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Interspecies interactions in biofilms: co-regulation of *Pseudomonas fluorescens* and *Listeria monocytogenes* isolated from chilled beef processing

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ABSTRACT: Mixed biofilms formed with the coexistence of spoilage bacteria and foodborne pathogens represent a significant cause for persistent microbial contamination in the food industry. Deciphering the interspecies interaction mechanisms is a critical prerequisite for developing effective control strategies. This study systematically investigated the interaction mechanisms within dual-species biofilms of *Pseudomonas fluorescens* and *Listeria monocytogenes*. Results found that the dual-species biofilms exhibited significantly enhanced tolerance to sodium hypochlorite, with a higher survival rate than mono-species biofilms after 20 min treatment ($P < 0.05$). Dual RNA-Seq analysis revealed that *P. fluorescens* activated pathways related to the two-component system, flagellar assembly, fatty acid metabolism, and quorum sensing (QS) in the co-culture system. In response, *L. monocytogenes* regulated numerous genes and pathways, such as chemotaxis, two-component system, flagellar assembly, phosphotransferase system, and amino acid biosynthesis. The cell-free supernatant (CFS) from the co-culture significantly promoted biomass accumulation, increased matrix thickness, and reduced roughness in mono-species biofilms ($P < 0.05$), suggesting that metabolites could act as positive signaling molecules in this process. The autoinducer-2 (AI-2) concentration in the co-cultured CFS was significantly higher than that in mono-cultured CFS ($P < 0.05$). In addition, exogenous addition of AI-2 enhanced the biofilm formation of *P. fluorescens* and dual-species in a concentration-dependent manner ($P < 0.05$), confirming the key regulatory role of QS in this process. This study provided new insights into the synergistic mechanisms of dual-species biofilms and the development of control strategies.

Keywords: *Listeria monocytogenes*, *Pseudomonas fluorescens*, dual-species biofilms, Dual RNA-Seq, AI-2, disinfectants resistance

1. Introduction

As a predominant phenotype of microbial life activity, biofilms provide biological barriers with self-secreted extracellular polymeric substances (EPS) against external adverse effects^[1]. The inter-kingdom interactions between different species in mixed biofilms, consisting of two or more microorganisms, serve as

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the foundation to modify the microbial ecological function, biomass production and matrix structure^[2]. From the perspective of food industry, mixed biofilms were more resistant to conventional cleaning measures or chemical disinfectants in comparison to mono-species biofilms and planktonic bacteria, thus worsening the cross contamination of spoilage or pathogenic bacteria at food contact surfaces^[3-4]. In the meat industry, biofilms formed by pathogenic and spoilage bacteria could create a persistent source of product contamination, leading to serious hygienic problems and also economic losses due to food spoilage^[5].

Pseudomonads is not only well-known for its ability to cause spoilage to animal origin foods, but also is a prevalent microorganism found on many food processing industry environments^[6-7]. Additionally, *Pseudomonads* serves as a coexisted organism in the context of mixed biofilms with pathogenic bacteria e.g., *Listeria monocytogenes*^[8-9]. *L. monocytogenes*, the causal agent of foodborne listeriosis, is a serious disease most often associated with certain high-risk groups, including pregnant women, neonates, and the elderly, as well as individuals with compromised immune systems^[10]. In the annual EU report, listeriosis had been incriminated to both animal and non-animal origin ready to eat foods, resulting in 1330 hospitalizations and 286 deaths with a notification rate of 0.62 per 100,000 population, which was an increase of 15.9% compared with 2021 (0.53 cases per 100,000 population) and the highest rate and number of cases reported since 2007^[11].

Microbial coculture system has been considered a promising strategy to understand biofilms, since microbial communication regulates microbial behaviour, such as growth rate, production of certain metabolites and virulence^[12]. Cocultured metabolites are the basis of bacterial interactions, which affect the physiological processes of adjacent bacteria. Studies had illustrated interspecific interactions in biofilms about the metabolic level, including signal communication^[12-13]. Among them, autoinducer-2 (AI-2) is a universal signalling molecule of quorum sensing (QS) in Gram-negative and Gram-positive bacteria, and functions as the “common language” for bacterial communication^[13]. The coexisting appearance of *Pseudomonas* and *L. monocytogenes* in biofilms under different temperature conditions had been extensively studied^[6,14-17]. Our previous studies found that *P. fluorescens* and *L. monocytogenes* interacted synergistically to enhance biofilm formation in a dual-species model^[18], whose resistance could be similar to that observed in other studies^[19]. This finding suggests that metabolites such as AI-2 may function as signalling molecules during the formation of dual-species biofilms. However, the co-regulated mechanisms of gene networks and signalling metabolites within dual-species biofilms of *P. fluorescens* and *L. monocytogenes* remain unclear.

Therefore, building upon prior phenotypic studies^[18], this study elucidated the transcriptional profiles using dual RNA-seq and deciphered the co-regulation mechanism of dual-species biofilms formed with *P. fluorescens* and *L. monocytogenes*. It assessed the impact of co-cultured metabolites on biofilm formation and architecture, prospectively to identify key regulatory genes and pathways, and further elucidated the role of AI-2 in dual-species biofilms of *Pseudomonas* and *L. monocytogenes*. These findings provided mechanistic foundation for the development of novel strategies to control biofilms in food processing.

2. Materials and Methods

2.1 Bacterial strains

P. fluorescens and *L. monocytogenes*, selected based on a previous study^[18], were inoculated and activated with continuous shaking for 18 h at 28 °C and 37 °C, respectively. Pre-activated suspensions underwent a second round of activation to ensure the stability and vitality of the organisms at the same culture conditions as mentioned above. The suspensions were then diluted to approximately 6 log CFU/mL in LB broth and set aside for subsequent experiments.

2.2 Biofilm resistance to sodium hypochlorite

Mono-specie biofilms were incubated on polystyrene coupons (50 mm × 25 mm × 1 mm) at 25 °C for 72 h. Using the same culture method, *P. fluorescens* and *L. monocytogenes* were mixed at a 1:1 ratio to incubate dual-species biofilms. and then the coupons were immersed in sterile saline solution and subjected to three rounds of washing to obtain adherent cells within the biofilms. Following this, the coupons were exposed to sodium hypochlorite (100 µg/mL) for 0, 3, 6, 9, 12, 15, 20 min, respectively^[20]. Sodium hypochlorite was then removed, and the coupons were placed into centrifuge tubes containing D/E broth to neutralize residual sterilizing agent on the surface of the biofilms. Furthermore, the biofilm was vortexed for 5 min to dissolve the biofilm cells. Mono-species biofilms were cultured using LB agar, while cocultured biofilms were cultured using Pseudomonas CFC Selective medium and Listeria Chromogenic Medium selective media (Hopebio, Qingdao, China) at 28 °C and 37 °C for 48 h, respectively^[21].

2.3 Transcriptome analysis

2.3.1 RNA extraction, library preparation and sequencing

Mono- and dual-species biofilms were cultured for 72 h in polystyrene cell culture plates at 25 °C. Sterile saline was used to wash the plates three times to remove planktonic cells. Adherent cells from the biofilms were collected using a sterile scraper. The UID Dual RNA-seq were performed by Seqhealth Technology Co., LTD (Wuhan, China). The TRIzol Reagent (Invitrogen, USA) was utilized to extract total RNAs from both mono- and dual-species biofilm cells. The process of DNA digestion was conducted subsequent to the extraction of RNA using DNaseI. Qualified RNAs were quantified using the Qubit3.0 and the Qubit™ RNA Broad Range Assay kit for measurement purposes (Life Technologies, India). For the preparation of stranded RNA sequencing library, a total of 2 µg RNA was utilized. The depletion of rRNA was carried out using DSN duplex-specific nuclease. Subsequently, the KC-Digital™ Stranded mRNA Library Prep Kit (Seqhealth, China) was employed following the manufacturer's guidelines. The utilization of a unique molecular identifier consisting of 8 random bases in the pre-amplified cDNA molecules labeling process effectively eliminates any potential duplication bias during PCR and sequencing steps. Library products within the range of 200-500 bps were selectively enriched, accurately quantified, and ultimately subjected to sequencing on DNBSEQ-T7 sequencer (MGI Tech Co., Ltd. China) utilizing the PE150 model. The RNA was extracted from three independent cultures.

2.3.2 RNA-Seq data analysis

The initial step involved the application of fastp to filter out raw sequencing data, removing low-quality reads and trimming those contaminated with adaptor sequences. Subsequently, any potential errors or biases arising from PCR amplification or sequencing were effectively eliminated. The consensus sequences, after undergoing deduplication, were utilized for conducting standard RNA-seq analysis. The identification of differentially expressed genes between groups was performed using the edgeR package. The differentially expressed genes (DEGs) were determined based on P -values < 0.05 and a fold-change > 1.5 . The KOBAS software was utilized to perform Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis on differentially expressed genes. The raw sequencing data was deposited in the NCBI SRA under the accession number PRJNA1377323.

2.4 Effect of cocultured cell-free supernatants (CFS) on biofilm formation

The supernatants from the coculture were introduced to individual biofilms to further illustrated the enhanced formation of dual-species biofilms. Primarily, a mixture of *P. fluorescens* and *L. monocytogenes* in a 1 : 1 ratio was incubated at 25 °C for 72 h. The cocultured suspension was centrifuged at 4 °C and 12,000 r/min for 20 min. The resulting supernatant was then filtered through sterile 0.22 µm needle filters to obtain CFS of the coculture, which was subsequently stored at -80 °C after confirming its sterility. The mono-species biofilms consisting of *P. fluorescens* or *L. monocytogenes* were cultivated in polystyrene 96-well plates. The resulting cell suspensions (200 µL) were prepared with CFS concentrations of 0, 1/4, and 1/2. The ability of biofilm formation was subsequently evaluated using crystal violet staining^[18].

2.5 Micromorphology of biofilms

2.5.1 Scanning electron microscope (SEM)

The mono and dual-species cell suspensions of *P. fluorescens* and *L. monocytogenes* were inoculated into 48-well plates, with each well containing 450 µL of the suspension. Subsequently, 150 µL CFS (1/4 CFS) was added to each well, while LB broth was added as a control. Sterile polystyrene coupons (10 mm × 10 mm × 1 mm) were vertically inserted into the microtiter wells and incubated for 72 h. The biofilm coupons were subsequently dehydrated in a gradient of alcohol concentrations (50%, 70%, 80%, 90%, and 100%). Following critical point drying and gold coating, the samples were meticulously examined at 5000 × using a SU8020 SEM (Hitachi, Tokyo, Japan).

2.5.2 Atomic force microscope (AFM)

AFM morphology was measured using a Bruker Dimension Icon (Germany) in tapping mode with an Otespa-R3 probe. The main parameters included a scan rate of 1 Hz and a resolution of 512 * 512. NanoScope Analysis 1.8 was used to analysis AFM images.

2.6 Essential DEGs validation using reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

In the KEGG pathway, 9 DEGs of *P. fluorescens* and 12 DEGs of *L. monocytogenes* were selected. RT-qPCR was used to verify the reliability of transcriptome data. The SteayPure Universal total RNA Extraction Kit (Accurate Biotechnology, Changsha, China) was utilized to extract total RNA from each sample in accordance with the manufacturer's guidelines. The sample culture conditions (25 °C, 72 h) were consistent with those in Section 2.3.1, and the RNA were obtained from three independent cultures. The RNA concentration and purity were assessed using a nucleic acid detector (Implen, Germany) that was calibrated with enzyme-free water. Following this, the purified RNA underwent reverse transcription into cDNA using an Evo M-MLV RT Mix Kit (Accurate Biotechnology, Changsha, China) for subsequent analysis through RT-qPCR. For gene expression quantification during the RT-qPCR assay, the SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biotechnology, Changsha, China) was utilized. The specific primers sequences were listed in Table S1 and Table S2. The quantification of mRNA was conducted utilizing a real-time PCR platform (CFX96, Bio-Rad, USA), and included three technical replicates. The $2^{-\Delta\Delta C_t}$ method was used to analyze the expression of the relevant genes obtained according to the melting curve.

2.7 AI-2 determination

The relative concentrations of AI-2 in mono- and dual-species supernatants of *P. fluorescens* and *L. monocytogenes* were determined using the *Vibrio harveyi* BB170 bioluminescence assay^[22]. Briefly, *V. harveyi* BB170 was inoculated into Autoinducer Bioassay (AB) medium (Topbio, China) and incubated at 28 °C with shaking at 90 r/min for 18 h. The suspension was adjusted to optical density (OD₆₀₀) range of 0.8–1.0, and then diluted at the ratio of 1 : 5,000. AB medium, LB broth and *V. harveyi* CFS were individually mixed with the above diluted suspension in the ratio of 1 : 100 and incubated at 28 °C with shaking. The aforementioned samples were utilized consecutively as negative control, medium control, and positive control. On the other hand, the supernatants of mono- and dual-species biofilms were used as treatment groups. Each sample was tested with 6 replicate wells and the experiment was repeated 5 times. The AI-2 fluorescence intensity values were measured using a multimode microplate reader (Enspire, Perkin Elmer, USA) at the time point where the fluorescence intensity values of the medium control group reached the minimum within a 6-h period.

2.8 Exogenous addition of AI-2

In 96-microtiter plates, mono- and dual-species suspension were added to each well, followed by the addition of DPD (4, 5-dihydroxy 2, 3-pentanedione, AI-2 precursor) to achieve a concentration of DPD/AI-2 in the suspension at 1, 10, 50 ng/ mL, respectively. Additionally, sterile water was added as the black control group and the incubation period lasted for 24 h. The violet crystal staining method was used to assess biofilm forming ability.

2.9 Statistical analysis

The experiment was designed with three biological replicates, each comprising three technical replicates at least. Statistical analysis was conducted using Statistical Analysis System (SAS, Version 9.2. Cary, USA).

One-way analysis (ANOVA) was applied to compare the exogenous CFS effect on biofilms, and Tukey's test was used for comparing means between different groups. Data from resistance to sodium hypochlorite, AI-2 relative light units, biofilm roughness, and biofilm thickness were analyzed by two-way analysis (ANOVA) using the MIXED procedure. The mode, time and their interactions fitted as fixed effects, and independent repetition as a random effect. Statistical significance was defined as a P -value < 0.05 and all data met the homogeneity of variance assumption ("sig. > 0.05).

3. Results

3.1 Disinfectant resistance

The effect of sodium hypochlorite on mono- and dual-species biofilms were assessed in Table 1. There was a significant interaction effect of mode and time on the sodium hypochlorite resistance of biofilm ($P < 0.01$). After 3 min treatment, the cell number of *P. fluorescens* reduced about 0.9 and 0.4 log CFU/cm² in mono- and dual-species biofilms compared to control, respectively, though there were no significant differences ($P > 0.05$). The cell number gradually decreased with the prolongation of sterilization time. At 6 min of sterilization treatment, the cell count in the coculture was significantly higher than that in the single biofilm by approximately 1.4 log CFU/cm² ($P < 0.05$), and the difference even reached 2.0 log at 15 min. On the other hand, while *L. monocytogenes* survival counts were not significantly different at 12 min, the mono-species biofilms exhibited a much greater reduction in viable cells (3.7 log CFU/cm²) compared to the coculture (1.7 log CFU/cm²). The interspecies interactions within the dual-species biofilm reduced the susceptibility of both *P. fluorescens* and *L. monocytogenes* to disinfectants.

Table 1 Resistance to sodium hypochlorite of mono- and dual-species biofilms (log CFU/cm²)

Species	Mode	Time (min)							Mode $\times$ Time	
									SE	P value
		0	3	6	9	12	15	20		
<i>P. fluorescens</i>	Mono	6.6 ^a	5.7 ^b	4.1 ^{ef}	3.2 ^g	2.7 ^h	1.9 ⁱ	1.5 ^j	0.21	< 0.001
	Dual	6.3 ^a	5.9 ^b	5.5 ^{bc}	4.7 ^d	4.3 ^c	3.9 ^f	3.6 ^f		
<i>L. monocytogenes</i>	Mono	6.4 ^a	5.1 ^b	4.2 ^c	3.4 ^{de}	2.7 ^f	1.9 ^h	1.4 ⁱ	0.19	< 0.001
	Dual	4.5 ^c	4.2 ^c	3.5 ^d	3.2 ^e	2.8 ^f	2.3 ^g	1.9 ^h		

Letters a - j indicate significant differences at differences groups ($P < 0.05$).

3.2 Biofilm formation ability

It investigated the effect of cocultured CFS on the biofilm formation of mono-species, as showed in Fig. 1. The biomass of *P. fluorescens* was significantly higher than that of control group at different CFS concentrations ($P < 0.05$). Similarly, although *L. monocytogenes* had a lower biofilm-forming ability without the addition of CFS, the biomass under the influence of 1/4 CFS and 1/2 CFS were about 1.62 and 2.96 times in comparison with the control group, respectively. It could be considered that exogenous addition of cocultured CFS could promote biofilm formation of *P. fluorescens* and *L. monocytogenes*, and the biomass dynamics were also enhanced with the increase of CFS concentration. This suggested that metabolites from the co-culture acted as potential mediators promoting interspecies interactions.

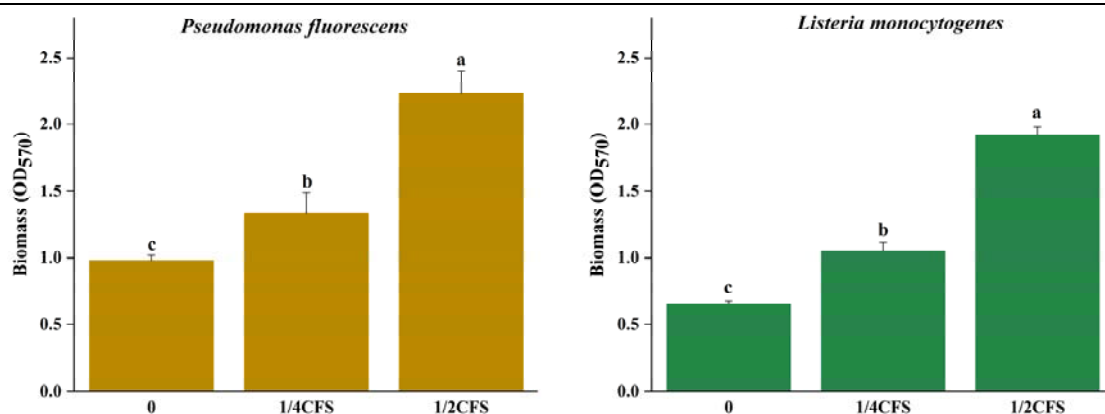


Fig. 1 Effect of adding cocultured CFS on mono-species biofilms. Different letters (a -c) indicate significant differences between different groups ($P < 0.05$).

3.3 Biofilm morphology

The SEM (Fig. 2) and AFM (Fig. 3 and Fig.S1) images depicted the morphological characteristics of mono- and dual-species biofilms in LB and 1/4 CFS conditions. The cells in single *P. fluorescens* biofilms were sparsely distributed, however, upon addition of CFS, it formed an aggregated, stacked and dense matrix. Meanwhile, the thickness of *P. fluorescens* biofilms in the CFS group was significantly increased to 182.5 nm, compared to that of the control group (125.4 nm), and its roughness was notably reduced ($P < 0.05$, Fig. 3 B, C). Obviously, in the CFS group, a substantial quantity of flocculent EPS enveloping the cells could be clearly observed in the *L. monocytogenes* biofilms. Although CFS had no significant impact on the thickness of *L. monocytogenes* biofilm ($P > 0.05$), it exhibited a reduction in its roughness ($P < 0.05$). In the coculture scenario, the exogenous addition of CFS had no significant effect on the structural parameters. Nonetheless, compared to the mono-species biofilms, the cocultured biofilms demonstrated a significant increase in thickness ($P < 0.05$), further indicating that coculture facilitated biofilm formation.

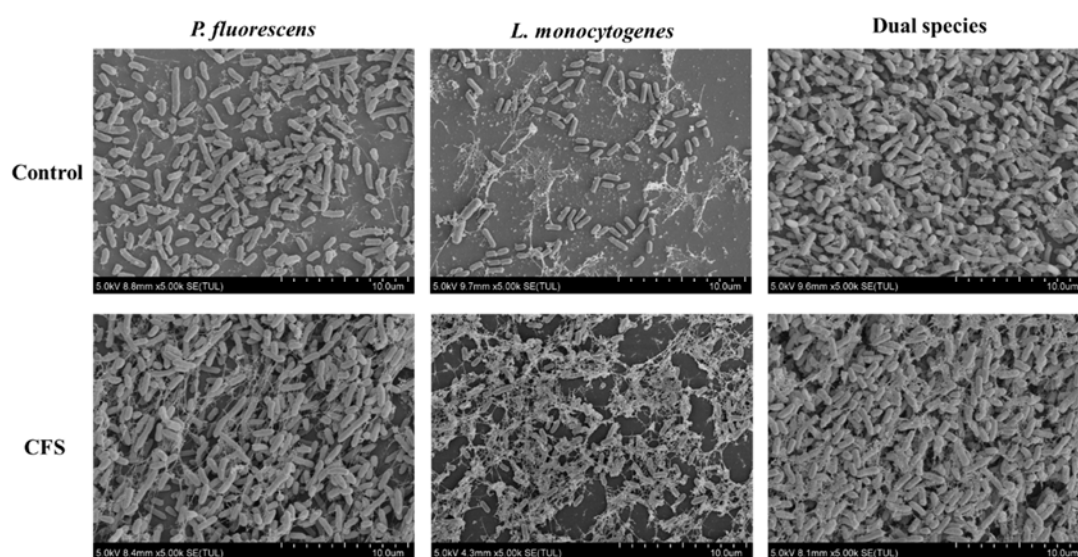


Fig. 2 SEM micromorphology of mono- and dual-species biofilms with *P. fluorescens* and *L. monocytogenes* cultured in control and CFS groups.

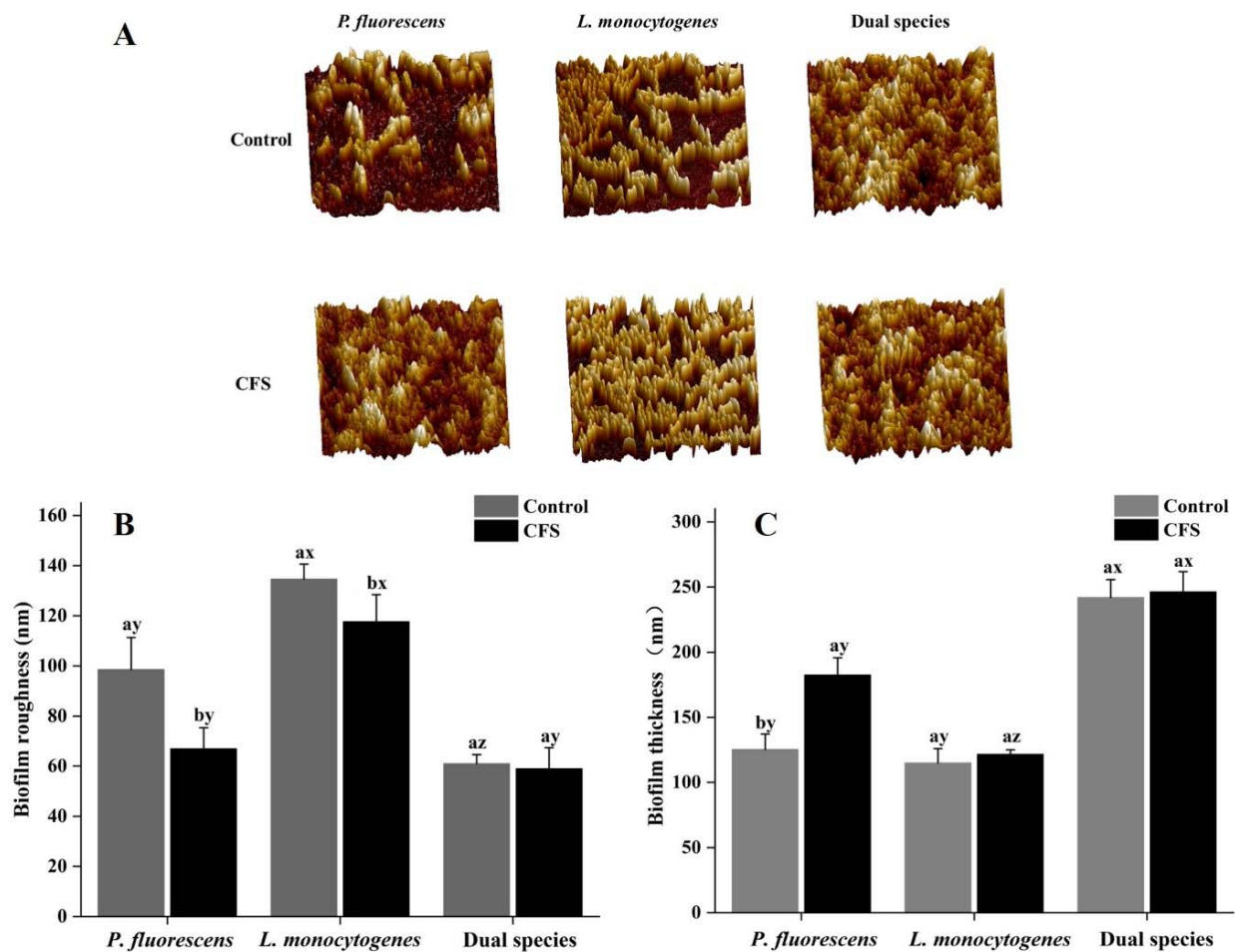


Fig. 3 AFM micromorphology of mono- and dual-species biofilms with *P. fluorescens* and *L. monocytogenes* cultured in control and CFS groups, respectively. 3D images (A), biofilm roughness (B) and thickness (C). Different letters (a - b) indicate significant differences between control and CFS groups in same species ($P < 0.05$). Different letters (x - z) indicate significant differences between different species in same columns ($P < 0.05$).

3.4 Dual RNA-seq

3.4.1 Transcriptomic analysis of *P. fluorescens*

The Dual RNA-seq was utilized to explore regulatory pathways and genes of dual-species biofilms (Fig. 4). A total of 679 DEGs of *P. fluorescens* were screened out, among which 409 genes were down-regulated and 270 genes were up-regulated (Fig. S2). GO functional enrichment analysis was conducted on DEGs. Specifically, 153 GO terms were enriched in dual-species biofilms. Among these, 123 were linked to biological process (BP), primarily involved in cellular metabolism, biosynthesis, nitrogen compound metabolism and amide biosynthesis. Cellular components (CC) were associated with 18 terms, such as intracellular part, organelle, and cytoplasm. Meanwhile, 12 terms were assigned to molecular functions (MF), including organic cyclic compound binding and structural molecule activity. The KEGG enrichment pathway of *P. fluorescens* demonstrated the DEGs that were linked to alterations in biofilm formation and interspecies interactions primarily involving two-component system, QS, flagellar assembly, fatty acid metabolism, biosynthesis of amino acids, bacterial secretion system and ATP binding cassette (ABC) transporters (Fig.

4C). Among enriched biological pathways of down-regulated DEGs, the foremost pathways were shown in Fig. 4D, such as ribosome, pyrimidine metabolism, lipopolysaccharide biosynthesis, fatty acid metabolism and oxidative phosphorylation.

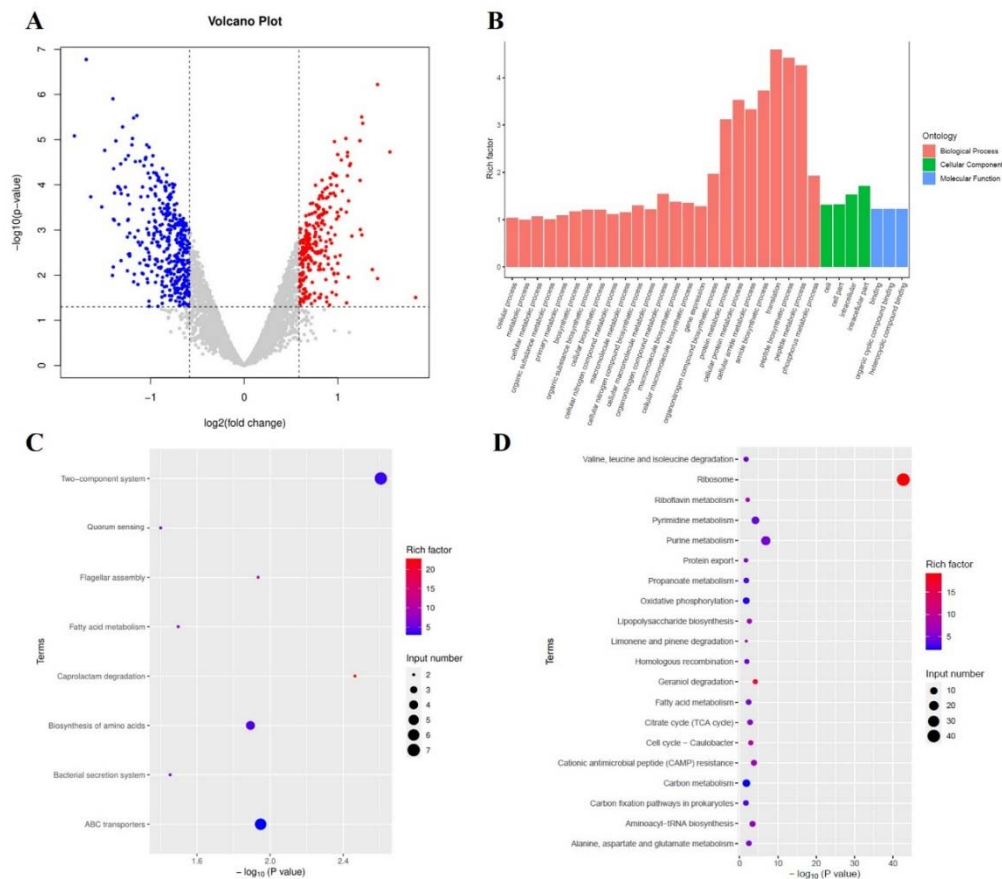


Fig. 4 Dual RNA Seq analysis of *P. fluorescens*. A. volcano map of different genes (gray dots represent non-differentially expressed genes, blue dots represent downregulated genes, and red dots represent upregulated genes). B. GO pathway terms of DEGs. C-D. KEGG pathway terms of up- and down-regulated DEGs ($P < 0.05$).

3.4.2 Transcriptomic analysis of *L. monocytogenes*

Interspecies interactions induced a substantial upregulation of gene expression in *L. monocytogenes* (Fig. 5A). There were 959 genes up-regulated and 807 genes down-regulated in the transcriptome of *L. monocytogenes* (Fig. S3). According to GO enrichment analysis, it exhibited enrichment across 13 GO terms in cocultured system. The multi-organism processes were associated with BP, and 5 terms related to CC, such as bacterial flagella and cellular projections, and 7 MF terms linked to carbohydrate-derived binding, ribonucleotide binding, motility and inorganic anion transmembrane transport activity. To further analyze the promotional response of coculture on *L. monocytogenes* biofilm, the up-regulated differential genes were subjected to KEGG bioinformation analyses and the top 20 most significant KEGG terms were demonstrated in Fig. 5 C, D. The KEGG pathways in DGEs identified were significant enrichment related to the flagellar assembly and bacterial chemotaxis pathways. In addition, the coculture also significantly promoted the *L. monocytogenes* biofilm-associated pathway involving the phosphotransferase system (PTS), peptidoglycan biosynthesis, biosynthesis of amino acids, aminoacyl-tRNA biosynthesis and ABC transporters. In addition,

L. monocytogenes down-regulated pathway of biosynthesis of amino acids, ribosome, pantothenate and CoA synthesis, fatty acid biosynthesis, aminoacyl-tRNA biosynthesis, and etc.

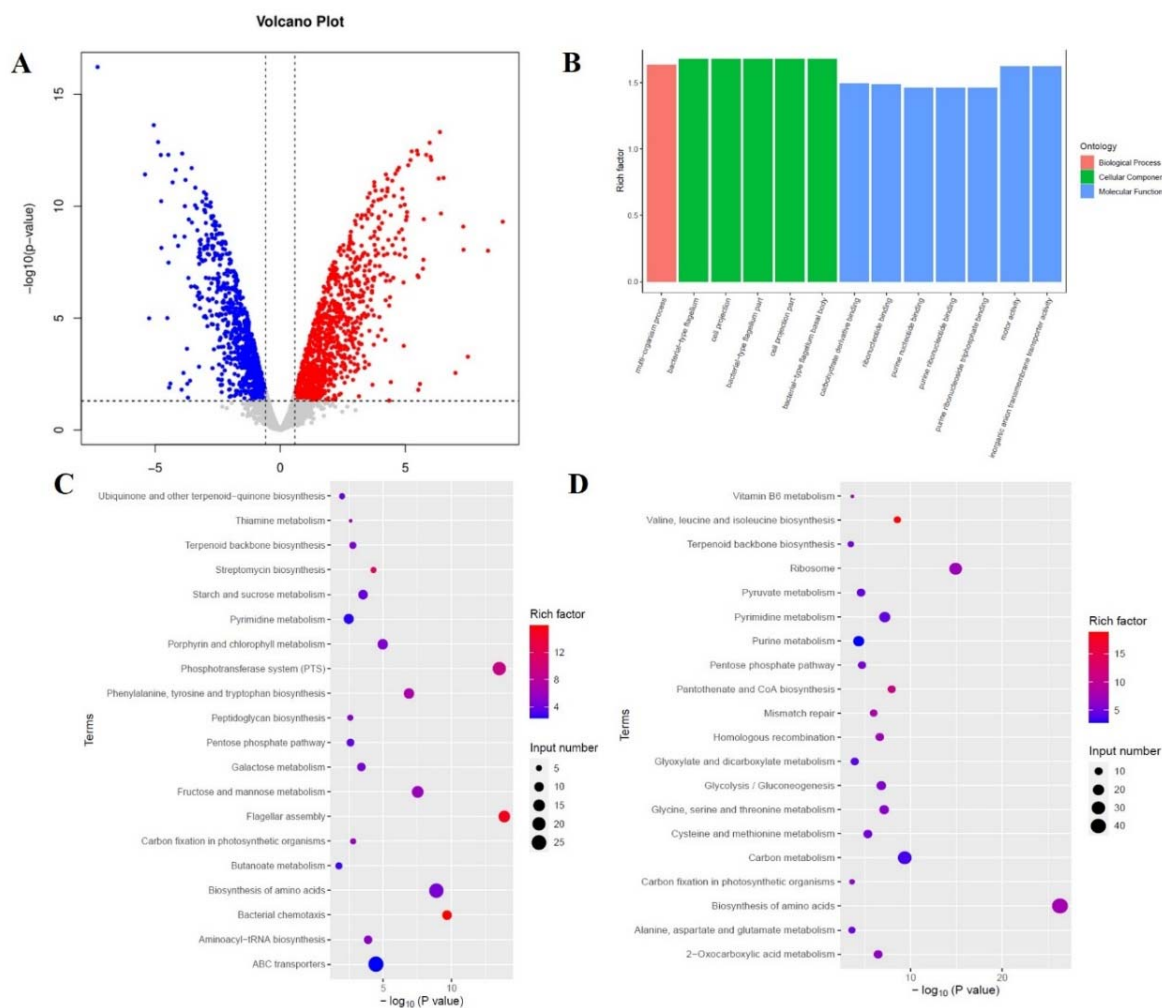


Fig. 5 Dual RNA Seq analysis of *L. monocytogenes*. A. volcano map of different genes (gray dots represent non-differentially expressed genes, blue dots represent downregulated genes, and red dots represent upregulated genes.). B. GO pathway terms of DEGs. C-D. KEGG pathway terms of un- and down-regulated DEGs ($P < 0.05$).

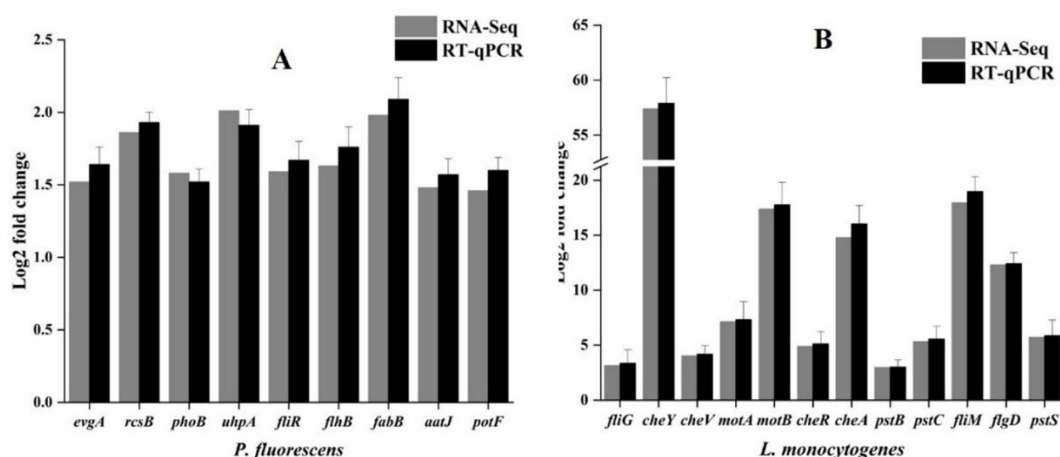
3.5 RT-qPCR

The significant KEGG pathways associated with dual-species biofilm formation were listed in Table 2 ($P < 0.05$). In *P. fluorescens*, genes related to the two-component system, including *evgA*, *rcsB*, *uhpA*, and *phoB*, exhibited significantly upregulated expressions. There was an observed upregulation of genes involved in flagellar assembly (*flhR*, *flhB*), ABC transporters (*aatJ*, *potF*) and fatty acid metabolism (*fabB*). The KEGG pathways of *L. monocytogenes* in cocultured system were found to be associated with 5 principal biological pathways. Among these, flagellar assembly (*flgK*, *flgG*, *motA*, *motB*, *flgE*), ABC transporters (*pstB*, *pstC*) and two-component system (*cheY*, *cheV*, *motA*, *cheR*, *cheA*) were significantly enriched ($P < 0.05$). *flhG*, *cheY*, *cheV*, *motA*, *motB*, *cheR*, *cheA* and *flhM* were key genes in bacterial chemotaxis genes. In addition, the expression of 20 DEGs related to the PTS was significantly upregulated in dual-species biofilms. The aforementioned genes were selected randomly for further RT-qPCR evaluation to validate the reliability of Dual RNA-Seq, which was consistent with the transcriptome data in Fig. 6.

Table 2 Analysis of KEGG pathways in dual-species biofilms.

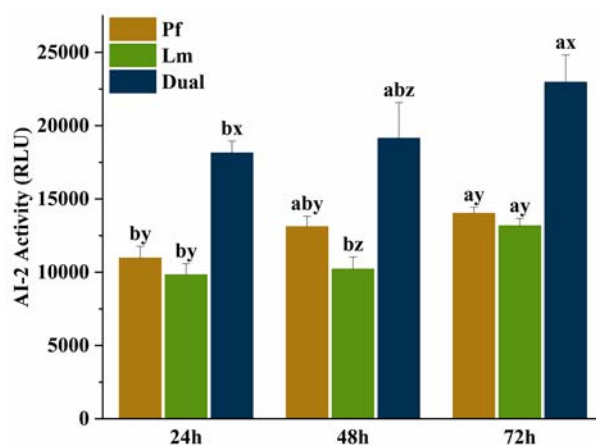
	Biological pathway	KEGG ID	DEGs numbers	P Value
<i>P. fluorescens</i>	Two-component system	ko02020	7	< 0.01
	ABC transporters	ko02010	6	0.01
	Flagellar assembly	ko02040	2	0.01
	Fatty acid metabolism	ko01212	2	0.03
<i>L. monocytogenes</i>	Flagellar assembly	ko02040	15	< 0.01
	Phosphotransferase system	ko02060	20	< 0.01
	Bacterial chemotaxis	ko02030	10	< 0.01
	ABC transporters	ko02020	18	< 0.01
	Two-component system	ko01212	27	< 0.01

The KEGG pathway was significantly enriched ($P < 0.05$).

**Fig. 6** RT-qPCR validation of *P. fluorescens* (A) and *L. monocytogenes* (B)

3.6 AI-2 assay with *V. harveyi* BB170

The relative concentrations of AI-2 in the supernatants of mono- and dual-species biofilms were quantified with a *V. harveyi* bioluminescence sensor (Fig. 7). AI-2 concentrations increased progressively during cultivation, reaching a maximum at 72 h in both mono- and dual-species biofilms, demonstrating the capability of both species to generate this signal. Strikingly, the concentration was significantly higher in the dual-species biofilms throughout the experiment (24, 48, and 72 h; $P < 0.05$). This enhancement was most evident at 72 h, suggesting a synergistic interaction that amplifies AI-2 production.

**Fig. 7** AI-2 relative concentrations of mono- and dual-species biofilms of *P. fluorescens* and *L. monocytogenes*. Different letters (a - c) indicate significant differences between same column within the different incubation time ($P < 0.05$). Different letters (x - z) indicate significant differences between same time points with different columns.

3.7 Effect of exogenous AI-2 on biofilm formation

To validate the regulatory role of the QS system, the regulatory effect of the AI-2 signaling molecule on biofilms was investigated in Fig 8. The biofilm-forming ability of both *P. fluorescens* and the dual-species system increased in a dose-dependent manner with the addition of AI-2. At a concentration of 10 ng/mL, the biofilm biomass reached 1.46 for *P. fluorescens* and 2.63 for the dual-species biofilm, both of which were significantly greater than those of the untreated controls ($P < 0.05$). When the AI-2 concentration was increased to 50 ng/mL, the biomass of *P. fluorescens* and cocultured biofilms increased by approximately 0.74- and 1.25-fold, respectively, compared to the controls ($P < 0.05$). In contrast, AI-2 supplementation at any concentration did not significantly affect the biofilm formation of *L. monocytogenes* ($P > 0.05$). These results suggested that AI-2 enhanced dual-species biofilm development primarily by regulating the behavior of *P. fluorescens*. It also verified the transcriptomic results of QS.

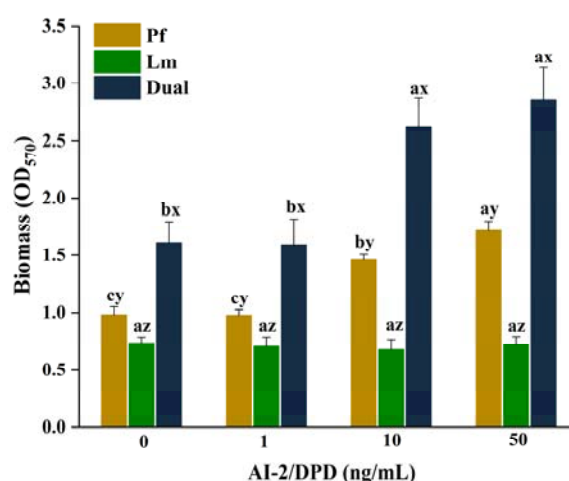


Fig. 8 Effect of exogenous addition of AI-2 on biofilm formation ability in mono- and dual-species biofilms. Different letters (a - c) indicate significant differences between same column within the same incubation time period ($P < 0.05$). Different letters (x - z) indicate significant differences between same time point with different columns.

4. Discussion

In food processing environments, microorganisms rarely exist in isolation, typically forming complex communities composed of multiple species^[23]. In meat processing, *P. fluorescens* and *L. monocytogenes* were frequently co-isolated from food and equipment surfaces^[8,24]. The two species exhibited high detection and contamination rates, demonstrated strong persistence, and were difficult to eradicate even with standard cleaning and disinfection protocols^[25-26]. Previous studies indicated that dual-species of *P. fluorescens* and *L. monocytogenes* exhibited synergistic interactions and resulted in a robust three-dimensional architecture^[18]. Its structure consisted of a densely interwoven EPS matrix, which could provide a protective shelter for the embedded bacterial cells^[27]. In this study, the enhanced tolerance of the dual-species biofilms to sodium hypochlorite further supported the above hypothesis (Table 1). Meanwhile, previous studies showed that *L. monocytogenes* localizes to deeper layer of dual-species biofilm formed with *P. fluorescens*^[28]. The biofilm formed by *P. fluorescens* provided a shelter for *L. monocytogenes*, which may be the reason that *P. fluorescens* was preferentially inactivated. Namely, the interspecies cooperation between *P. fluorescens* and *L.*

monocytogenes enhanced resistance to environmental stresses, which could explain long-term persistence and recurrent contamination. So, disrupting microbial interactions and interrupting the close association represent a promising strategy for controlling mixed biofilm formation. In other words, deciphering the molecular mechanisms underlying the interspecies interactions between *P. fluorescens* and *L. monocytogenes* will be a crucial prerequisite for controlling the contamination and ensuring food safety.

Dual RNA-seq simultaneously captures the transcriptomes of two interacting species by direct joint library preparation and sequencing of co-cultured samples, by passing separation-induced biases and retaining valid reads while filtering cross-mapping reads to reveal their concurrent transcriptional mapping^[29]. Dual RNA-seq results revealed significant enrichment in motility-related pathways, including flagellar assembly and bacterial chemotaxis, suggesting a regulatory role in mixed biofilm development. Flagellar assembly is known to be crucial for motility, adhesion, and biofilm formation in both Gram-negative and Gram-positive bacteria^[30]. For instance, flagellar motility facilitated the transition of bacteria from a planktonic state to the attachment mode, with the early stages of biofilm formation typically characterized in terms of the upregulation of flagellar genes. Conversely, during the intermediate phase, bacteria within the biofilms became attached to biotic or abiotic surfaces, exhibiting reduced motility alongside the downregulation of flagellar genes. Finally, the expression of flagellar-associated genes gradually resumed, aiding in the escape from the biofilm structures^[31]. More interestingly, *P. fluorescens* forms biofilms faster than *L. monocytogenes* in the co-culture system, and flagellum-mediated motility serves as a vital strategy for *L. monocytogenes* to invade the biofilms formed by *P. fluorescens* and colonize the deeper layers of the co-cultured aggregate^[32]. The upregulation of the flagellar assembly pathway in *L. monocytogenes* may contribute to the formation of co-cultured aggregates^[33], which was consistent with the observations from SEM (Fig. 2). Bacterial chemotaxis refers to reactive movement of bacteria in response to chemical gradients, enabling microorganisms to seek beneficial conditions, and avoids harmful ones, such as starvation, disinfectants, and antibiotics^[34]. Research indicated that *cheA*, *cheV*, and *motB* positively upregulated aggregation and biofilm formation of *L. monocytogenes*^[35].

Concurrently, pathways including PTS, fatty acid metabolism or synthesis, the two-component system, and ABC transporters, along with the associated genes, also participated in regulating the dual-species biofilm formation of *P. fluorescens* and *L. monocytogenes* in this study (Table 2). Among the *L. monocytogenes* in co-culture, 20 PTS-related genes could potentially contribute directly or indirectly to biofilm formation. For instance, PTS affected the expression of the virulence regulator PrfA by modulating carbon metabolism, thereby affected biofilm adhesion^[36]. However, the role of PTS pathway in biofilm formation still needs further verification. The composition of cell membrane fatty acids is one of the critical strategies for bacteria to adapt to fluctuating environments and form biofilms. It maintains the optimal fluidity and permeability of the cell membrane, which not only regulates the nutrient transport but also reduces energy consumption, thereby significantly enhancing bacterial survival rates^[37-38]. The two-component regulatory system acts as a pivotal regulator in environmental adaptation, biofilm formation, QS, flagellar motility, and metabolic

control^[39]. In *L. monocytogenes*, the CheA/Y two-component chemotactic system can regulate flagellar motility, which receives external signals through CheA and regulates responses to these signals through CheY^[34]. The overexpression of the *cheY* gene enhanced cell motility and hydrophobicity to regulate biofilm formation^[40]. In addition, the ABC transport system serves as a regulator of cellular homeostasis and acts a vital role in signal transduction, antibiotic resistance, chemotaxis, and biofilm formation^[33]. The ABC transport of *P. fluorescens* and *L. monocytogenes* was highly expressed during this interaction, indicating that species exhibited a high level of transmembrane transport activity during co-culture growth. The *aatJ* participated in glutamate and aspartate metabolism^[41], whilst *potF* is associated with putrescine transport system and QS system^[42]. The aforementioned analysis revealed that the formation mechanism of dual-species biofilms between *P. fluorescens* and *L. monocytogenes* constituted a complex regulatory network.

The CFS of coculture in this study significantly enhanced the biomass of individual *P. fluorescens* and *L. monocytogenes* biofilms (Fig. 1), which was further confirmed by observing the variations in biofilm microstructure using AFM and SEM (Figs. 2, 3). Considering the potential impact of nutrient dilution on biofilm formation, the addition of a sterile water volume equivalent to that of CFS was confirmed to have no impact on biofilm formation (data were not shown). AI-2 signaling has an essential role in interspecies interactions between *P. fluorescens* and *Staphylococcus aureus*, facilitating the construction of a matrix of dual-species biofilms and EPS production^[43]. Similarly, the involvement of *Streptococcus mitis* AI-2 in interspecies interactions with *Pseudomonas aeruginosa* was also significant^[44]. However, to the best of author's knowledge, the role of AI-2 in dual-species biofilm of *P. fluorescens* and *L. monocytogenes* has been rarely reported. The QS pathway was significantly enriched in the interactome transcriptome analysis of dual-species biofilms, which predicted that QS signaling molecules could be involved in regulating this process. Based on the transcriptomic findings, the promotional effect of QS signaling molecules on dual-species biofilm formation was characterized by quantifying the relative concentration of AI-2 in the supernatant (Fig. 7). Simultaneously, we supplemented the cultural systems with exogenous signaling molecules to further corroborate the transcriptomic results and confirmed the promotional role of AI-2 (Fig. 8). Interestingly, while the co-culture system exhibited a concurrent increase in both dual-species biofilm biomass and AI-2 levels, this does not necessarily imply that a larger number of biofilm cells were actively contributing to AI-2 production. In contrast, consistent with our previous findings, this synergistic interaction of biofilm formation was attributed to the substantial formation of EPS (proteins and polysaccharides), rather than a simple summation of cell numbers^[18]. That was to say, the accumulation of AI-2 served as a contributing factor to the proliferation of biofilm cells. Additionally, previous studies had demonstrated that the exogenous AI-2 exhibited a dose-dependent manner effect on the regulation of *P. aeruginosa* biofilm formation^[45]. Meanwhile, AI-2 also exerted a similar effect on the biofilm formation of *P. fluorescens* in this study. However, the biofilm biomass of *L. monocytogenes* was not affected by exogenous AI-2 molecules. The pathway for the synthesis of the AI-2 signaling molecule is usually as follows: S-adenosylhomocysteine, an intermediate of S-adenosylmethionine, is catalyzed by S-adenosylhomocysteine ribonuclease (Pfs) to generate S-ribose

homocysteine (SRH). Next, the S-ribosylhomocysteine lyase (LuxS) converts SRH to the AI-2 precursor molecule DPD and homocysteine, followed by spontaneous cyclisation of DPD to form an AI-2 type signaling molecule^[46]. AI-2 molecules were not detected in the luxS mutants; however, the biofilms were denser and adhered than those formed by wild *L. monocytogenes*. It was noteworthy that the relationship between gene transcription and protein expression in interacting strains were not a simple linear correlation, while the transcription level of luxS could influence AI-2 concentration to some extent^[47]. For instance, following a dietary shift, AI-2 concentrations increased while luxS gene expression exhibited a downward trend^[48]. In the present study, determining AI-2 metabolic levels was, to a certain extent, more practically relevant than measuring gene transcription. Additionally, further studies revealed that the addition of AI-2 did not stimulate biofilm formation in *L. monocytogenes*, whereas the inclusion of SRH enhanced cellular adhesion. This implied that *L. monocytogenes* could lack an AI-2 receptor and instead relies on metabolizing the precursor substance in biofilm forming^[49]. In addition, other metabolites may also affect the physiological activity and biofilm formation of *L. monocytogenes*. For example, metabolic volatile metabolites of *P. fluorescens* can be potential signaling molecules in the regulation of *L. monocytogenes* biofilm formation^[50]. The growth of *L. monocytogenes* could also be facilitated by *Pseudomonas* through the provision of essential intermediate metabolic substrates, such as protein hydrolysis products^[13].

5. Conclusions

In this study, *P. fluorescens* and *L. monocytogenes* formed robust dual-species biofilms through synergistic interaction, which enhanced the resistance to sodium hypochlorite and undoubtedly heightened food safety concerns. During this synergistic process, one strain activated complex gene regulatory networks, including multiple key pathways and genes such as QS, to adapt to the presence of the other strain, promoting biofilm formation and thereby better coping with the survival environment. Furthermore, the stimulatory effect of metabolites from co-culture on bacterial biofilms, along with the detection of high levels of the QS signal molecule AI-2 and supplementary exogenous validation, collectively elucidated the molecular mechanisms underlying the interspecies interactions. This study prospectively deciphered the formation of dual-species biofilms from both transcriptional and metabolic perspectives, providing a reference for the development of effective control strategies in the food industry.

Conflicts of interest

The authors declare no conflict of interest.

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