protein and muscle fibrin. The main component of the protein extracted by water extraction is sarcoplasmic protein, and the main component of protein extracted by salt extraction is muscle (Sathivel, Yin, Bechtel, & King, 2009). According to the different extraction methods, we divided the grub protein into water-soluble protein and salt-soluble protein, and cultivated LP45 with different amounts of different proteins to compare its effects on the growth and proliferation of probiotics. Fig. 3A shows the growth curves of LP45 cultured in each experimental group, in which the final OD values of three experimental groups of *Ilisha elongata* salt-soluble protein were similar to the final OD values of MRS experimental group, and the final OD values of three experimental groups of *Ilisha elongata* water-soluble protein were lower than the final OD values of MRS experimental group. As can be seen from Fig. 3B, only the experimental groups of MRS + 0.5 mg/mL *Ilisha elongata* salt-soluble protein (MRS + 0.5 mg/mL Ie-S) and MRS + 1 mg/mL *Ilisha elongata* salt-soluble protein (MRS + 1 mg/mL Ie-S) significantly reduced the growth generation time of LP45 compared with the MRS experimental group. The experimental group in which MRS (peptone in MRS was replaced by *Ilisha elongata* water-soluble protein in equal amount(Ie-W er pep)), the experimental groups of MRS + 0.5 mg/mL *Ilisha elongata* water-soluble protein (MRS + 0.5 mg/mL Ie-W),MRS + 1 mg/mL *Ilisha elongata* water-soluble protein (MRS + 1 mg/mL Ie-W) and the experimental group in which MRS (peptone in MRS was replaced by *Ilisha elongata* salt-soluble protein in equal amount(Ie-S er pep)) all increased the growth generation time of LP45, that is, reduced the proliferation rate of LP45.As shown in Fig. 3C, compared with the MRS experimental group, other experimental groups significantly increased the total number of colonies of LP45 at 24 h of fermentation, except for the experimental group MRS(Ie-W er pep). Among them, the experimental group (MRS + 1 mg/mL Ie-S) had the highest LP45 colony count of 2.385×10^{10} CFU /mL at 24 h of fermentation.The effect of *Ilisha elongata* salt-soluble protein on LP45 growth and proliferation was more significant than that of *Ilisha elongata* water-soluble protein as screened by LP45 growth model in which the addition of 1 mg/mL *Ilisha elongata*
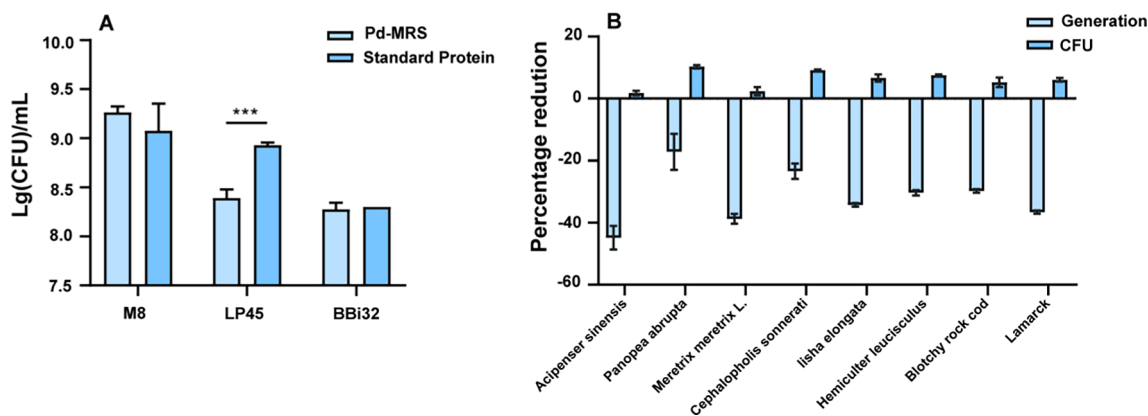
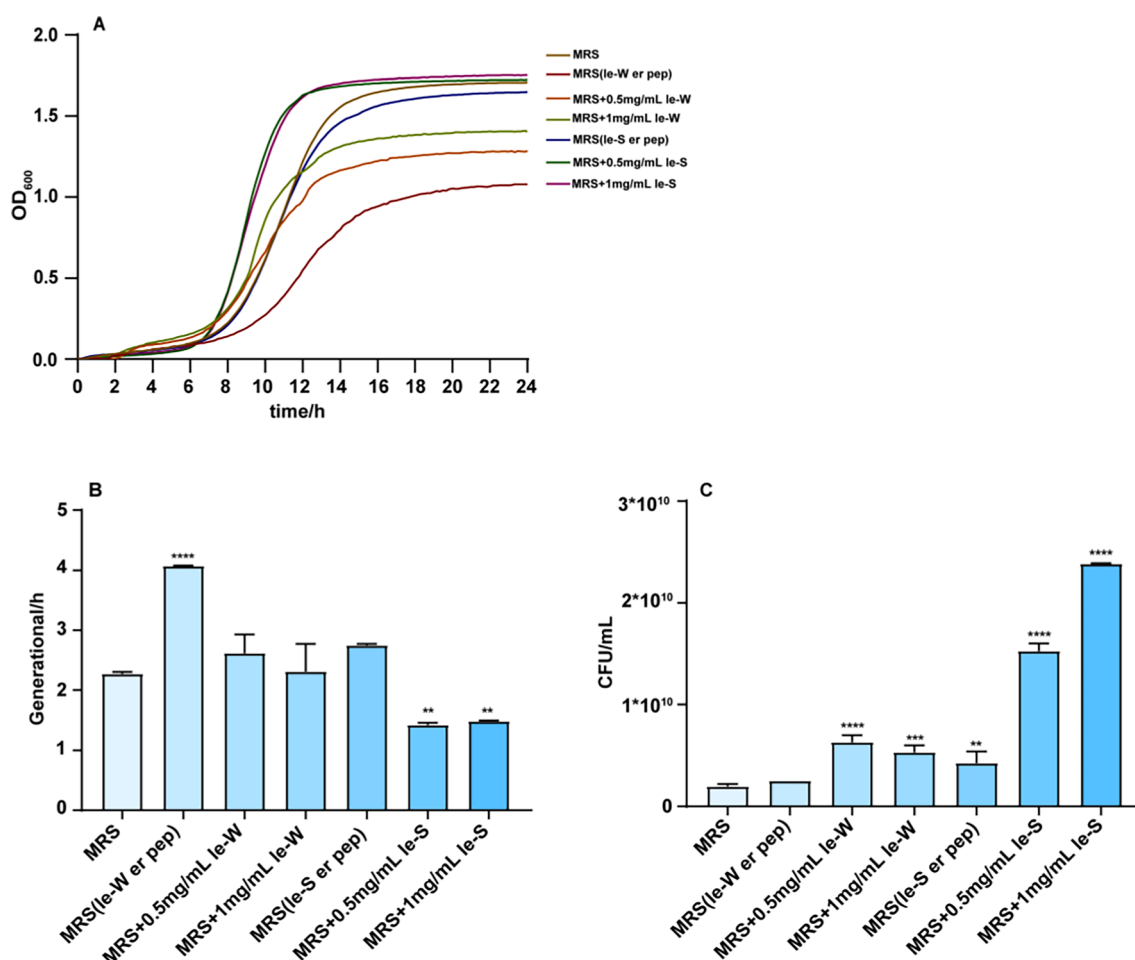**Fig. 2.** The total number of colonies (A);Percentage change compared to control (B).***p < 0.005.

Fig. 3. Growth curve of LP45 (A); Growth time of LP45 (B); Total number of colonies of LP45 (C). **p < 0.01, compared with the MRS group;***p < 0.005, compared with the MRS group;****p < 0.001, compared with the MRS group.

salt-soluble protein to MRS significantly promoted the growth of LP45.

Although there are more and more studies focus on the relationship between proteins and probiotics have been reported/published in recent years, there is no consistent conclusion on the mechanisms of the interaction between them. Some researchers believe proteins provide act as nitrogen sources, and can enhance bacterial proliferation, (Juillard, Bars, Kunji, Konings, & Richard, 1995), while others have suggested that proteins can act as initiators of the polypeptide transport system sub. Zhang, et al.(Zhang, Zhang, Li, & Liu, 2020)found that the mechanisms of action of proteins on probiotics include providing

essential amino acids to probiotics, improving the resistance of strains to acidic environments, promoting amino acid and protease activity and other beneficial effects on probiotics.Some researchers monitored the consumption of peptides and amino acids by Lactobacillus lactis and showed that the growth of L.lactis was dependent on oligopeptides as a nitrogen source(Juillard, Bars, Kunji, Konings, & Richard, 1995).Researchers added protein hydrolysates from poultry bone and meat to the culture medium and found that the ability of these hydrolysates to promote the growth of Lactobacillus was significantly higher than that of two common commercial hydrolysates(Meli, Lazzi, Neviani, & Gatti,

2013). Zhang et al. showed that soybean peptides promote the proliferation of probiotic bacteria. Researchers explored the effects of soy peptides on the proliferation of common probiotic bacteria (fecal streptococcus, wax-like bacilli, stalactites, yeast) and showed that soy peptides promote the proliferation of these four bacteria, and the addition of soy peptides inhibited the growth of Staphylococcus aureus. Ding, et al. (Ding, & Li, 2021) found that the walnut oligopeptide (WOPs) not only promoted bacterial growth and reproduction but also reduced the number of dead cells. The addition of WOPs significantly increased the secretion of bacterial biofilm and EPS, which suggested that the promotion of WOPs on the growth of L. plantarum Z7 might be related to the quorum sensing system of bacteria.

3.3. The effect of Ilisha elongata protein on the intestinal adhesion adhesion of LP45

The adhesion adhesion of probiotics contributes to their colonization in the intestine; it can also enhances the signal communication between lactobacilli and intestinal cells and inhibits the colonization of pathogenic bacteria in the intestine and finally improves the immunity of the organism (Juge, 2012, Baarlen, et al., 2009, Kaushik, et al., 2009). Lactobacillus adhesion helps to maintain the normal intestinal flora structure and maintain the morphological and functional integrity of the intestinal mucosa. In maintaining the normal intestinal flora structure, Lactobacillus achieves its purpose mainly through the spatial site-blocking effect. The adhesion of pathogenic bacteria to intestinal
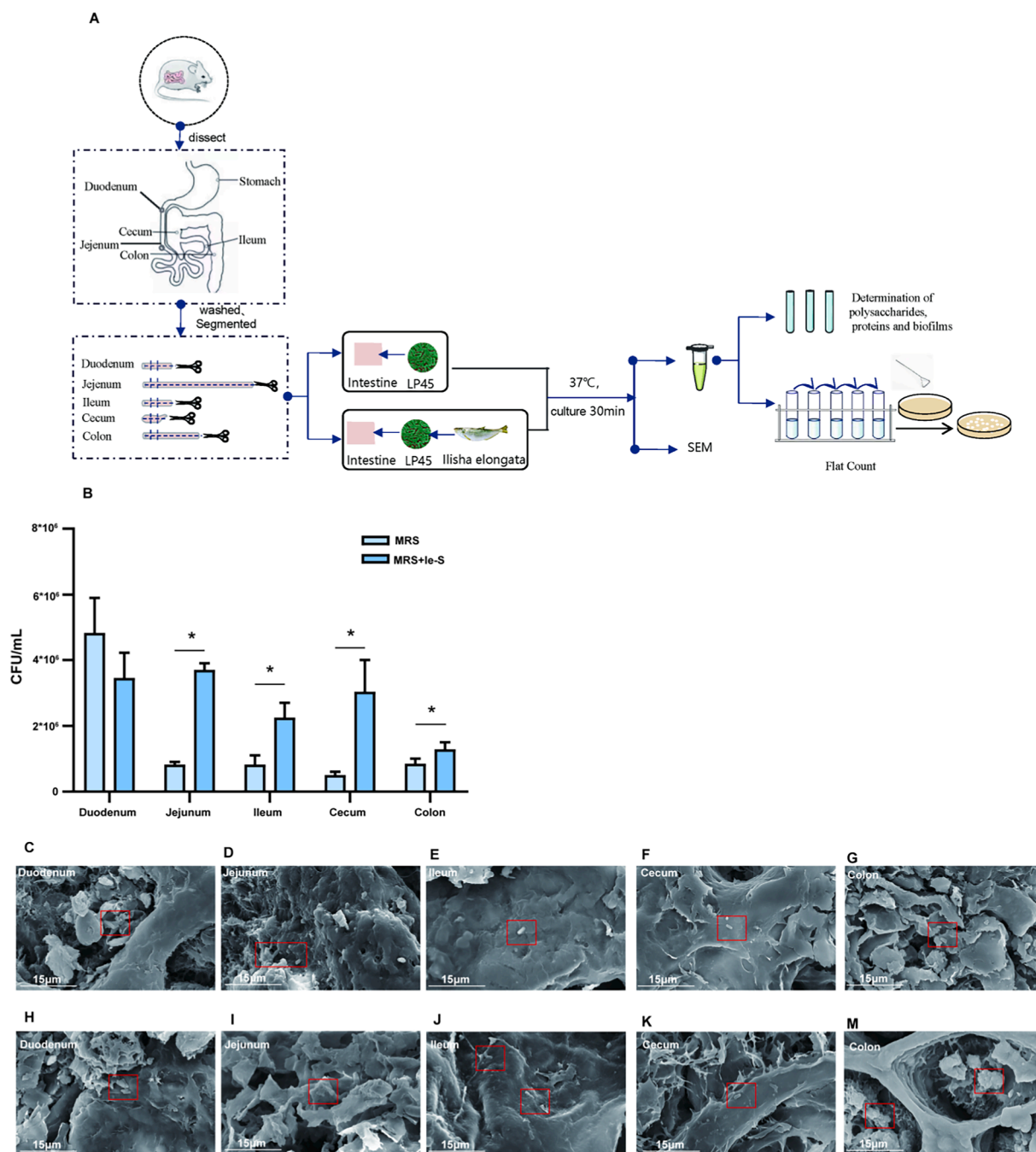

Fig. 4. Schematic diagram of the mouse ex vivo intestinal tissue adhesion model(A)The number of viable bacteria of LP45 adhering in the intestine (B); Scanning electron micrograph of LP45 adhering in the intestine of MRS experimental group (C-G); Scanning electron micrograph of LP45 adhering in the intestine of MRS + IeP experimental group (H-M). *$p < 0.05$.

epithelial cells is considered to be the first step in their pathogenesis, and when highly adherent lactobacilli colonize the intestine before pathogenic bacteria, they can form a physical barrier on the intestinal surface, thus blocking the contact between pathogenic bacteria and intestinal mucosa. (Zhang et al., 2010) The adhesion of different strains varies greatly, and even if the strains are the same, the adhesion varies due to differences at the strain level, and the adhesion of Lactobacillus is strain specific. For the same strain, the final adhesion results may also be different due to the different intestinal locations selected for adhesion. (Argyri, et al., 2013) The adhesion adhesion of probiotics is often used as an essential evaluation criterion of probiotics by researchers (Blum, et al., 1999, Yadav, Tyagi, Kaushik, Saklani, Grover, & Batish, 2013, Duary, Rajput, Batish, & Grover, 2011). Common models for assessing the adhesion adhesion of probiotics are in vitro cell adhesion model, in vitro mucin adhesion model, in vitro intestinal tissue model, and in vivo adhesion model. (Laparra, & Sanz, 2010) The in vitro intestinal tissue model can better simulate the actual environment in vivo in a short period of time.

Fig. 4A shows a schematic diagram of the mouse isolated intestinal tissue adhesion model. The number of viable bacteria is an important indicator that directly reflects the adhesion adhesion of a probiotic. As shown in Fig. 4B, the ability of Lactobacillus plantarum LP45 to adhere to different intestinal segments differed, with the highest number of viable bacteria adhering to the duodenal segment and the lowest number adhering to the cecum segment. The addition of 1 mg/mL *Ilisha elongata* protein to the MRS medium significantly increased the number

of viable bacteria adhering to the jejunum, ileum, cecum and colon segments for LP45. As shown in Fig. 4C-4 M, The internal structure of intestinal tissue, including intestinal villi, colonic crypts, and mucus, was clearly observed under electron microscopy. The probiotic bacteria were observed to adhere tightly to the inner layer of the intestinal segments under scanning electron microscopy, forming a biofilm-like state. The results of electron microscopy and the results of plate count of adherent viable bacteria reflected the adhesion of Lactobacillus plantarum in the intestine both qualitatively and quantitatively, and the results of colony count indicated that *Ilisha elongata* protein promoted the adhesion of Lactobacillus plantarum in the jejunum, ileum, cecum and colon segments.

3.4. Role of Ilisha elongata protein on changes in intestinal polysaccharides, proteins, and biofilms caused by LP45

The gut mucosa forms a protective barrier for epithelial cells and also provides an ecological niche for the microbiota (Belzer, 2021). This research quantified the changes in polysaccharide, protein, and biofilm induced by LP45 adhesion to the intestine, as shown in Fig. 5B. Compared to the blank intestinal segment control, the two experimental groups in the colon segment only significantly increased the intestinal polysaccharide content, and the experimental group with *Ilisha elongata* protein addition also increased the polysaccharide content relative to the MRS experimental group, but not statistically significant. The polysaccharide content was significantly reduced in the MRS
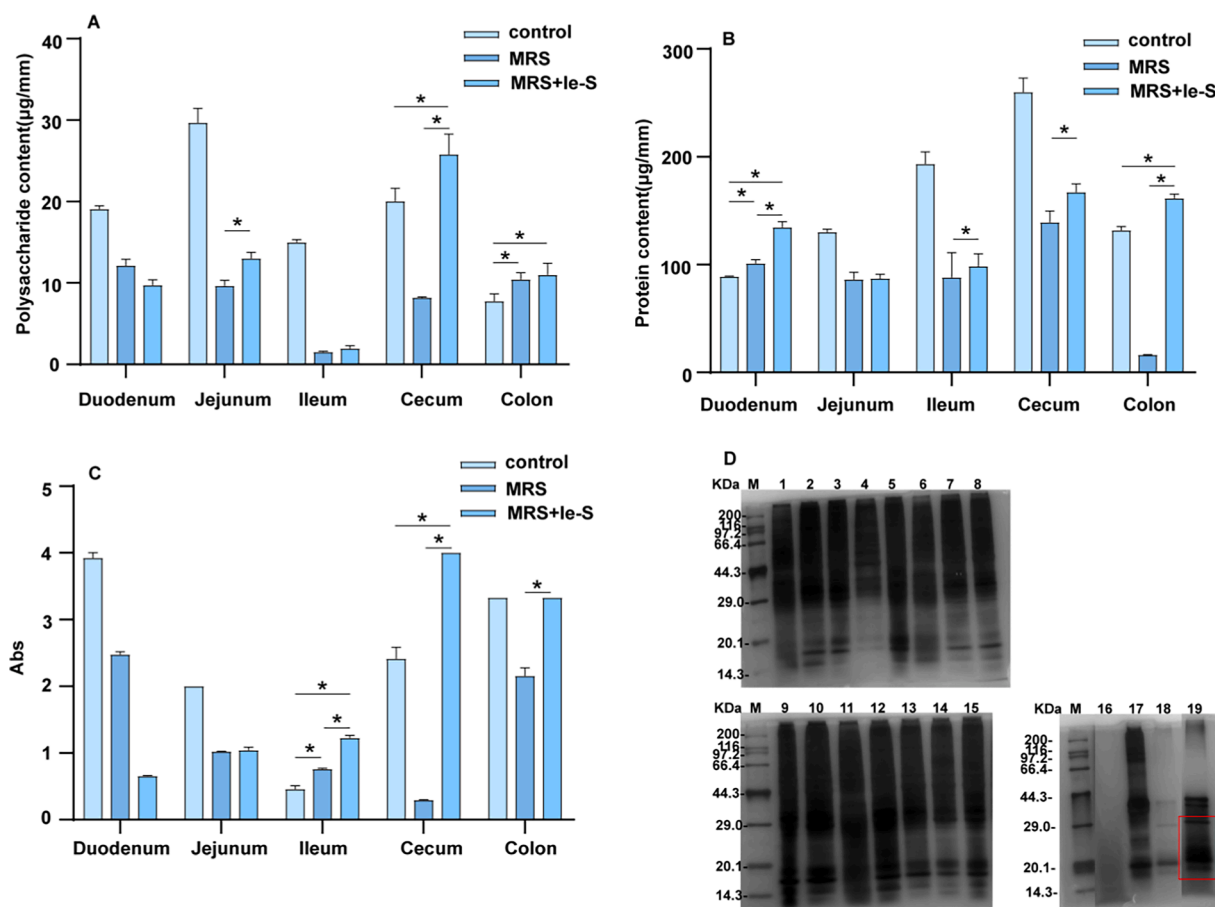

Fig. 5. Changes in sugar content in each intestinal segment (A); changes in protein content in each intestinal segment (B); changes in biofilm biomass in each intestinal segment (C);Protein molecular weight distribution of each intestinal segment (D); 1–5: protein molecular weight distribution of duodenum, jejunum, ileum, cecum, and colon in control group, respectively. 6–10: protein molecular weight distribution of duodenum, jejunum, ileum, cecum, and colon in MRS group, respectively. 11–15: protein molecular weight distribution of duodenum, jejunum, ileum, cecum, and colon in MRS + IeP group, respectively distribution. 16: protein molecular weight distribution of MRS medium. 17: protein molecular weight distribution of *Ilisha elongata* protein. 18: protein molecular weight distribution of LP45. 19: protein molecular weight distribution of *Ilisha elongata* protein culture LP45. control-blank intestinal segment control, IeP-*Ilisha elongata* protein.

experimental group compared to the blank cecum control group, but the *Ilisha elongata* protein-added experimental group significantly increased the polysaccharide content relative to both the control and MRS groups. It is possible that the *Ilisha elongata* protein promoted the secretion of extracellular polysaccharides when LP45 adhered to the cecum and colon. Extracellular polysaccharides may be involved in the biological activity of prebiotics, probiotics (Silva, Lopes Neto, & Cardarelli, 2019). The Exopolysaccharides (EPS) produced by probiotic bacteria have various health-improving effects such as antibacterial, immunomodulatory, anti-inflammatory, antioxidant, anti-tumor, anti-viral, antidiabetic, anti-ulcer, and cholesterol-lowering.

As shown in Fig. 5B, only the two duodenal segment experimental groups significantly increased the intestinal protein content compared to the blank intestinal segment control, and the experimental group with *Ilisha elongata* protein addition also significantly increased the protein content relative to the MRS experimental group. The protein content was significantly increased in the *Ilisha elongata* protein-added group compared to the cecum MRS group, suggesting that *Ilisha elongata* protein promoted the protein content produced by LP45 during its adhesion to the duodenum and cecum. Schroeder, B. O. et al. found that the addition of Bifidobacterium longum increased the growth of mucus in the intestinal mucosa and that the addition of probiotics stimulated the production of mucin in the intestinal mucosa. (Schroeder et al., 2017). It is hypothesized that *Ilisha elongata* protein can promote LP45 stimulated the production of mucin in the duodenum and cecum. Mucus and mucin glycans act as receptors for gastrointestinal microorganisms (Juge, 2012). Mucus-binding proteins (MUBs) have been revealed as one class of effector molecules involved in mechanisms of the adherence of lactobacilli (an important commensal bacteria in the gastrointestinal tract) to the host. (Boekhorst et al., 2006). Adhesins of Lactobacillus species are also derived from surface proteins and mediate the colonization of gastrointestinal epithelial cells (Yadav, Tyagi, Kaushik, Saklani, Grover, & Batish, 2013).

There are five stages for biofilm formation (Thien-Fah, et al.,2001). Stage I: initial attachment of bacterial cells to the surface substrate; Stage II: secretion of extracellular polymers by the attached bacteria, thereby promoting irreversible attachment of bacteria to the surface substrate/ the attached bacteria secret the extracellular polymers and thereby promoting the irreversible adhesion of bacteria to the surface substrate Stage III: initial formation and development of biofilm structural morphology; Stage IV: maturation of biofilm structural morphology; Stage V: Shedding of individual cells from the biofilm, the shed cells can re-attach and form a new biofilm. The structure of the biofilm appears to depend heavily on the production of a mucus-like extracellular polymer matrix (EPS) since it provides structural support for the biofilm. Biofilms play an important role in (defending?) inflammatory bowel disease, because of that the bacteria growing in biofilms are more resistant to antibiotics than independent organisms, (Macfarlane, & Dillon, 2007). As shown in Fig. 5B, two experimental groups with ileal intestinal segments only significantly increased intestinal biofilm biomass compared to the blank intestinal segment control group, and the experimental group with *Ilisha elongata* protein addition significantly increased intestinal biofilm biomass relative to the blank intestinal segment control group, MRS The experimental group with *Ilisha elongata* protein addition significantly increased the intestinal biofilm biomass compared to the blank intestinal segment control group and MRS group. Only the *Ilisha elongata* protein-added group significantly increased the intestinal biofilm biomass compared to the blank cecum control group, and the *Ilisha elongata* protein-added group also significantly increased the intestinal biofilm biomass compared to the cecum MRS group. This indicates that *Ilisha elongata* protein promoted the biofilm produced by LP45 when it adhered to ileum and cecum. Bacterial extracellular polysaccharides promote the formation of bacterial cell surface biofilms and act as a protective barrier (Das, Baruah, & Goyal, 2014, Hussain, Zia, Tabasum, Noreen, & Zuber, 2017, Angelin, & Kavitha, 2020). The changes of polysaccharides in duodenum, jejunum

and cecum showed the same trend with the changes of biofilm, but the changes of polysaccharides in ileum and colon did not show exactly the same trend with the changes of biofilm, which might be caused by other factors.Lactobacillus S-layer proteins are composed of protein or glycoprotein subunits in a regular crystalline arrangement, forming a solid surface layer capable of covering the entire cell surface, with molecular weights ranging from 25 to 71 KDa. As shown in Fig. 5D, the blank intestinal tissue was more complex with more large molecular weight proteins and fewer proteins less than29 KDa, and the distribution of small molecular weight proteins could be clearly seen. Compared with the control group of blank intestinal segment, the probiotic addition of small molecular weight proteins increased. The experimental group with *Ilisha elongata* protein addition increased the content of protein with molecular weight around 29 KDa. Compared with the protein molecular weight distribution of *Ilisha elongata* protein and LP45, several protein bands were increased in LP45 cultured with added *Ilisha elongata* protein, with molecular weights ranging from 20.1 to 29 KDa. Therefore, we hypothesized that the experimental group with *Ilisha elongata* protein addition promoted the production of probiotic S-layer protein.

4. Conclusion

In this study, eight different type of aquatic proteins with specific intestinal probiotics were analyzed to compare the protein-loving characteristics of different probiotics and the proliferation effects of aquatic proteins on LP45. The results showed that the addition of 1 mg/mL of *Ilisha elongata* salt-soluble protein to MRS can significantly reduce the growth generation time of LP45 and significantly increased the total number of colonies of LP45 after fermentation. In this study, we established an intestinal adhesion model with mice to evaluate the effect of *Ilisha elongata* salt soluble protein on the intestinal adhesion of LP45. The results showed that the number of adherent live bacteria in jejunum, ileum, cecum and colon segments was significantly increased by the addition of *Ilisha elongata* salt-soluble protein, the biomass of biofilm in ileum and cecum segments was significantly increased, the polysaccharide content in cecum and colon segments was significantly increased, and the protein content in duodenum and cecum segments was significantly increased. This study provides a basis for the production of extracellular polysaccharide, secretion of mucin, production of S-layer protein and biofilm formation in the process of probiotic adhesion promoted by *Ilisha elongata* protein. This study revealed the probiotic pro-value-added and adhesion-enhancing effects of *Ilisha elongata* salt-soluble protein, provided strong evidence of the promotional effect of aquatic proteins on intestinal health and their potential as active proteins. The mechanism of action of aquatic proteins for beneficial intestinal health gut homeostasis will be further investigated in our following research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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