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Fructooligosaccharides Modulate Quorum-Sensing–Mediated Biofilm Formation in *Lactiplantibacillus plantarum* C8 via Amino Acid and Carbohydrate Metabolic Pathways

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ABSTRACT: Oligosaccharides can support gut health by promoting the growth of beneficial bacteria like *Lactobacillus*, to help maintain gut balance and boost the immune system of the host. During the intestinal colonization, the forming biofilms can improve its adhesion and protect it from immune attacks and environmental stress. However, how oligosaccharides affect *Lactobacillus* biofilm formation and its probiotic effects remain unclear. This study examined how oligosaccharides influence *L. plantarum* C8 biofilm formation, growth, and function, using metabolomics to uncover underlying regulatory mechanisms. FOS markedly enhanced *L. plantarum* C8 self-aggregation (79.12%), surface hydrophobicity (32.82%), and adhesion (43.26%). Both FOS and MOS significantly upregulated biofilm-related genes, promoting biofilm maturity and stability. Quorum sensing analysis further revealed that FOS addition increased AI-2 production compared with planktonic and other biofilm samples. Furthermore, Non-targeted metabolomics has revealed that carbohydrate-related pathways, together with quorum sensing, constitute an integrated regulatory network governing biological membrane formation. Valine, leucine, and isoleucine biosynthesis pathway, was linked to enhanced extracellular polysaccharide (EPS) production. In conclusion, oligosaccharides, especially fructooligosaccharides, play a significant role in regulating the formation of biofilms mediated by quorum induction through multiple metabolic pathways.

Keywords: *Lactiplantibacillus plantarum* C8; biofilm; oligosaccharides; metabolomics

1. Introduction

Lactobacillus is a Gram-positive, non-spore-forming, rod-shaped bacterium that constitutes a substantial component of the normal human microbiome. Within this species, *Lactiplantibacillus plantarum* (*L. plantarum*) is distinguished by its multifaceted functional properties. It is morphologically characterized by a long, uniformly thick rod with blunt termini and is devoid of flagella and spores. Widely distributed in natural environments, *L. plantarum* is recognized as a typical facultative anaerobe, primarily producing lactic acid

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during fermentation ^[1]. Meanwhile, *L. plantarum* is extensively employed in the industrial production of fermented foods, including but not limited to sauerkraut, pickles, and various dairy products such as yogurt and cheese ^[2-4].

L. plantarum demonstrates robust carbohydrate fermentation capabilities alongside marked salt tolerance. In fermented food matrices, biofilms formed by *L. plantarum* have demonstrated notable resilience under harsh processing conditions. Additionally, previous investigations have indicated that the biofilm formed by *Lactobacillus paracei* on chitosan-modified polypropylene surfaces facilitates lactic acid fermentation. The findings suggest that this biofilm-based fermentation system significantly enhances lactic acid productivity. Additionally, previous investigations have indicated that the biofilm formed by *Lactobacillus paracei* on chitosan-modified polypropylene surfaces facilitates lactic acid fermentation. The findings suggest that this biofilm-based fermentation system significantly enhances lactic acid productivity. The system is characterized by high yield, operational continuity, and sustained stability, thereby maintaining elevated production levels over prolonged periods ^[5].

Biofilm is a complex, multilayered, aggregative community of microorganisms that adheres to both biotic and abiotic surfaces, enabling these organisms to persist within self-produced or acquired extracellular polymeric substances. In natural environments, microorganisms predominantly exist not as isolated planktonic cells but rather as constituents of biofilm ecosystems or as organisms attached to surfaces ^[6]. Compared with their planktonic counterparts, cells embedded in biofilms exhibit enhanced resistance to environmental stressors and antimicrobial agents. The extracellular polymers constituting biofilms principally comprise proteins, lipids, extracellular polysaccharides, extracellular DNA (eDNA), and extracellular RNA (eRNA) ^[7]. Notably, extracellular polysaccharide (EPS) synthesized by microorganisms is a critical component of biofilms; It not only facilitates the effective storage of water and nutrients but also augments the mechanical integrity of the biofilm. Moreover, exopolysaccharides enable the formation of a three-dimensional, highly adherent, and stable scaffold on surfaces, thereby immobilizing cells within the biofilm, enhancing intercellular adhesion and interactions, and ultimately contributing to a more compact biofilm structure ^[8].

This resilience of lactic acid bacteria (LAB) is primarily attributed to the robust extracellular matrix, which mitigates desiccation and minimizes nutrient depletion during fermentation ^[9]. It can also exhibit synergistic interactions when co-cultured with other LAB. However, in industrial environments, especially under conditions of acidic, carbohydrates sources, penetrating stress, and mechanical shear forces, these factors impose severe physiological limitations on lactic acid bacteria. To overcome these stress factors, lactic acid bacteria adopt a biofilm lifestyle, which is a crucial adaptive strategy. It goes beyond simple physical aggregation and ensures the survival and metabolic stability of the bacteria. As carbon sources, oligosaccharides also outperform their monosaccharide counterparts in promoting *Lactobacillus* biofilm formation, such as agave-derived fructans in particular drive robust exopolysaccharide production and dense biofilm assembly ^[10]. Synbiotic combinations of GOS with *Lactobacillus* starters have been demonstrated to optimize fermentation kinetics and catalytic efficiency ^[11]. Non-targeted metabolomics has revealed

significant differences in metabolite expression between biofilm-associated and planktonic cells of *Bifidobacterium spp* ^[12]. Metabolomics enables the characterization of the diversity and abundance of small molecules produced by microorganisms during processes such as biofilm formation. Previous studies employing non-targeted metabolomic analysis of *L. plantarum* L-ZS9 biofilm cells have demonstrated enhanced metabolic activity, particularly in amino acid and carbohydrate metabolism pathways, compared to their planktonic counterparts ^[13].

Lactiplantibacillus plantarum is metabolite expression between biofilm-associated, this article aims to explore whether oligosaccharides can influence the formation of biofilms by regulating the metabolic pathways and quorum sensing of *Lactiplantibacillus plantarum*. Various functional oligosaccharides were screened as the sole carbon source for the functional characteristics of the strain. Subsequently, the influence of these selected oligosaccharides on biofilm formation by *L. plantarum* C8 was systematically evaluated, and non-targeted metabolomics analysis was employed to identify and compare differential metabolites across experimental groups to elucidate the metabolic mechanisms underlying oligosaccharide-mediated biofilm development and survival enhancement under the harsh environment.

2. Material and methods

2.1. Bacterial and Materials

Lactiplantibacillus plantarum C8 (CGMCC No. 30504) was isolated from traditional fermented pickles in Zhoushan (Zhejiang, China) and is preserved at the Laboratory of Animal Product Processing, Ningbo University (Ningbo, China). MRS broth, MRS agar, and beef infusion powder were obtained from Qingdao Haibo Biotechnology Co., Ltd (Qingdao, China). Galactooligosaccharides (GOS) were provided by Beijing Coolaber technology Co., Ltd (Beijing, China) ($\geq 90\%$), mannose oligosaccharides (MOS) ($\geq 93\%$), fructooligosaccharides (FOS) ($\geq 95\%$) and isomaltooligosaccharides (IMO) were provided by Shanghai Yuanye Biotechnology Co., Ltd (Shanghai, China). Additional reagents including glucose, erythritol, raffinose, xylooligosaccharides, Tween 80, n-butanol, xylene, anhydrous ethanol, sodium chloride, and uranyl acetate were sourced from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Improved MRS medium was prepared using the following components per 100 mL of distilled water: 10 g peptone, 4 g yeast extract, 8 g beef extract, 5 g sodium acetate, 2 g potassium dihydrogen phosphate, 2 g diammonium hydrogen citrate, 0.2 g anhydrous magnesium sulfate, and 0.04 g anhydrous manganese sulfate. Additionally, 80 mL of Tween 80 and 20 g/L of the corresponding sugar (depending on the experimental condition) were added. The final pH was adjusted to (5.7 ± 0.02) before sterilization.

For the cell culture, Caco-2 human colorectal adenocarcinoma cells were purchased from Wuhan Bode Biological Co., Ltd (Hubei, China). Complete DMEM medium was composed of 45 mL Dulbecco's Modified Eagle Medium (DMEM), 5 mL fetal bovine serum (FBS), and 500 μ L penicillin-streptomycin solution. Pancreatin was obtained from Gibco (Thermo Fisher Scientific, Waltham, American), and the CCK-8 assay kit was purchased from Shanghai Shenger Biotechnology Co., Ltd (Shanghai, China). Peptone, anhydrous manganese sulfate, dimethyl sulfoxide (DMSO), fluorescein isothiocyanate (FITC), trypsin, bovine bile salts,

and pepsin were sourced from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China).

2.2. determination of self-aggregation ability of lactic acid bacteria

L. plantarum C8 was activated and inoculated into freshly prepared improved MRS broth at a 1% (v/v) inoculation ratio, followed by incubation at 37 °C for 10-12 hours. The culture was then transferred into sterile centrifuge tubes, and cells were harvested by centrifugation and washed three times with sterile PBS. The washed cell suspension was adjusted with PBS to an optical density at 600 nm (OD₆₀₀) of (1.00 ± 0.01), designated as A0. The suspension was gently mixed and allowed to stand at room temperature for 12 hours. During this period, the OD₆₀₀ of the upper phase was measured every 2 hours using a microplate reader, and recorded as A1. The self-aggregation rate was determined using the following equation:

$$\text{Self-aggregation rate(\%)} = (1 - A1/A0) \times 100$$

2.3. Determination of cell surface hydrophobicity of lactic acid bacteria

L. plantarum C8 was activated and inoculated into freshly prepared improved MRS broth at a 1% (v/v) inoculation ratio, followed by incubation at 37 °C for 10-12 hours. The culture was centrifuged, and the cells were washed three times with sterile PBS. The resulting bacterial suspension was adjusted with PBS to an OD₆₀₀ of (1.00 ± 0.01), referred to as B0. To assess cell surface hydrophobicity, 1 mL of xylene (organic phase) was added to 3 mL of the bacterial suspension, followed by thorough mixing. The mixture was allowed to stand undisturbed at room temperature for 4 hours until phase separation. The aqueous phase was collected and the OD₆₀₀ was measured as B1. The hydrophobicity rate was determined using the following equation.

$$\text{Hydrophobic rate(\%)} = (1 - B1/B0) \times 100$$

2.4 Determination of adhesion properties of LAB

2.4.1 Cytotoxicity evaluation on caco-2 cells

The cytotoxicity of *L. plantarum* C8 was evaluated using the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instructions. Caco-2 cells in good condition were seeded into 96-well plates and pre-incubated at 37 °C with 5% CO₂ for 24 hours to allow for adherence. After discarding the spent medium, pure DMEM medium was added, followed by 10 µL of bacterial suspension at different concentrations (1 × 10⁷, 1 × 10⁸, and 1 × 10⁹ CFU/mL) to each well. Each group included six replicates. The plates were incubated for an additional 2 hours, after which 10 µL of CCK-8 reagent was added to each well. The plates were then incubated for another 4 hours. The absorbance was measured at 450 nm using a microplate reader. Among them, the experimental group A1: Caco-2 cells, CCK-8, bacterial solution, pure DMEM medium; the control group A2: Caco-2 cells, CCK-8, pure DMEM medium; the blank group A3: CCK-8, pure DMEM medium. The Cell viability rate was determined using the following equation.

$$\text{Cell viability rate(\%)} = [(A1 - A3)/(A2 - A3)] \times 100$$

2.4.2 Determination of adhesion properties of *L. plantarum* c8 to caco-2 cells

Caco-2 cells in optimal growth condition were seeded into 6-well plates and incubated overnight at 37 °C with 5% CO₂ for monolayer formation. *L. plantarum* C8 was activated in improved MRS broth (1% inoculum)

and incubated at 37 °C for 10-12 hours. The bacterial culture was then centrifuged, washed three times with sterile PBS, and resuspended in PBS to achieve final concentrations of 1×10^7 , 1×10^8 , and 1×10^9 CFU/mL. The bacterial suspensions were stained with fluorescein isothiocyanate (FITC) for 30 minutes at room temperature, and excess dye was removed by repeated washing with PBS. The initial fluorescence intensity of the stained bacteria was recorded as C0. The labeled bacteria were pelleted by centrifugation (6000 rpm, 4 °C, 5 min), resuspended in an equal volume of pure DMEM medium, and 2 mL of the bacterial suspension was added to each well containing the Caco-2 monolayer. The plates were incubated in the dark for 2 hours under standard cell culture conditions. Following incubation, non-adherent bacteria were removed by gentle washing with sterile PBS. The wells were observed and imaged using an inverted fluorescence microscope. Subsequently, the adherent cells were digested using pancreatin, and the released bacterial fluorescence in the digestion supernatant was measured using a microplate reader to obtain the post-adhesion fluorescence intensity, C1. The adhesion rate was determined using the following equation.

$$\text{Adhesion rate(\%)} = \left(1 - \frac{C1}{C0}\right) \times 100$$

2.5 Preparation of *L. plantarum* C8 biofilm (BF) and planktonic (PL) cell samples

The method was slightly modified according to the a previous study ^[14]. To obtain biofilm samples, 10 mL of modified MRS broth containing a 1:100 dilution of *L. plantarum* C8 was added into sterile high-borosilicate glass culture dishes (diameter: 90 mm) and incubated statically at 37 °C for 24 hours to facilitate biofilm formation. Following incubation, the loosely associated planktonic cells and residual medium were gently removed by washing with sterile PBS. A small volume of PBS was then added, and the surface-adhered biofilm was carefully detached using a sterile cell scraper. The collected suspension was subjected to low-speed centrifugation (4000 rpm, 4 °C, 8 min), and the resulting pellet was retained as the biofilm (BF) sample. For preparation of planktonic (PL) cells, the activated *L. plantarum* C8 was inoculated into fresh modified MRS broth at a 1% inoculum, followed by static incubation at 37 °C for 24 hours. An appropriate volume of the culture was transferred into sterile centrifuge tubes, washed three times with sterile PBS, and resuspended to an OD₆₀₀ of (1.00 ± 0.01) to yield the PL sample. Both BF and PL samples were subsequently used for further experimental analysis.

2.6 Determination of the biofilm formation of *L. plantarum* C8

The method was slightly modified according to a previous study ^[15]. To evaluate biofilm formation, 200 µL of modified MRS broth containing a 1:100 dilution of *L. plantarum* C8 was inoculated into each well of a sterile 96-well microtiter plate. The plate was incubated statically at 37 °C for 24 hours to allow biofilm development. Following incubation, the supernatant containing planktonic cells and residual medium was carefully removed. Each well was gently washed three times with 250 µL of sterile PBS to eliminate non-adherent bacteria. Subsequently, 200 µL of 90% methanol was added to each well and left to fix the biofilm for 30 minutes. The methanol was then aspirated, and the plate was air-dried at room temperature. Thereafter, 100 µL of 0.1% crystal violet solution was added to each well for 15 minutes to stain the adherent biofilm.

Excess dye was removed by washing the wells three times with PBS, followed by air-drying. To solubilize the bound dye, 200 μ L of 33% glacial acetic acid was added to each well and incubated at 37 °C for 20 minutes. The optical density of the solubilized dye, indicative of biofilm biomass, was measured at 595 nm (OD₅₉₅) using a microplate reader.

2.7 Determination of live cell content in *L. plantarum* C8 biofilm

Biofilm samples were pretreated in accordance with the instructions provided in the Vazyme Annexin V-FITC/PI Apoptosis Detection Kit (Nanjing, China). Following staining, samples were subjected to flow cytometric analysis within one hour to ensure data accuracy and signal stability. Flow cytometry was performed using an Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA, USA). The acquired data were analyzed using FlowJo software version 10 (FlowJo LLC, Ashland, OR, USA), and the proportions of cells positive for FITC and/or PI staining were calculated to assess membrane integrity and cellular viability.

2.8 Quantitative analysis of the major constituents in the biofilm of *L. plantarum*

2.8.1 Determination of total sugar content in biofilm matrix

The method was slightly modified according to a previous study^[16]. EPS from *L. plantarum* C8 biofilms were extracted using an ultrasonic-assisted method. Biofilm samples were subjected to sonication at 30-70% amplitude and 20 kHz frequency for 5-10 minutes. To prevent thermal degradation of EPS, ultrasonic treatment was applied intermittently. Post-sonication, samples were cooled to room temperature and centrifuged at 8000 rpm (4 °C, 10 min). The supernatant was collected and sterilized through a 0.22 μ m membrane filter. Total carbohydrate content was determined using the phenol-sulfuric acid method. Glucose standard solutions (0 mg/L-100 mg/L) were prepared, and 1 mL of each was mixed with 1 mL of 60 g/L phenol solution, followed by rapid addition of 5 mL concentrated sulfuric acid. After thorough mixing, the mixtures were incubated in a 30 °C water bath for 30 min. Absorbance was measured at 490 nm using a spectrophotometer. Distilled water served as the blank control.

2.8.2 Determination of protein content in biofilm matrix

The detection of extracellular protein concentration was carried out in accordance with the instructions of the BCA protein concentration determination Kit (absin Biotechnology Co., Ltd., Shanghai, China).

2.9 Determination of the relative expression levels of genes in the biofilm of *L. plantarum* C8

Total RNA from both planktonic bacterial suspensions and biofilm samples of *L. plantarum* C8 was extracted using the HiPure Bacterial RNA Kit (Magen, Guangzhou, China), following the manufacturer's instructions. After that, the reverse transcription reaction was carried out according to the instruction manual of the Vazyme HiScript III All-in-one RT SuperMix Perfect for qPCR kit (Nanjing, China). Using a 20 μ L reaction system, cDNA was synthesized by reverse transcription with the extracted RNA as a template. The entire process was carried out using sterile and enzyme-free pipette tips and centrifuge tubes. The reverse transcription system was prepared on ice and gently pipetted 8-10 times to ensure thorough mixing of the reaction system. Then, it was briefly centrifuged and collected to the bottom of the tube.

2.10 Relative quantification of *LuxS* gene and *eps*-associated genes expression

RT-qPCR was conducted using the Vazyme ChamQ Universal SYBR qPCR Master Mix (Nanjing, China). The synthesized complementary DNA (cDNA), derived from total RNA extracted from biofilm and planktonic cells, served as the template. The 16S rDNA gene of *L. plantarum* C8 was employed as the internal reference gene for normalization of gene expression. Gene-specific primers targeting the *LuxS* gene and EPS-Associated gene were designed and synthesized by Hangzhou Youkang Biotechnology Co., Ltd (Hangzhou, China). The sequences of the primers are provided in Table 1. The RT-qPCR reaction system was prepared on ice, using sterile, RNase-free consumables to avoid contamination. After gentle mixing and brief centrifugation, amplification was performed under the RT-qPCR reaction procedures. The relative expression of the *LuxS* gene and EPS-Associated gene was quantified using the $2^{-\Delta\Delta C_t}$ method.

Table 1. Primers required for RT-qPCR reactions

primer name	Primer sequence(5' to 3')	Base number
<i>LuxS</i> -F	CAACCTAACAAGACCGCTAT	20 bp
<i>LuxS</i> -R	AACCACTCCGACAACCAA	18 bp
1818-F	GCGCTATTTTGGCGTCACG	20 bp
1818-R	AATATCGGCCACCCGTCGA	19 bp
1820-F	AAGTTCCTCGTAAGTATTTAACGTGACAAGTG	33 bp
1820-R	CATCAGACTATGGTAGATTGGTGTAATAAATGTG	34 bp
1825-F	CGTTGCCGAGCTCCAGG	17 bp
1825-R	CCGGAGTCGTCCCTTGCT	18 bp
1826-F	CTGCAAGTCTTACTTGTCGATGCTG	25 bp
1826-R	TGCAACCGTTAAAACTCGGTCA	23 bp
1827-F	ATTCGCAGGTGTTTCAATCGGT	23 bp
1827-R	CCCCCCACGAAACCAA	17 bp
16S rRNA-F	GCTCAACCGAAGAAGTGC	18 bp
16S rRNA-R	TAATCCTGTTGCTACCCATA	21 bp

2.11 Determination of production of AI-2 signaling molecules in the biofilm of *L. plantarum* C8

The method was slightly modified according to a previous study [17]. *Lactiplantibacillus plantarum* C8 preserved at -80 °C was revived by inoculation (1%, v/v) into 100 mL sterile MRS broth and incubated statically at 37 °C for 10-12 h. The strain was subcultured under identical conditions for three successive generations to ensure stable physiological status. Cells were harvested by centrifugation (6,000 rpm, 4 °C, 5 min), washed three times with sterile PBS, and resuspended in sterile distilled water to an optical density of OD₆₀₀ of (1.00 ± 0.05). The standardized suspension was subsequently inoculated (1%, v/v) into fresh MRS broth under co-culture conditions and incubated at 37 °C for 12 h. After incubation, cultures were centrifuged (6,000 rpm, 4 °C, 5 min), and the supernatant was collected for further analysis. For biofilm samples, 10 mL of diluted culture (1:100) was added to sterile borosilicate dishes (90 mm diameter) and incubated at 37 °C for 24 h to allow biofilm formation. After PBS washing to remove Planktonic cells and culture medium, the biofilm was scraped into sterile PBS and collected by centrifugation (4000 rpm, 4 °C, 8 min). *Vibrio harveyi* BB170 was activated in AB medium (1% v/v) and cultured (30 °C, 120 rpm) to an OD₆₀₀ of 0.6-0.8, then diluted 1:5000 into fresh AB medium. A total of 1 mL of either planktonic or biofilm-derived supernatant was added to 100 mL of the *V. harveyi* BB170 assay culture. Fluorescence intensity (excitation/emission: 485/538

nm) was measured using a microplate reader every hour for the first 6 h, and every 3 h thereafter. AI-2 activity was inferred from the fluorescence curve.

2.12 Determination of the microscopic morphological characteristics of the biofilm of *L. plantarum* C8

2.12.1 The formation of biofilms was observed by scanning electron microscopy (SEM)

Biofilm samples were prepared for scanning electron microscopy (SEM) by cultivating *L. plantarum* C8 in modified MRS Broth (1:100 dilution) on sterile glass coverslips (14 mm diameter) placed in 24-well plate. Following incubation at 37 °C for 24 h, Planktonic cells and culture were removed, and the biofilms were fixed with 4% glutaraldehyde at 4 °C for 12 h. After fixation, samples were rinsed with PBS and dehydrated through a graded ethanol series (30%–100%), followed by critical point drying with liquid CO₂. The dried specimens were sputter-coated with gold and observed under a Hitachi S-3400N SEM in high vacuum mode.

2.12.2 The formation of biofilms was observed by laser confocal scanning microscopy (CLSM)

2 mL modified MRS broth containing a 1:100 diluted bacterial suspension was added to a confocal culture dish and incubated statically at 37 °C for 24 h to allow biofilm formation. After removing planktonic cells and washing with sterile PBS, the biofilm was fixed with 4% glutaraldehyde for 20 min. Following additional PBS washes, the biofilm was stained with FITC (0.1 mg/mL) in the dark for 20–30 min and rinsed thoroughly to eliminate excess dye. The three-dimensional architecture of the biofilm was visualized using a Zeiss LSM880 confocal laser scanning microscope.

2.13 Non-targeted metabolomics analysis of the effect of oligosaccharides on the biofilm of *L. plantarum* C8

2.13.1 Sample preparation

L. plantarum C8 was activated and inoculated into fresh modified MRS broth at 1% (v/v), followed by static incubation at 37 °C for 24 h. For PL samples, the bacterial suspension was collected, washed three times with sterile PBS, and adjusted to an OD₆₀₀ of (1.00 ± 0.01). For BF samples, 10 mL of modified MRS broth containing a 1:100 dilution of the bacterial suspension was added to a sterile borosilicate culture dish (90 mm diameter) and incubated under the same conditions. After removing planktonic cells with PBS, the adhered biofilm was collected using a sterile cell scraper and harvested by centrifugation (4000 r/min, 4 °C, 8 min). Six representative groups, selected from the eight groups described in Section 2.5, were subjected to non-targeted metabolomics analysis. The experimental design is summarized in Table 2.

Accurately weighed PL and BF samples were transferred to 2 mL centrifuge tubes and extracted with 600 µL methanol containing 4 ppm 2-amino-3-(2-chlorophenyl)-propionic acid. After vortexing for 30 s, 100 mg of glass beads were added, followed by homogenization at 60 Hz for 90 s and ultrasonic extraction at (24 ± 1) °C for 15 min. The samples were then centrifuged at 16,000 g, 4 °C for 10 min, and the supernatants were filtered through 0.22 µm membranes prior to LC-MS analysis.

Table 2. List of related groups used in this experiment

group	Sample type
GLU-PL	Planktonic sample
FOS-PL	Planktonic sample

MOS-PL	Planktonic sample
GLU-BF	Biofilm sample
FOS-BF	Biofilm sample
MOS-BF	Biofilm sample
QC	Equal amounts of sample samples are mixed

2.13.2 Chromatographic conditions

Metabolite separation was performed using a Thermo Vanquish UHPLC system (Waltham, USA) equipped with an ACQUITY UPLC® HSS T3 column (2.1 × 100 mm, 1.8 μm; Waters, Waltham, USA). The injection volume was 2 μL, with a flow rate of 0.25 mL/min and a column temperature of 40 °C. For LC-ESI(+)-MS analysis, the mobile phases were 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). The gradient elution was as follows: 2% A (0–1 min), 2–50% A (1–9 min), 50–98% A (9–12 min), 98% A (12–13.5 min), 98–2% A (13.5–14 min), and 2% A (14–20 min). For LC-ESI(–)-MS analysis, mobile phases were acetonitrile (C) and 5 mM ammonium formate in water (D), with the same gradient profile applied.

2.13.3 Mass spectrometry conditions

Mass spectrometric analysis was conducted using a Thermo Q Exactive Focus system (Waltham, USA) equipped with an electrospray ionization (ESI) source operating in both positive and negative ion modes. Spray voltages were set at +3.5 kV and –2.5 V, respectively. Sheath gas and auxiliary gas pressures were maintained at 30 and 10 arb. Full MS scans (m/z 81–1000) were acquired at a resolution of 70,000 with a capillary temperature of 325 °C. Data-dependent MS/MS acquisition employed higher-energy collisional dissociation (HCD) at 30% collision energy, with a resolution of 17,500. The top three most intense ions were selected for fragmentation with dynamic exclusion to minimize redundant MS/MS acquisition.

2.14 Statistical Analysis

All statistical analyses were performed using one-way ANOVA in SPSS version 26 to test for significance, and multiple comparisons were performed using LSD and Duncan's tests. The P -value < 0.05 was considered statistically significant. All values were expressed as mean ± standard deviation ($\bar{X} \pm SD$). Metabolite identification was performed based on accurate mass measurements ($\text{ppm} < 30$) and MS/MS spectral matching against databases including MassBank, LipidMaps, mzCloud, KEGG and HMDB. Data preprocessing and multivariate statistical analyses were conducted using Ropls, with differential metabolites screened based on $VIP > 1$ and $P < 0.05$. The difference in letters among different groups indicates significant variations.

3. Results

3.1 The influence of oligosaccharides on the adhesion related properties of *L. plantarum* C8

Auto-aggregation of *L. plantarum* C8 depended strongly on the carbon source (Fig. 1A). Although GOS induced the fastest initial aggregation between 2 and 6 h (25.88 % → 44.20 %, $P < 0.05$), FOS overtook GOS thereafter and continued rising steadily to reach 79.12 % at 12 h, the highest of all substrates ($P < 0.05$). Surface hydrophobicity mirrored this trend: cells grown on FOS exhibited the greatest hydrophobicity

(32.82 %, $P < 0.05$), compared with GLU (29.11 %), MOS (30.25 %) and GOS (26.70 %) (Fig. 1B), supporting a mechanistic link between hydrophobicity and self-aggregation. Cytotoxicity assays against Caco-2 monolayers confirmed negligible toxicity of C8 at all tested concentrations (Fig. 1C), indicating safety for epithelial applications. Finally, adhesion assays demonstrated that FOS- and MOS-grown *L. plantarum* C8' s cells adhered significantly better to Caco-2 cells (43.26 % and 40.63 %, respectively, $P < 0.05$) than GLU controls (28.38 %), whereas GOS reduced adhesion to 18.82 % ($P < 0.05$, Figs. 1D and E). Collectively, FOS as a sole carbon source significantly enhances *L. plantarum* C8' s aggregation, surface hydrophobicity and intestinal-cell adhesion ($P < 0.05$).

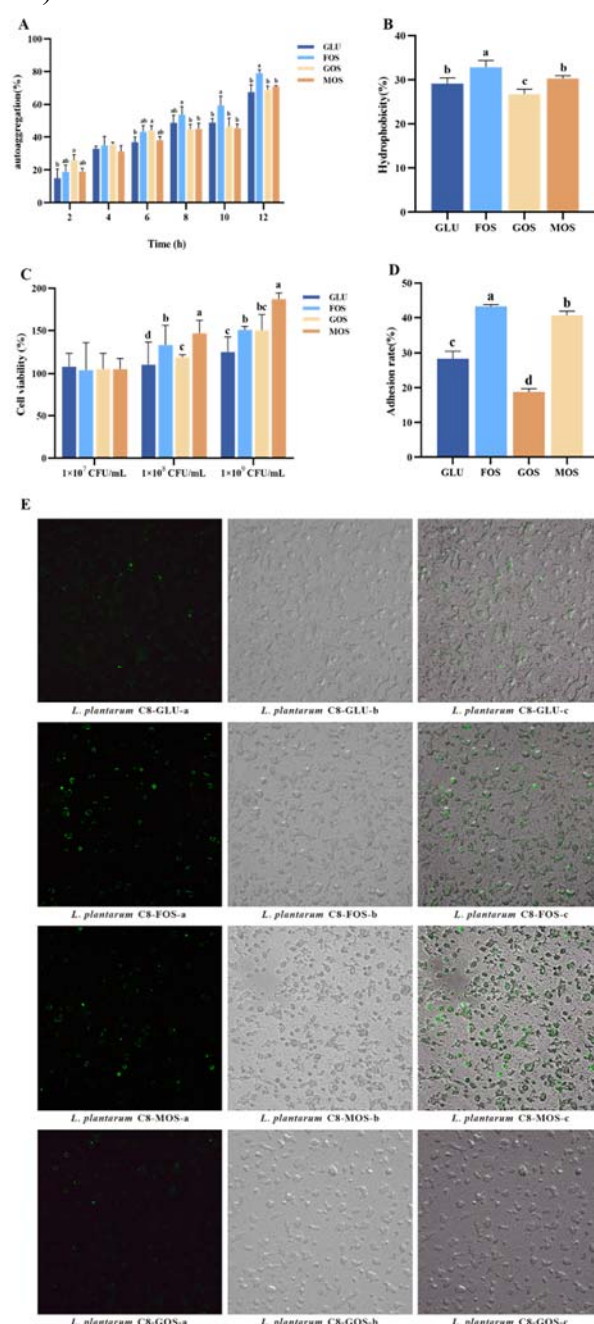


Figure 1. (A) Effect of different carbon source culture conditions on the self-aggregation ability of strains. (B) Effect of different carbon source culture conditions on the hydrophobicity of strains. (C) Toxic effects of strains with different carbon sources culture conditions on Caco-2 cells. (D) Effect of strains with different carbon source culture on the adhesion properties of Caco-2 cells. (E) Fluorescence of Caco-2 cell adhesion by strains with different carbon source culture conditions. Among them, a: Fluorescence plot, b: bright field plot, and c: superimposed plot. Three in each group are parallel. The difference in letters among different groups indicates significant variations.

3.2 Effect of oligosaccharides on biofilm formation by *L. plantarum* C8

Crystal violet staining revealed distinct differences in biofilm formation under different carbon sources. The GOS group exhibited the weakest biofilm formation, evidenced by the lightest staining, whereas the FOS group demonstrated the most complete biofilm morphology and darkest staining, indicating enhanced biofilm development (Fig. 2A). Quantitative analysis further confirmed these observations. Following staining and dissolution with 33% glacial acetic acid at 37 °C for 20 minutes, the OD₅₉₅ values were measured: the MOS group showed the highest absorbance at 3.41 ($P < 0.05$), followed by the FOS group at 3.35 and the GLU control group at 3.05 (Fig. 2B). These results were consistent with the staining intensity observed visually, underscoring the superior biofilm-forming capacity associated with MOS and FOS as carbon sources.

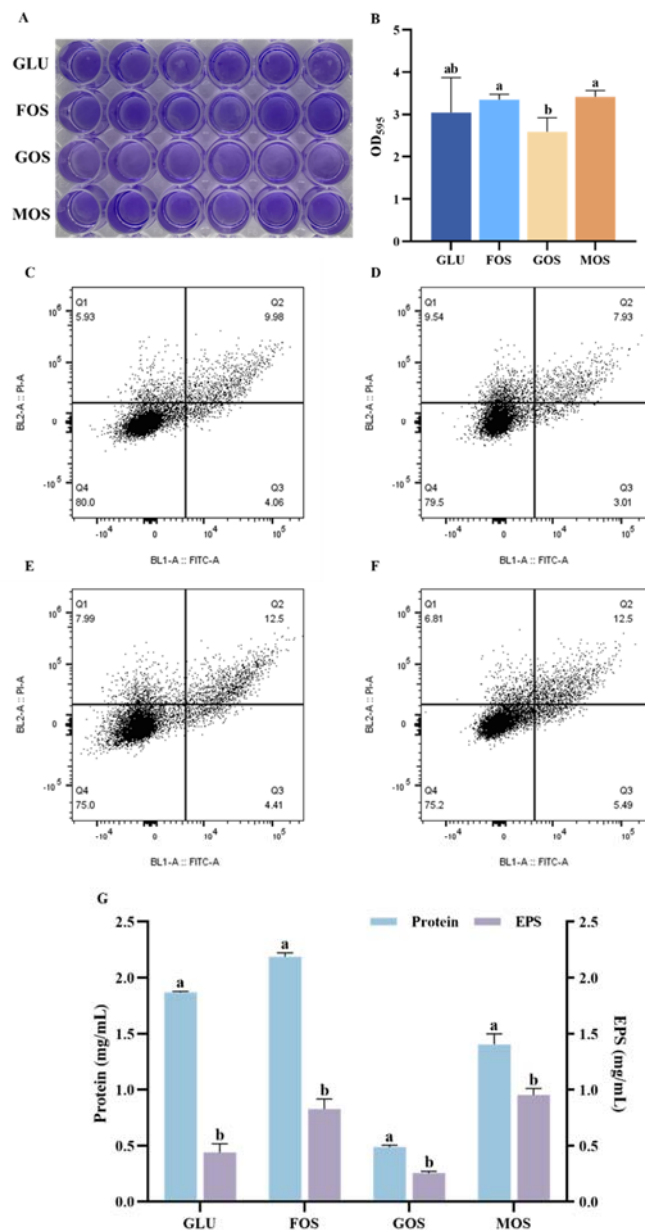


Figure 2. (A) Effect of different carbon source culture conditions on biofilm formation of bacterial strains. The biofilm sample stained with crystal violet. (B) Quantitative analysis of crystal violet staining of biofilms. Effect of different carbon source culture conditions on viable cells in biofilms of bacterial strains. (C) GLU, (D) FOS, (E) GOS, and (F) MOS. (G) Content of components in biofilm under different carbon source culture conditions. Three in each group are parallel. The difference in letters among different groups indicates significant variations.

3.3 Effect of oligosaccharides on the viable cell density within the biofilm of *L. plantarum* C8

In this study, FITC and PI were employed as DNA-binding fluorescent probes to differentiate cell viability within biofilms: green fluorescence indicated live cells, red denoted dead cells, and dual-stained cells represented mechanically damaged cells. Live cell proportions in the GLU and FOS groups reached 80.0% and 79.5%, respectively, with corresponding dead cell populations of 9.98% and 7.93%. In contrast, the GOS and MOS groups exhibited lower viability, with live cell proportions of 75.0% and 75.2% (Fig. 2C to F). It is noteworthy that in all groups, the proportion of mechanically damaged cells (Q1 region) exceeded 5%, suggesting that cell death and lysis may contribute to biofilm-associated biological processes. Previous studies have demonstrated that explosive cell lysis can release intracellular components, including cytosolic proteins, extracellular DNA (eDNA), and membrane vesicles, which serve as key constituents of the *Pseudomonas aeruginosa* biofilm matrix [18]. Moreover, the lysis of *P. aeruginosa* cells and the subsequent release of DNA within biofilms have been reported to occur in a spatially regulated manner, indicating a programmed cell death-like process. This regulated lysis facilitates the accumulation of eDNA, an important matrix component that plays a critical role in maintaining the structural integrity and stability of the biofilm [19].

3.4 The impact of oligosaccharides on the key components of *L. plantarum* c8 biofilm formation

The changes in protein and EPS contents of *L. plantarum* C8 biofilms under different carbon source conditions are shown in Figure 2g. When GOS served as the sole carbon source, both protein and EPS contents were significantly lower than those observed in the other groups ($P < 0.05$), which may be attributed to the limited capacity of GOS to support biofilm formation, thereby leading to reduced biofilm production. Correspondingly, the overall biofilm yield under this condition was also markedly low. In contrast, the FOS group exhibited the highest protein content, which was significantly greater than that of the GLU group ($P < 0.05$). Although the protein content in the MOS group was relatively low, the EPS content was the highest and significantly exceeded that of the other groups ($P < 0.05$). This phenomenon may be attributed to the upregulated expression of EPS-related genes, which facilitated biofilm formation and enhanced polysaccharide accumulation.

3.5 The effect of oligosaccharides on the relative expression levels of *eps* genes in *L. plantarum* C8 biofilms

Whole-genome analysis of *L. plantarum* C8 identified genes associated with EPS synthesis. Figure 3A illustrates the complete gene cluster involved in extracellular polysaccharide production, highlighting the arrangement and functions of the relevant genes within the cluster. Five key genes from the EPS gene cluster were selected for RT-qPCR analysis (Figs 3B and C). In PL samples, the presence of oligosaccharides as the sole carbon source led to increased expression of EPS-related genes compared to the GLU control group ($P < 0.05$). However, oligosaccharides exert a limited effect on the expression of EPS-related genes, with generally low relative expression levels ($P < 0.05$). This suggests that EPS synthesis is not an active process in planktonic bacteria. In contrast, BF samples exhibited significantly higher expression of EPS-related genes, particularly under MOS treatment, indicating a critical role for EPS synthesis in biofilm formation ($P < 0.05$).

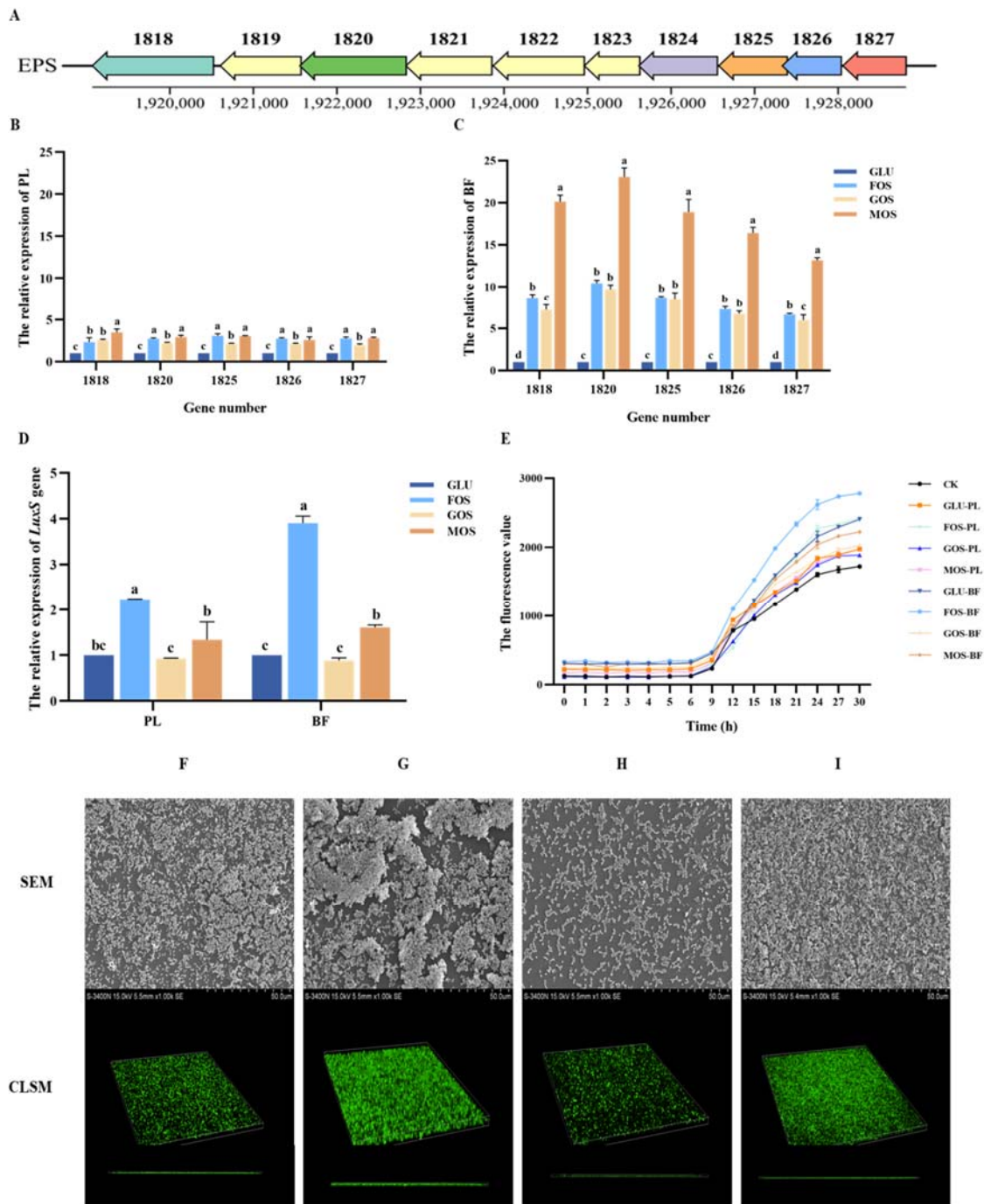


Figure 3. (A) Cluster of genes related to EPS synthesis. (B) The expression levels of EPS-related genes in the state of planktonic-state bacteria. (C) The relative expression level of EPS genes in the biofilm state. (D) Effects of different carbon source culture conditions on the relative expression of *LuxS* gene in strain. (E) Effect of different carbon source culture conditions on signalling molecules of strain AI-2. Scanning electron microscopy results of biofilm morphology under different carbon source culture conditions and CLSM results of biofilms under different carbon source culture conditions. (F) GLU, (G) FOS, (H) GOS, and (I) MOS. Three in each group are parallel. The difference in letters among different groups indicates significant variations.

3.6 The effect of oligosaccharides on the relative expression levels of the *LuxS* gene and AI-2 signaling molecules in *L. plantarum* C8 biofilms.

LuxS-mediated quorum sensing in *L. plantarum* C8 is markedly influenced by the available carbon source ($P < 0.05$, Fig. 3D). Under biofilm (BF) conditions, *LuxS* transcript levels were consistently higher than in planktonic (PL) cultures ($P < 0.05$), confirming its role in biofilm maturation. FOS induced the greatest up-

regulation of *LuxS* ($P < 0.05$), followed by MOS ($P < 0.05$), whereas galactooligosaccharides GOS slightly repressed expression relative to GLU controls ($P < 0.05$). Parallel measurements of AI-2 activity using *V. harveyi* BB170 revealed low signal in PL cultures but substantially elevated levels in BF samples, with a clear rise from 12 to 24 h ($P < 0.05$, Fig. 3E). Again, FOS-supplemented cultures produced the strongest AI-2 signal (PL > 2200 RLU, BF > 2500 RLU, $P < 0.05$), underscoring FOS as the optimal carbon source for enhancing both *LuxS* expression and autoinducer secretion in *L. plantarum* C8 biofilms.

3.7 The influence of oligosaccharides on the microscopic morphological characteristics of *L. plantarum* C8 biofilm

Biofilm formation by *L. plantarum* C8 after 24 hours of incubation was analyzed using SEM and CLSM (Figs. 3F-I). SEM images show that under GOS treatment, biofilm formation was limited, with sparse and small bacterial communities. In contrast, GLU-treated samples exhibited distinct grooves and localized bacterial clusters, indicating biofilm maturation. When MOS served as the sole carbon source, a dense and uniformly distributed bacterial layer with numerous surface protrusions was observed, consistent with crystal violet staining results.

CLSM analysis at 40× magnification further confirmed the impact of different carbon sources. FOS-treated samples exhibited the highest fluorescence intensity, indicating dense bacterial aggregation. The MOS group also showed considerable fluorescence, though less than the FOS group. In comparison, both GLU and GOS groups demonstrated weaker fluorescence signals. Vertical cross-sectional views revealed that the FOS group developed the thickest biofilm (8.14 μm), followed by MOS (5.52 μm), while GLU and GOS groups formed significantly thinner biofilms (4.72 μm and 4.28 μm , respectively). These findings suggest that carbon source type markedly influences the biofilm structure and biomass of *L. plantarum* C8.

3.8 Multivariate statistics and analysis of overall metabolites

Hierarchical clustering (Figs. 4A and B) revealed tight intra-group metabolite concordance — GLU-PL and FOS-PL samples co-cluster with only minor deviations, whereas MOS-PL forms a separate, more heterogeneous clade. The GLU-BF is most uniform, FOS-BF shows moderate dispersion and MOS-BF highest heterogeneity. PCA (Figs. 4C and D) confirmed analytical stability and captured 53.9 % (positive mode) and 54.7 % (negative mode) of variance (R^2X), with clear separation of all six treatment groups. PLS-DA (Figs. 4E-H, Table 3) further discriminated treatments: score plots partition intra- versus inter-group variance, and models achieved R^2Y/Q^2 of 0.998/0.992 (positive) and 0.996/0.983 (negative), indicating excellent fit and predictive robustness.

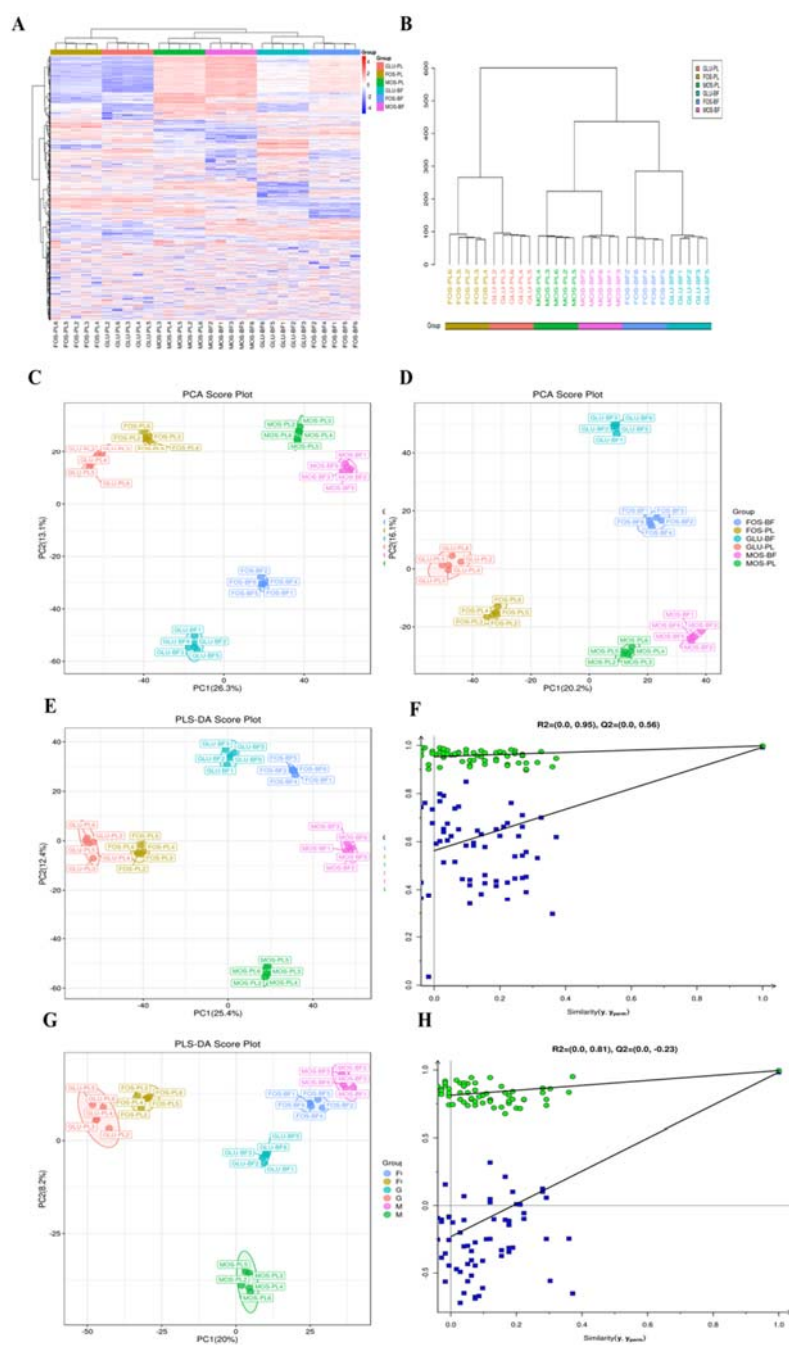


Figure 4. (A) Heat map of metabolite clustering. (B) Tree map of metabolite clustering. (C) PCA score graph in the positive ion mode. (D) PCA score graph in negative ion mode. (E) PLS-DA score graph of positive ion mode. (F) PLS-DA displacement test graph of positive ion mode. (G) PLS-DA score graph of negative ion mode. (H) PLS-DA displacement test graph of negative ion mode.

Table 3. List of PLS-DA model parameter

Comparable group	Mode	R2X(cum)	R2Y(cum)	Q2(cum)
GLU-PL vs FOS-PL vs MOS-PL vs GLU-BF vs FOS-BF vs MOS-BF	Positive ion	0.454	0.998	0.992
	Negative ion	0.282	0.995	0.983

3.9 Primary differential metabolite identification-*ms* and secondary differential metabolite identification-*MS/MS*

Differential metabolites were identified from the primary substance list of the samples and screened using a preset *P*-value threshold (≤ 0.05) and VIP threshold (> 1.0) in statistical tests. A total of 7,476 differential

metabolites were identified across all groups, with significant variability in the number of differential metabolites between comparison groups (Table 4 and Fig. 5F). The GLU-BF vs MOS-BF comparison exhibited the highest number of differential metabolites (3594, 48.1% of all metabolites), indicating the most substantial metabolic differences between these groups. Conversely, the MOS-PL vs MOS-BF comparison showed the least difference (2958 metabolites), suggesting minimal metabolic fluctuation. The overlap of differential metabolites among multiple sets was visualized using an Upset Venn diagram, facilitating the analysis of complex intersections across groups. The Upset Venn diagram highlights common and specific metabolites across the experimental groups (Figs. 5A-E). Chemical classification of the identified metabolites (Table 5) revealed that lipids and lipid-like molecules (192), organic acids and derivatives (169), and organic heterocyclic compounds (138) were the most abundant. Nucleosides, nucleotides, and their analogues (36) were also notable, contributing to the formation of lactic acid bacteria biofilms through roles in energy supply, signal transduction, surface adhesion, EPS synthesis, and stability. Organic nitrogen compounds (11) may be involved in intercellular signal transduction, particularly regulating quorum sensing.

Table 5. Types and numbers of differential metabolites

Types	count
Lipids and lipid-like molecules	192
Organic acids and their derivatives	169
Organic heterocyclic compounds	138
Organic oxygen compound	99
Benzene derivatives	85
Nucleosides, nucleotides and their analogues	36
Phenylpropyl and polyketone compounds	27
Organic nitrogen compounds	11
Hydrocarbon compounds	11
Alkaloids and their derivatives	8
Organic sulfur compounds	5
Organic halogen compounds	3
Homogeneous non-metallic compounds	3
Metal/non-metal mixed compounds	2
Lignans, neolignans and related compounds	2
Organic metal compounds	2
Hydrocarbon derivatives	1
Homogeneous metal compounds	1
Organophosphorus compounds	1
Organic 1, 3-dipole compounds	1

Table 4. Statistical table of differential metabolites

Group	Total metabolites	Increase the number of differential metabolites	Reduce the number of differential metabolites	Total number of differential metabolites
GLU-BF vs GLU-PL	7476	2233	1122	3355
FOS-BF vs FOS-PL	7476	2204	1304	3508
MOS-BF vs MOS-PL	7476	1608	1350	2958
FOS-BF vs GLU-BF	7476	1922	1267	3189
FOS-BF vs GLU-BF	7476	2324	1270	3594

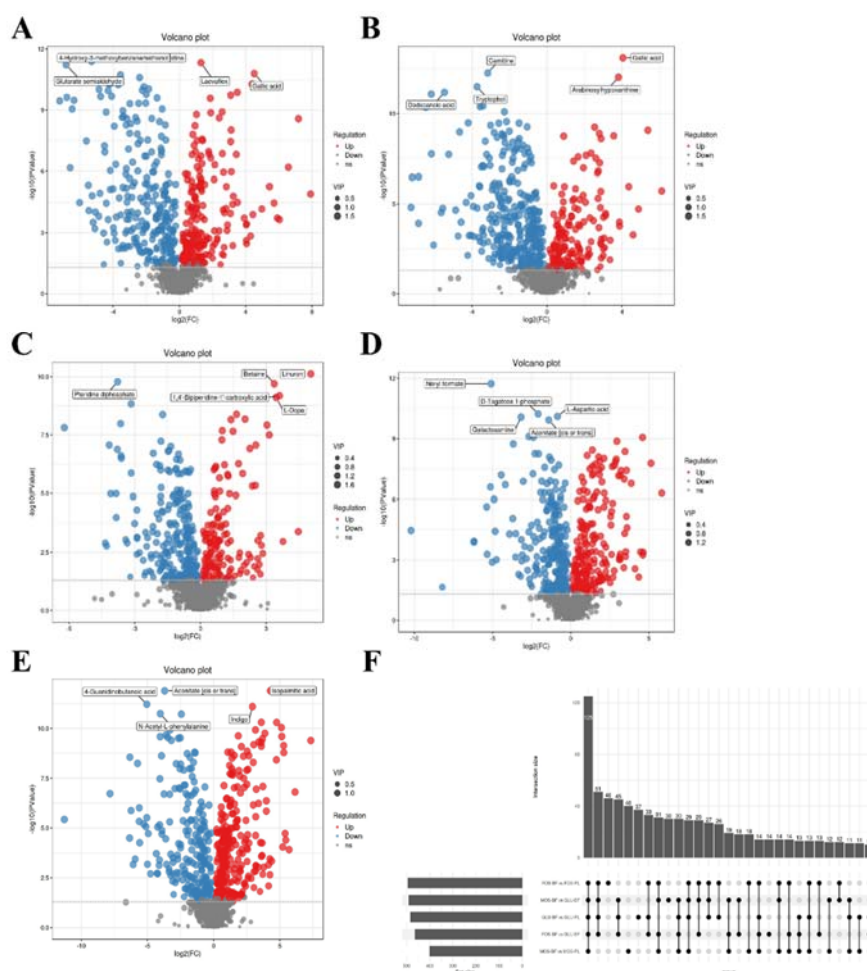


Figure 5. Volcano map of differential metabolites. (A) GLU-BF vs GLU-PL, (B) FOS-BF vs FOS-PL, (C) MOS-BF vs MOS-PL, (D) FOS-BF vs GLU-BF, and (E) MOS-BF vs GLU-BF. (F) Venn diagram of differential metabolites.

3.10 Analysis of secondary differential metabolites in the metabolic pathways

Six groups were selected and differential metabolite analysis was conducted between each pair. Figures 5A-E show a total of 484 differential metabolites in the GLU-BF vs GLU-PL group, 495 in the FOS-BF vs FOS-PL group, and 400 in the MOS-BF vs MOS-PL group, indicating significant differences across treatment groups. Cluster analysis of the top 50 metabolites with significant differences between biofilm and planktonic states. Figure 6A revealed notable metabolic differences. In the GLU-BF group, L-lactic acid, a major metabolite of lactic acid bacteria, helps lower environmental pH, activate H^+ -ATPase, and induce an acid stress response, indirectly promoting biofilm formation. Intermediates such as D-tagatose-1-phosphate, DHAP, glyoxylic acid, and succinic acid provide carbon sources for EPS synthesis and impact membrane formation, with succinic acid contributing energy through the tricarboxylic acid cycle (TCA). Cluster analysis of the top 50 metabolites in the FOS-BF vs FOS-PL group, Figure 6B highlighted increased abundance of fatty acids (lauric acid, citric acid, valenyl carnitine) and derivatives, along with glycerophosphate choline, a phospholipid component likely involved in biofilm construction and stabilization. Similarly, Figure 6C shows that in the MOS-BF group and the MOS-PL group, the expressions of intermediates such as succinic acid, α -ketoglutaric acid and DHAP were upregulated. This indicates that the energy metabolism activities within the biofilm were significantly enhanced, and the generation levels of ATP and NADH increased, thereby providing

sufficient energy support for the formation and maintenance of the biofilm.

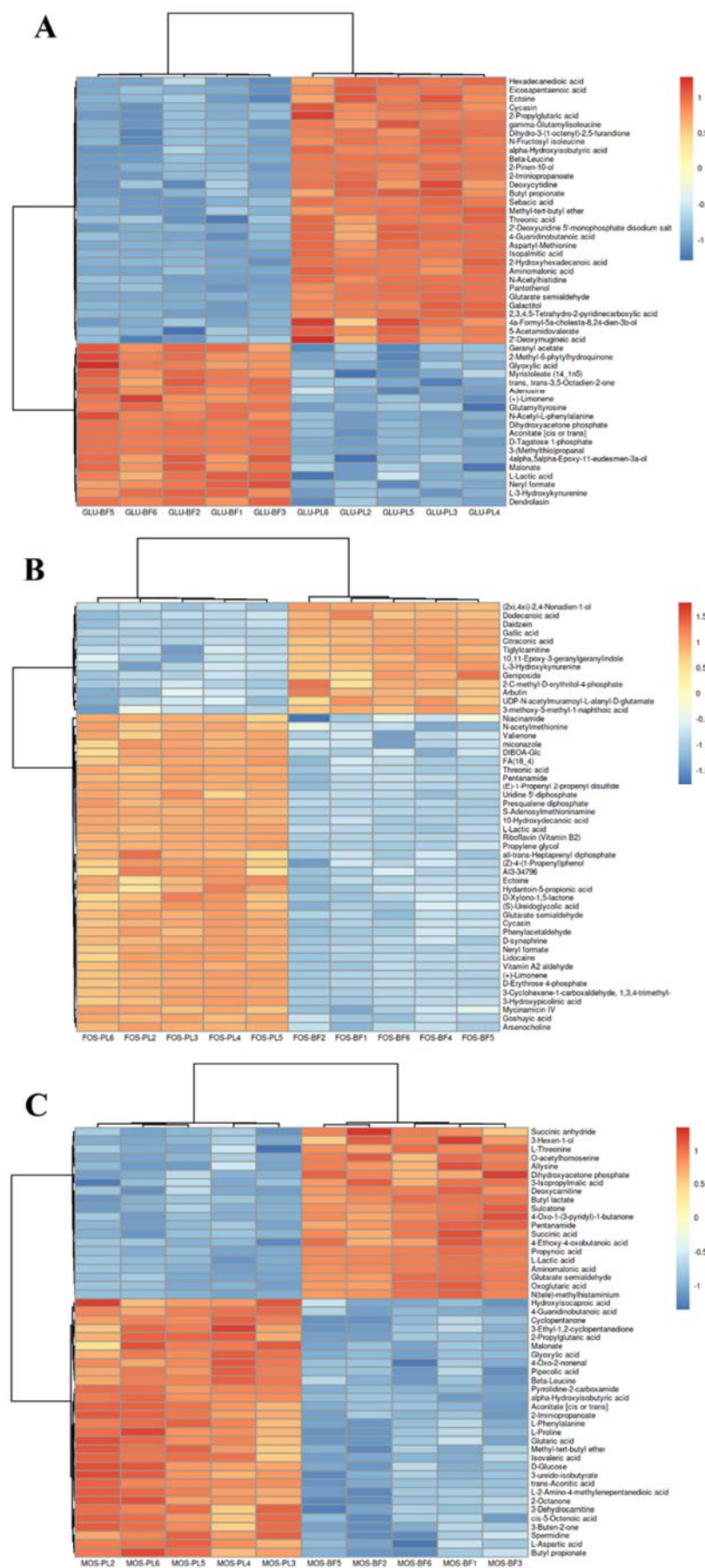


Figure 6. Heat map of clustering of selected differential metabolites. (A) GLU-BF vs GLU-PL, (B) FOS-BF vs FOS-PL, and (C) MOS-BF vs MOS-PL.

3.11 Analysis of differential metabolic pathways

Differential metabolites were annotated in KEGG, revealing 187 associated pathways (Fig. 7A). Pathways related to Metabolism predominated (116). Within the metabolic category, the most frequent subclasses were amino-acid metabolism (21), terpene/polyketide metabolism (13), carbohydrate metabolism (12), lipid metabolism (12), signal transduction (7), and glycan biosynthesis/metabolism (3). From the above, it can be concluded that amino acid metabolism and carbohydrate metabolism play a key role in the formation, stability and function of biological membranes. Metabolic pathway analysis identified 21 amino acid-related pathways involving both biosynthesis and degradation processes (Fig. 7B). Additional pathways included metabolism of cysteine, methionine, etc. Notably, biofilm cells exhibited significant upregulation of metabolites such as L-aspartate, L-valine, L-lysine, L-glutamate, L-threonine, tryptophan, O-acetyl-L-homoserine, and N-acetyl-L-histidine. These changes support enhanced energy production and extracellular polysaccharide (EPS) synthesis, crucial for biofilm structural integrity. Carbohydrate metabolism analysis revealed 12 significantly enriched pathways, including the pentose phosphate pathway, glycolysis/gluconeogenesis, TCA cycle, etc. (Fig. 7C). The metabolites of biofilm cells, such as 3-ketone- β -D-galactose, glucosides, 2-phosphoglyceric acid, and dihydroxyacetone phosphate, are significantly elevated. Among them, 2-phosphoglyceric acid and dihydroxyacetone phosphate are bridge substances connecting glycolysis and fatty acid metabolism, while galactose derivatives and glycosides provide carbon for EPS biosynthesis, promoting the formation and adhesion of biofilms.

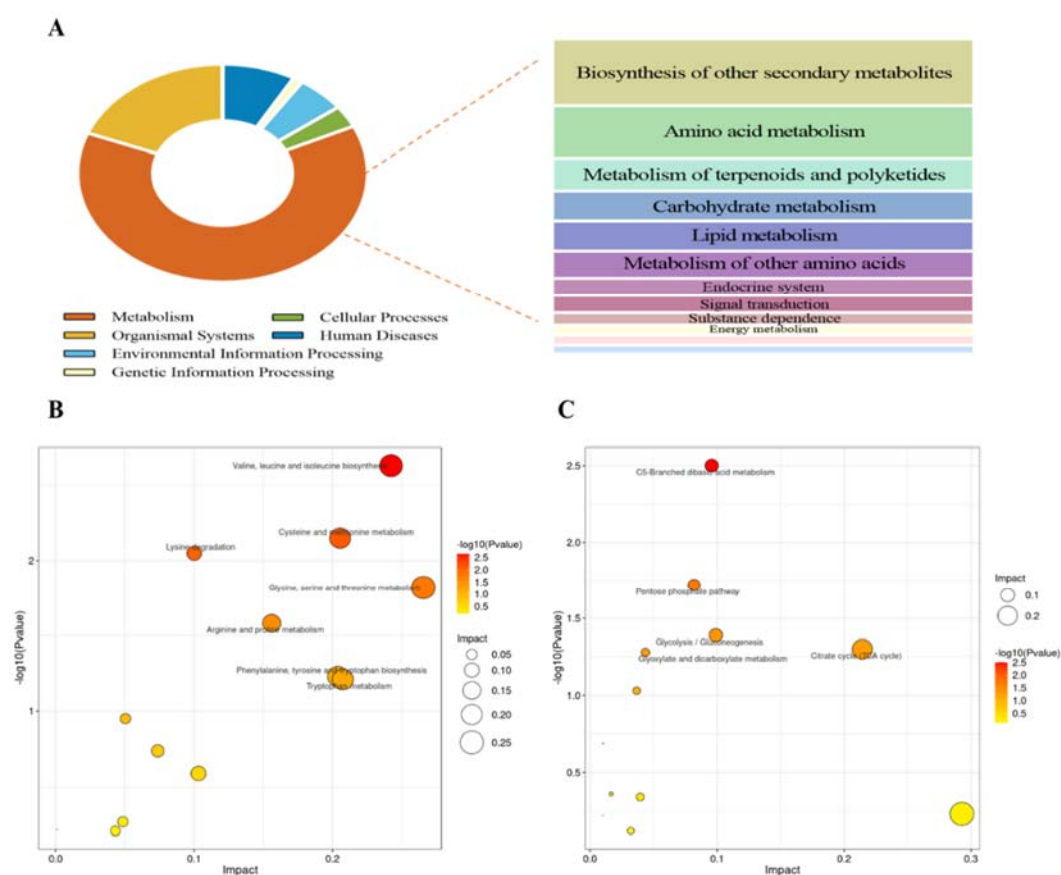


Figure 7. (A) Differential Metabolic Pathways Statistical Chart. (B) Amino acid metabolic pathway bubble diagram. (C) Bubble diagram of metabolic pathways of carbohydrate compounds.

4. Discussion

Bacteria in biofilms are subjected to environmental stresses (e.g., oxygen, nutrients, antibiotics), making EPS vital for adhesion, protection, and tolerance. Conversely, EPS is less essential for planktonic bacteria, which exist in a free-floating state. These findings suggest that different carbon sources influence EPS gene expression, underscoring their regulatory role in biofilm formation.

An important criterion for choosing probiotics is their ability to adhere to the intestinal mucosa of the human body. The interactions of mucus-binding proteins, lipophosphonic acid, extracellular polysaccharides and other components on the surface of lactic acid bacteria with recipient cells determine different adhesion mechanisms^[20]. Self-aggregation is an important characteristic of the surface properties of lactic acid bacteria, involving mutual attraction and reversible adsorption among bacteria. This characteristic can protect lactic acid bacteria from extreme conditions such as oxidative stress and nutritional deficiency^[21]. Through this experimental study, using glucose as the control, we systematically evaluated the auto-aggregation property, surface hydrophobicity and Caco-2 cell adhesion characteristics of *L. plantarum* C8 when different oligosaccharides were used as the sole carbon source. In the evaluation of the self-aggregation ability and surface hydrophobicity of the strains, *L. plantarum* C8 in the FOS group had good self-aggregation (79.12%) and surface hydrophobicity (32.82%). This shows that bacteria with strong hydrophobicity may have enhanced their self-aggregation to a certain extent because their surfaces also have a high affinity.

Studies have found that after culturing *Lactobacillus acidophilus* NCFM with raffinose, it was discovered that its adhesion to mucin and intestinal HT-29 cells increased, and significant changes occurred in 14 unique proteins of the surface subproteome^[22]. Furthermore, our analysis of the adhesion ability of Caco-2 cells showed that the adhesion ability of *L. plantarum* C8 in the FOS group and the MOS group on the cell surface was significantly enhanced compared with the control GLU group, with adhesion rates of 43.26% and 40.63%, respectively. The adhesion rate is consistent with the results of self-aggregation and hydrophobicity, and there is a certain positive correlation among the three. Strong self-aggregation and hydrophobicity can enhance the surface adhesion ability of bacteria, thereby increasing their adhesion rate.

Biofilms are complex microbial consortia composed of bacteria embedded within a matrix of EPS, proteins, nucleic acids, lipids, and water^[23]. These components synergistically confer structural integrity, environmental resilience, and metabolic functionality. EPS and proteins, in particular, are critical to biofilm formation and stability. EPS facilitates bacterial adhesion and nutrient retention by forming a three-dimensional matrix that protects cells from environmental stress^[24]. Oligosaccharides promoted *L. plantarum* C8 biofilm formation, with different types significantly affecting biofilm thickness and density. This was reflected in increased bacterial aggregation, extracellular matrix accumulation, and stronger biofilm structure. The living cell content in biofilms treated with oligosaccharides was comparable to the control GLU group, suggesting that oligosaccharides provide a similar growth environment. Experimental results indicate that FOS and MOS significantly enhance biofilm development in *Lactobacillus*, likely by providing nutritional support and promoting intercellular interactions. SEM and CLSM observations confirmed that FOS and MOS-

treated biofilms had denser, more complex three-dimensional structures, consistent with crystal violet staining results. These findings suggest that oligosaccharides not only impact biofilm physical structure but also regulate biofilm formation through gene expression and quorum sensing pathways.

Comparison of PL and BF samples showed that FOS and MOS upregulated key biofilm-related genes, such as EPS and *LuxS* genes, enhancing biofilm formation and maturation. The GOS group, however, exhibited inhibited *LuxS* gene expression. Quorum sensing analysis revealed that oligosaccharides regulate AI-2 signaling molecule synthesis and release, influencing biofilm stability. AI-2 production was higher in biofilm samples than planktonic ones. SEM and CLSM observations confirmed that FOS and MOS-treated biofilms had denser, more complex three-dimensional structures, consistent with crystal violet staining results. These findings suggest that oligosaccharides not only impact biofilm physical structure but also regulate biofilm formation through gene expression and quorum sensing pathways. Through non-targeted metabolomics, identified significant metabolic distinctions between biofilm and planktonic states of *L. plantarum* C8. Differential metabolites included lipids, organic acids, benzene derivatives, nucleosides, and nucleotides, primarily comprising carbon and nitrogen sources (e.g., glucose, fructose, galactose, L-lysine). These compounds are integral to cell wall, membrane, and extracellular matrix synthesis and are involved in key metabolic pathways such as carbohydrate, amino acid, and lipid metabolism, thereby facilitating the production of essential biofilm components including EPS and proteins. By regulating the membrane structure, these fatty acids support the integrity and maintenance of biofilms. Analogous findings in *Helicobacter pylori* biofilm formers have revealed elevated levels of lipid classes, including glycerophospholipids, fatty acyls, and sterol lipids. This lipid enrichment is associated with increased membrane rigidity and reduced fluidity, largely attributed to phospholipids and sphingolipids, thereby conferring enhanced membrane stability and resilience under stress conditions ^[25]. Beyond providing structural support, they are actively involved in mediating interactions between bacterial cells and their surrounding environment ^[26]. Specifically, the interplay between membrane lipids and EPS enhances bacterial adhesion and contributes to the structural cohesion of the biofilm matrix, thereby promoting environmental adaptability and communal bacterial survival. Regarding the failure of GOS, this might be due to the lack of specific transport proteins or β -galactosidase activity in *L. plantarum* C8. Studies have indicated that through molecular analysis methods, it was discovered that *L. plantarum*'s utilization of GOS involves various regulatory factors, including galactosidase, which are predicted to be involved in the utilization of higher degree of polymerization constituents in GOS ^[27]. Additionally, related research has also found that the catabolite control protein A (CcpA) plays a crucial role in the carbohydrate metabolism regulation of *L. plantarum*. This study found that the growth conditions of *L. plantarum* on GOS and glucose were different, and CcpA was the key factor causing this difference. Moreover, in the comparison between wild-type and CcpA mutant strains under GOS conditions, the number of differentially expressed genes was the lowest ^[28].

Biofilms demonstrate enhanced EPS precursor synthesis relative to planktonic cells, promoting increased EPS secretion that is closely linked to the structural stability and maintenance of the biofilm ^{[29][30]}. In the

context of carbohydrate metabolism, glycolysis and glucose catabolism supply both ATP and precursors for EPS biosynthesis. Notably, elevated levels of 2-phosphoglycerate and dihydroxyacetone phosphate in FOS and MOS groups suggest that oligosaccharide supplementation enhances glycolytic flux, thereby facilitating biofilm development. Additionally, the enrichment of acetoin, a butyrate metabolism intermediate, within biofilms implies its involvement in membrane structure regulation. Similar findings in *Acinetobacter baumannii* reveal that biofilm-associated metabolic shifts enable fermentation of pyruvate, supporting bacterial survival under hypoxic or anaerobic conditions—critical for long-term persistence^[31]. Furthermore, nucleotide metabolites such as adenosine monophosphate and cytidine monophosphate may serve as precursors for EPS synthesis and polymerization, supporting biofilm development at the molecular level^[32]. In the amino acid metabolism related analysis of biofilm formation, the observed accumulation of L-glutamic acid, L-lysine, and L-proline within biofilm cells underscores the pivotal role in EPS and cellular integrity. This aligns with reports of elevated L-amino acid pools in *E. coli* biofilms, where L-enantiomers predominate over D-forms^[33], studies have shown that valine is continuously secreted within bacterial biofilms, with its accumulation likely attributed to limited diffusion within the biofilm matrix and elevated cell density^[34], as observed in *A. baumannii* biofilm cells. Furthermore, the significant upregulation of TCA cycle intermediates (including succinic acid and α -ketoglutaric acid) indicates enhanced energy production, which is crucial for the formation of biofilms and the survival of bacteria under adverse conditions. Studies have confirmed that oxaloacetic acid, ketoglutaric acid and succinyl-CoA are intermediates provided by the TCA cycle for the synthesis of biological membranes^[35]. Also, the content of adenosine diphosphate (ADP) was significantly increased in the biofilm of *L. plantarum*^[19].

Quorum sensing is a coordinated behavior in microbial communities, usually relying on the concentration changes of small molecule signaling molecules. Some nitrogen compounds, especially amino acid substances, may act as signaling molecules in the group behavior of lactic acid bacteria, promoting the aggregation of lactic acid bacteria on the surface and thereby forming biofilms^[36]. In this study, it was found that certain metabolites, such as abscisic acid and flavin adenine dinucleotide, are involved in cellular signal transduction and the regulation of stress responses, and may affect the formation of *Lactobacillus* biofilms by regulating the quorum sensing pathway. As a key regulatory mechanism for the development of biofilms, quorum sensing controls the behavior of bacteria and the maturation of biofilms. Abscisic acid may affect microbial growth, while flavin adenine dinucleotide plays a crucial role in intracellular signal transduction and may be involved in quorum regulation. In addition, fatty acids such as isopentyl laurate, lauric acid and α -linolenic acid play an important role in the formation and stability of bacterial cell membranes. These compounds affect the fluidity, structural integrity and interaction with the host or other microorganisms of the membrane.

5. Conclusions

In this study, oligosaccharides were used as the sole carbon sources to systematically evaluate their effects on the growth characteristics and biofilm-forming ability of *L. plantarum* C8. Concurrently, non-targeted metabolomics was employed to elucidate the metabolic regulatory mechanisms through which these

oligosaccharides mediate biofilm synthesis. FOS and MOS markedly enhance auto-aggregation, surface hydrophobicity, and epithelial adhesion, suggesting that increased surface affinity directly promotes bacterial clustering and attachment—key prerogatives for successful probiotic colonization. Meanwhile, supplementation with these two oligosaccharides can significantly upregulate biofilm-associated genes (e.g., EPS-biosynthesis loci and *LuxS*), accelerates AI-2 signal production, and yields denser, more complex three-dimensional biofilms. These findings demonstrate that oligosaccharide type governs biofilm architecture and stability through coordinated regulation of gene expression and quorum sensing pathways. Moreover, the influence of FOS on the biosynthetic pathways of valine, leucine and isoleucine may contribute to the production of EPS, while elevated central metabolites in the butyrate pathway (e.g., oxyglutaric acid, acetoin) underscore the role of FOS in biofilm regulation. Together, these pathways establish a biochemical framework for biofilm formation and bacterial resilience under environmental stress.

Acknowledgments

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Conflict of Interest Disclosure

The authors declare no competing financial interest.

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