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Fatty acid remodeling and flagellar motility orchestrate *Pseudomonas fluorescens* biofilm formation during chilled meat distribution

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ABSTRACT: The formidable biofilm-forming ability of *Pseudomonas fluorescens* enables its persistent contamination under distribution conditions (0 - 4°C) of chilled meat, yet the mechanisms governing its biofilm formation remain inadequately explored. In this study, with 25°C serving as the control, *P. fluorescens* developed more substantial and architecturally coherent biofilms at 4°C during 96 h-incubation, accompanied by increased extracellular polymeric substances synthesis. This response phenotype enhanced cell adhesion to facilitate persistence and increased the cross-contamination after multiple cell transfers. Transcriptomic analysis revealed that 514 differentially expressed genes (DEGs) were up-regulated and 327 DEGs were down-regulated, which were involved in fatty acid degradation, flagellar assembly, lysine degradation, pyruvate metabolism, etc. Notably, as primary regulatory pathways, flagellar assembly and fatty acid metabolism suggested they could facilitate microbial accumulation and the efficient conversion of fatty acids, respectively. Correspondingly, changes in flagellar assembly-related genes and fatty acid degradation-related genes were consistent with transcriptome results. In view of these, the apparent reduction in motility and the increase in unsaturated fatty acid content, along with the enhancement of cell surface hydrophobicity and auto-aggregation, further verified the above-mentioned pathways. This study provided insights into formation mechanism of *P. fluorescens* biofilm and the development of control strategies.

Keywords: Chilled meat condition, *Pseudomonas fluorescens* biofilms, Fatty acid, Flagellar assembly, Transcriptome

1. Introduction

Biofilms are typically characterized by microbiota embedded in extracellular polymeric substances (EPS), which consists mainly of proteins, polysaccharides, extracellular DNA (eDNA) and small amounts of lipids^[1]. As the “boundary” coating bacteria, EPS serves several important functions: (a) it can act as a protective barrier that confers on microorganisms a formidable capacity to resist adverse environmental conditions, such as disinfectants, antibiotics and UV radiation^[2-3]; (b) it is an “adhesive” contributing to

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bacterial adhesion onto surfaces, resulting in persistent bacterial residues and the potential for cross-contamination^[4].

Cold chain plays a vital role in meat distribution^[5], keeping low temperatures throughout the entire chain from slaughter, process, storage, transportation and sale can extend the shelf life of meat. While the cold chain effectively controls microbial growth and enzymatic activity to maintain meat quality, the presence of psychrophilic microorganisms (such as *Pseudomonas*) remains the primary cause of meat spoilage across the cold chain. Under aerobic conditions during chilled meat distribution, *Pseudomonas* emerge as the dominant spoilage bacteria^[6]. Notably, over 60% of the initial *Pseudomonas* population is comprised of *Pseudomonas fluorescens*^[7]. During meat spoilage, due to its exceptional metabolic aptitude in protein and lipid hydrolysis, even under chilled conditions, the lipases and proteases of *P. fluorescens* play a crucial role in the degradation for lipids and proteins into fatty acids, amino acids, etc., leading to the deterioration of color and texture, secretion of rancidity and off-odors^[8-9]. Relying on its “psychrophilic advantage”, *P. fluorescens* could form robust biofilms on the equipment and contact surfaces within food processing environments at chilled temperatures, and subsequently pose persistent contamination risks throughout food processing systems, even after rigorous sterilization procedures^[10,11].

Previous studies have demonstrated that chilled temperatures promoted the biofilm formation and adherence of *Pseudomonas*^[12-13]. Notably, studies had documented that at 4°C, the stress resistance of *P. fluorescens* was facilitated through secreting substantial EPS, which appeared to be associated with quorum sensing^[14]. The contribution of biofilms to meat spoilage at chilled temperatures is a significant concern that warrants further detailed investigation, particularly regarding the risk of meat contamination caused by *Pseudomonas*. Moreover, the underlying mechanisms of *Pseudomonas* biofilm formation at chilled temperatures are intricate, involving multiple genes and pathway networks^[12, 14, 15]. Given the meat safety concerns during cold chain distribution, it is essentially necessary to decipher the formation mechanisms of *Pseudomonas* biofilms at low temperatures.

Therefore, this study aimed to evaluate the formation capacity, microstructure, and transfer ability of *P. fluorescens* biofilms under chilled condition (4°C), with biofilms cultured at room temperature (25°C) serving as the control. Subsequently, transcriptomic analysis emphatically elucidated the underlying regulation pathways. The RT-qPCR results, bacterial motility, and fatty acid degradation, cell surface hydrophobicity and auto-aggregation were further validated. Such information is valuable for the development of prevent strategies against *P. fluorescens* biofilms at low temperatures in chilled meat distribution.

2. Materials and methods

2.1 Bacterial strains and culture conditions

The strain of *P. fluorescens* CICC 21620 used in this study was bought from China Center of Industrial Cultural Collection. The stain was cryopreserved in Luria-Bertani (LB, Beijing Land Bridge Technology, China) medium, comprising 25 % (v / v) glycerol at -80°C in the laboratory. Prior to experimentation, the

strains were continuously cultured for 18 h at 28°C in LB medium, with shaking at a speed of 150 r/min, and this process was repeated twice. The activated strains were diluted to a concentration of 6 log CFU/mL using LB medium for subsequent experiments.

2.2 Biofilm formation assay

The biomass was evaluated using crystal violet (CV) stains as previously described with some modifications^[16]. *P. fluorescens* suspension (200 µL) was added to the 96-well polystyrene microtiter plates, and the biofilms were cultured at 4°C and 25°C for 24, 48, 72, 96, and 120 h, respectively. The same experiment was repeated three times. At each time point, after the planktonic cells were poured out, the biofilms were rinsed thrice with sterile 0.85% NaCl solution and then dried for 30 min. Subsequently, the biofilms were stained with CV stain (2% w/v, 200 µL) for 30 min under dark conditions. The suspension was discarded from the plate wells and the biofilms were dried as previously described. Afterwards, 95 % (v/v) ethanol (200 µL) was added and dissolved for 30 min. The absorbance at 570 nm was measured using a microplate reader (Molecular Devices, USA) to determine the biomass of biofilms.

2.3 Cell counts

The biofilms of *P. fluorescens* were cultured at 4°C and 25°C, respectively, as described in section 2.2. The biofilm cells without plankton were collected at each time point using sterile scrapers and redissolved with 0.85% NaCl solution. The plate counting method was employed to enumerate cell colony after incubation for 48 h at 28°C. The microbial populations were expressed as log CFU/cm².

2.4 Micromorphology of biofilms

2.4.1 Field emission-scanning electron microscopy (FE-SEM)

The *P. fluorescens* suspension was added to 48-well polystyrene microtiter plates in aliquots of 600 µL^[17]. Disinfected polystyrene coupons (10 × 10 × 1 mm) were vertically positioned within each well. After incubation to each time point statically, the coupons were gently rinsed three times with sterile phosphate buffered saline (PBS, Servicebio, China), and then aired-dried. The coupons were immersed in 2.5% glutaraldehyde at 4°C for 24 h to facilitate the fixation of biofilms. The samples underwent dehydration using a gradient of ethanol and were subsequently critical-dried with CO₂, followed by gold spraying coating. Finally, samples were imaged with a FE-SEM SU8020 (Hitachi, Tokyo, Japan) at 5,000 × magnification.

2.4.2 Confocal laser scanning microscopy (CLSM)

Biofilm microstructure was also analyzed using confocal laser scanning CLSM following an adapted protocol from Qian et al.^[18]. FITC-Tomato lectin, Lycopersicon Esculentum lectin (FITC-EL, Shanghai yuanye Bio-Technology, China), was a stable single subunit glycoprotein, which could bind to the amidogen of proteins (soaked samples for 25 min; 10 µL/mL). iFluorTM 555-Concanavalin A Conjugate (Con A-iF555, Xi'an Biolite, Ltd., China), a kind of agglutinin, could detect polysaccharides (soaked samples for 25 min; 5

$\mu\text{L/mL}$). 4',6-diamidino-2-phenylindole (DAPI) was commonly used to be combined with DNA, detecting the live cells in the samples (soaked samples for 25 min; 10 $\mu\text{L/mL}$). The microstructure was visualized by using a high-resolution laser confocal microscope (Carl Zeiss MicroImaging, Göttingen, Germany). Under the 20 $\times$ objective lens, the biofilms were observed through excitation lasers of 488 nm for FITC, 561 nm for Con A-iF555, and 405 nm for DAPI, which emitted red laser light, green laser light, and blue laser light, respectively. Biofilm images were analyzed using ZEN 2.3 software (Carl Zeiss MicroImaging, Göttingen, Germany).

2.5 Quantification of EPS in biofilms

The collection of EPS was according to Han et al.^[19, 20], bacterial suspension was discarded and the wells were rinsed three times with PBS, and then enriched biofilms using sterile cell scraper especially sidewalls and bottom of the wells. The biofilms of three wells were resuspended to 3 mL of PBS and ultrasonic treated for 5 min. The supernatant was collected and filtrated with 0.22 μm filter membrane after centrifuging at 4°C for 20 min at 6000 g. The BCA protein assay kit (CWBIO, China) and the phenol-sulfuric acid method was used for quantitative analysis of extracellular proteins and extracellular polysaccharides^[21, 22].

2.6 Transfer of biofilm from polystyrene to beef

Beef *longissimus lumborum* muscles were purchased from Huasheng Halal Meat Co., Ltd. (Yangxin, China) and removed the surface muscles with a sterile scalpel before the experiment. According to Midelet et al.^[23] with minor modifications, the beef coupons (5 $\times$ 2.5 $\times$ 0.6 cm) were placed on a biosafety cabinet, and irradiated with UV lamp for 15 min on each side to minimize the initial microbial load (Anamul Hasan Chowdhury et al., 2024). Before the transfer experiments, polystyrene coupons (5 $\times$ 2.5 $\times$ 0.1 cm) were placed in centrifuge tubes containing 10 ml of LB medium, incubating until maturity stage at 25°C and 4°C, respectively. Then, beef coupons were rinsed three times with 0.85 % NaCl and loaded with 500 g weights to positioned on biofilms for 30 s. This procedure was repeated 10 times on different beef coupons to accurately characterize the successive transfer ability^[24]. After each repetition, the samples were homogenized with a blender (BagMix-erR®400, France) for 2 min, following by selecting appropriate dilution levels to enumerate adherent cells on CFC Selective medium (Hopebio, Qingdao, China).

2.7 Transcriptomics analysis

In accordance with the manufacturer's instructions in MiniBEAT Universal RNA Extraction Kit (Accurate Biotechnology, Changsha, China), RNA was isolated from 96-h biofilm of *P. fluorescens*. Then RNA sequencing (RNA-seq) was completed in Novogene Corporation (Tianjin, China). Before sequencing with the Illumina HiSeq™ 2000 platform, the sequencing libraries were prepared on the base of specification. The remanent sequences referred to genome of *P. fluorescens* using Bowtie2 (<http://bowtie-bio.sourceforge.net/index.shtml>). Later, the original gene expression was statistically analyzed and calculated by HTSeq 0.6.1p2 (<http://www-huber.embl.de/users/anders/HTSeq>). Finally, the differentially expressed genes (DEGs)

were picked out on the basis of following requirement: multiple expression differences $|\log_2 \text{Fold Change}| > 1$ and significance P value < 0.05 , so that DEGs could be enriched by the software Partek Genomics Suite based on Gene Ontology (GO) annotations, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway.

2.8 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Before validating the selected genes through RT-qPCR, cDNA was obtained using the PrimeScript™ RT Reagent Kit (Takara, Beijing, China) to facilitate subsequent quantitative gene expression analysis with SYBR® Premix Ex Taq™ (Takara, Beijing, China). Based on the omics results, 28 DEGs of biofilm formation were selected to be validated, whose primers were synthesized by BioSsune Co., Ltd. (Shanghai, China) (Table S1). In addition, the relative gene expression was determined using $2^{-\Delta\Delta C_t}$ method^[25].

2.9 Motility assay

The amount of 3 μL *P. fluorescens* suspension was inoculated in the center of the plates, and cultured at 4°C and 25°C for 24 - 120 h, respectively. The motility ability was measured with vernier caliper. The formulas of motility medium were as follows: prepared swarming agar (2.5% [w/v] LB, 0.05% [w/v] glucose and 0.5% [w/v] agar powder); prepared swimming agar (1 % [w/v] tryptone, 0.5 % [w/v] NaCl, 0.25 % [w/v] glucose and 0.3 % [w/v] agar powder); prepared twitching agar (2.5 % [w/v] LB and 1% [w/v] agar powder)^[13].

2.10 Fatty acid analysis of biofilm cells

According to the method described by Wang et al.^[26], the fatty acid from biofilm cells cultured for 96 h were extracted through the following three kinds of reagent: (I) saponification reagent (45 g NaOH; 150 mL methyl alcohol; 150 mL aqua pura); (II) methylation reagent (325 mL 6M HCl; 275 mL methyl alcohol); (III) cleaning reagent (10.8 g NaOH; 900 mL aqua pura). The fatty acids were analyzed by gas chromatography-mass spectrometry (GC-MS, QP-2010 Plus, Shimadzu Corporation, Kyoto, Japan). Chromatographic separation of fatty acids was carried out using DB-5 capillary columns (30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm film thickness). The program of column temperatures was set as follows: holding for 1 min at 50°C, increasing temperature to 150°C at 20°C/min, then elevating to 250°C at 4°C /min for 1 min. The percentages of the whole category of detected fatty acid were determined based on the peak areas of methyl esters standards (Sigma-Aldrich, Shanghai, China).

2.11 Cell surface hydrophobicity (CSH) and auto-aggregation (AAG) analysis

The biofilm cells of *P. fluorescens* were subjected to centrifugation (11,000 $\times$ g, 10 min, 4°C), and subsequently rinsed three times repeatedly with PBS. The initial OD₆₀₀ absorbance of the bacterial suspension was adjusted to 0.2 (H₀) with an enzyme marker (Epoch™2 Bio Tek, USA). Afterwards, the suspension and isopycnic xylene (2 mL) were added to each sample and vortexed for 2 min. After standing for 15 min to separate organic and aqueous phases, the absorbance (H) of water phase was measured under OD₆₀₀ according

to the following Equation (1), the bacterial affinity to the solvent could be calculated. The standard for evaluating hydrophobicity was the percentage: hydrophilic (values < 20%), moderately hydrophobic (values between 20% and 50%) and highly hydrophobic (values > 50%)^[27].

$$\text{CSH}\% = \left(\frac{H_0 - H}{H_0} \right) \times 100\% \quad (1)$$

Pretreatment operations were consistent with the described above, the initial OD₆₀₀ absorbance of bacterial suspension was adjusted to 0.2 (A₀). Subsequently, the samples were incubated at 25°C for 6 h, and then measured the final OD₆₀₀ absorbance(A)^[28]. The expressed auto-aggregation was utilized the Equation (2).

$$\text{AAG}\% = \left(\frac{A_0 - A}{A_0} \right) \times 100\% \quad (2)$$

2.12 Statistical analysis

In this study, statistical analyses were performed by the MIXED procedure of Statistical Analysis System (SAS, Version 9.2, Cary, USA). Data from Biofilm formation ability, biofilm cell transfer capacity, fatty acid composition, cell motility, CSH and AAG were analyzed by a two-way ANOVA with incubation temperature and time and interaction as the fixed effects, while independent repetition as the random effects. The PDIF option was employed to separate least squares means, with significance assigned when $P < 0.05$. The figures were graphed using Prism 8.0 software (GraphPad Software, La Jolla, CA, USA). And the Prism 8.0 software was used to fit the transfer curves, while the Pearson correlation coefficient was used to determine the correlation between RNA-seq and RT-qPCR. All experiments included three independent replicates and three technical parallels.

3. Results and discussion

3.1 Biofilm formation

Obviously, biofilm formation exhibited a significant interaction effect of incubation time and temperature in Fig. 1 ($P < 0.05$). On the whole, there was a trend of initial increase followed by a gradual decline as the incubation time extended on biofilm formation, namely, the entire process of biofilm formation. At the early stages of biofilm formation at 24 h, the biomass was 0.36 at 4°C, and significantly lower than that at 25°C ($P < 0.05$). Interestingly, the biomass amount reached 3.66 at chilled temperature, remarkably higher about 1.27 than cultured at 25°C after 48 h ($P < 0.05$). More importantly, the amount reached the maximum at 96 h, i.e. mature biofilms, with the OD₅₇₀ value of 3.67 at 4°C, which was 1.23 higher compared with the biofilms at 25°C ($P < 0.05$). And even at 120 h, the amount at 4°C was still significantly higher than at 25°C by about 1.77 unit ($P < 0.05$). This suggests chilled temperatures facilitated the biomass formation of *P. fluorescens* biofilms.

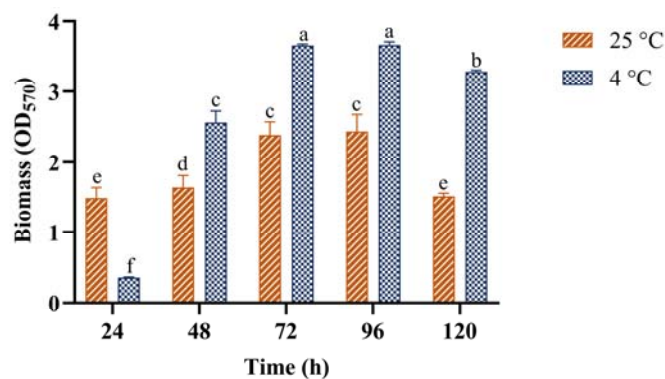


Fig. 1 Biofilm formation ability of *P. fluorescens* at 4°C and 25°C.

3.2 Cell counts

Similar trends as biomass were observed in biofilm cells population during early incubation stage at 25°C and 4°C (Fig. 2). The adherent cells at 4°C were 3.78 log CFU/cm² at 24 h, about 1.99 log CFU/cm² lower than that at 25°C. Under incubation conditions at 25°C, the cell counts of *P. fluorescens* biofilm emerged a progressive increase and reached a peak of 6.53 log CFU/cm² at 96 h. Nevertheless, the cell counts at 4°C adhered considerably from 48 h and reached 6.46 log CFU/cm² at 72 h, which was significantly higher than those at 25°C by approximately 0.5 log CFU/cm² ($P < 0.05$). Interestingly, there was no significant difference between the two incubation temperature conditions at 96 h ($P < 0.05$). In addition, the adherent cells at 4°C were 5.99 log CFU/cm², whereas at 25°C the amount was 6.54 log CFU/cm² after 96 h.

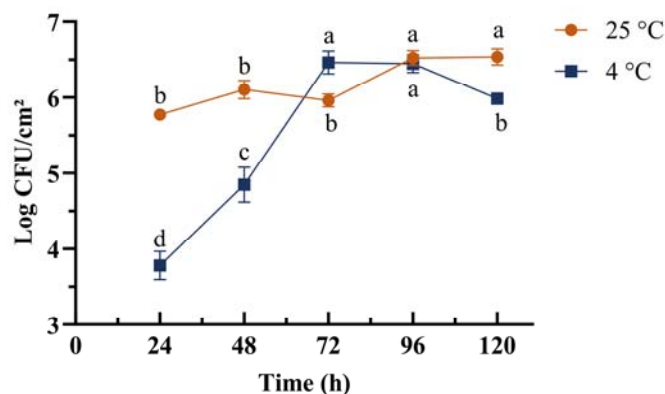


Fig. 2 Cell counts of *P. fluorescens* biofilms at 4°C and 25°C.

3.3 Microstructure

The FE-SEM (Fig. 3A) and CLSM (Fig. 3B) visualization provided evidence of the impact on the microstructural characteristics in *P. fluorescens* biofilms incubated at 25°C and 4°C. With the increase of incubation time, the biofilms at 4°C exhibited increased thickness and density during the maturation period at 96 h, whereas those at 25°C remained thin and loose (Fig. 3A). The positions of the three colored fluorescence were overlapping crossings. Meanwhile, the structure of the biofilms at 25°C was sparse and unevenly secreted, while the biofilms at 4°C were compact, especially at 72 h, 96 h, Fig. 3B), which exhibited lamellar fluorescence. In addition, more distinct green and red signals were observed at 4°C during the biofilm maturation period at 96 h (Fig. 3B), indicating that more extracellular polysaccharides and extracellular proteins were secreted and more biomass was produced.

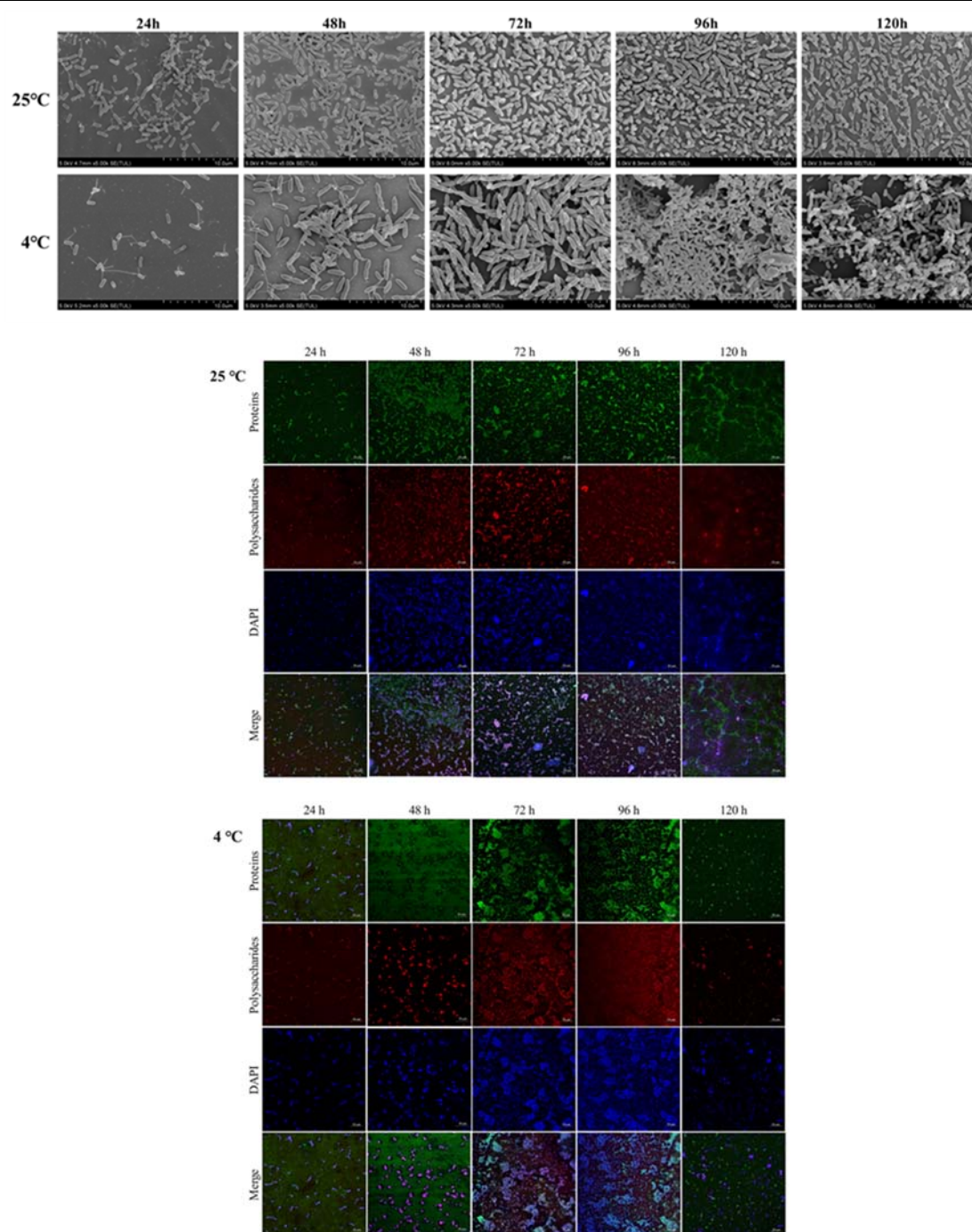


Fig. 3 Microstructure of *P. fluorescens* biofilms observed by field emission-scanning electron microscopy (A) and confocal laser scanning microscopy (B). The fluorescence includes FITC-EL (green) for protein, Con A-iF555 (red) for polysaccharide and 4', 6-diamidino-2-phenylindole (DAPI) (blue) for viable cells. The microstructure of biofilms was observed with a 20× object. Scale bar = 50 μm .

3.4 The components of EPS

The concentration of extracellular proteins (Fig. 4A) and extracellular polysaccharides (Fig. 4B) in *P. fluorescens* biofilms at 4°C and 25°C was quantified. Although less EPS were initially secreted at 4°C, the level increased remarkably at 72 h and reached the maximum value at 96 h, which was 1.57 mg/cm² (extracellular proteins) and 0.5 $\mu\text{g}/\text{cm}^2$ (extracellular polysaccharides). The secretion of extracellular proteins and polysaccharides increased 2.12-fold and 3.13-fold than those at 25°C (extracellular proteins: 0.74 mg/cm²; extracellular polysaccharides: 0.16 $\mu\text{g}/\text{cm}^2$), respectively indicating that the overproduction of EPS was

mainly due to the secretion of extracellular proteins. Additionally, both EPS remained significantly higher under the 4°C incubation condition compared with room conditions at 120 h.

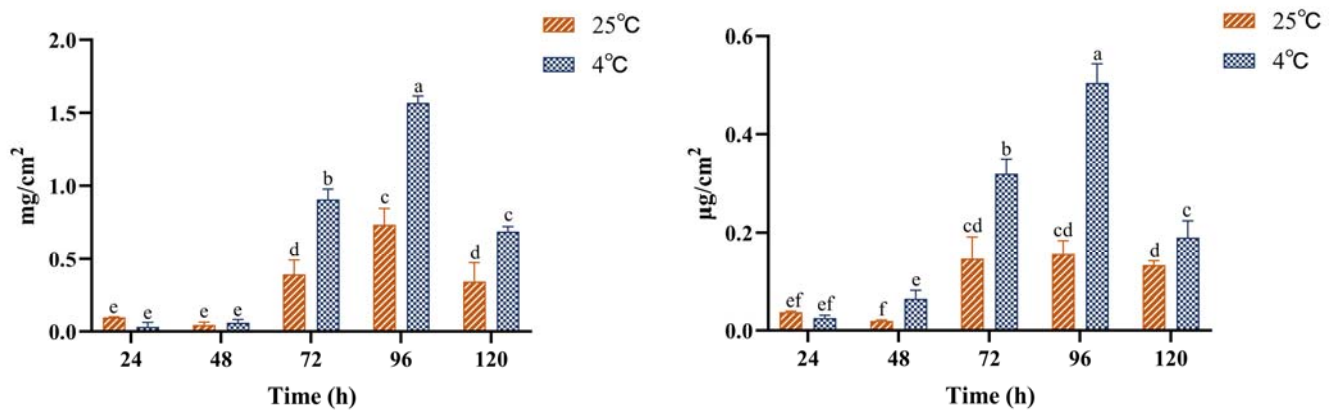


Fig.4 The concentration of extracellular polymer in *P. fluorescens* biofilms at 4°C and 25°C at 24 h to 120 h.

A. Extracellular proteins; B. extracellular polysaccharides. Mean with different letters(a-d) is significantly different in regard to the different incubation time ($P < 0.05$). ^{a-f} Means with different letters were significantly different at $P < 0.05$. The data is represented as the means $\pm$ standard errors.

3.5 Cell transfer ability on chilled beef

The transfer number of biofilm cells at 25°C and 4°C decreased gradually with the increase of transfer times, whereas the rate of decline gradually slowed down before levelling off (Fig. 5). Specifically, following the initial transfer, the viable number was 6.67 log CFU/cm² at 4°C, which was 0.41 log CFU/cm² pronouncedly higher than that incubated at 25°C ($P < 0.05$). After the second, third and fourth transfers, the number of biofilm cells transferred increased significantly by 0.68, 0.86 and 0.42 log CFU/cm², respectively, at 4°C compared to that at 25°C. The result indicates that cross-contamination risk posed by *P. fluorescens* biofilms could be worse at 4°C.

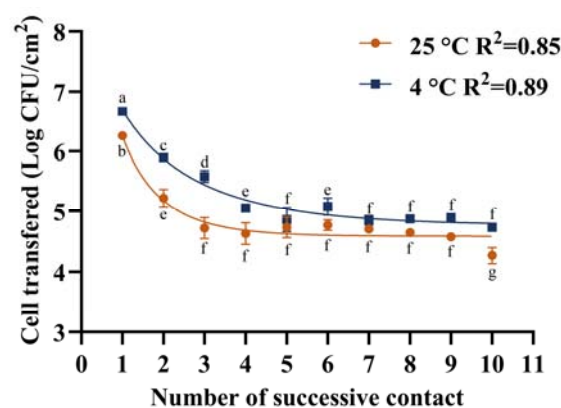


Fig. 5 The cells number of *P. fluorescens* biofilm after successive transfer from polystyrene surfaces to beef. ^{a-g} Means with different letters were significantly different at $P < 0.05$. The data is represented as the means $\pm$ standard errors.

3.6 Transcriptomics analysis

The transcriptome results indicated that a total of 841 DEGs in *P. fluorescens* biofilms at 4°C in comparison with that at 25°C, including 514 up-regulated genes and 327 down-regulated genes ($P < 0.05$, Fig. S1). These DEGs were categorized into 119 GO terms and divided into biological processes (BP), cellular

components (CC) and molecular functions (MF). Notably, alkyl hydroperoxide reductase complex (GO:0009321) was the most noticeable up-regulation in the CCs. In a series of MFs and BPs, oxidoreductase activity, acting on a sulfur group (GO:0016667) and response to oxidative stress (GO:0006979), response to oxygen-containing compound (GO:1901700) were the most significantly up-regulated regulatory GO items, respectively (Fig. 6A). In contrast, significantly down-regulated GO items at 4°C were mainly enriched in non-membrane-bounded organelle (GO:0043228), structural molecule activity (GO:0005198) and ribonucleoprotein complex biogenesis (GO:0022613) (Fig. 6B).

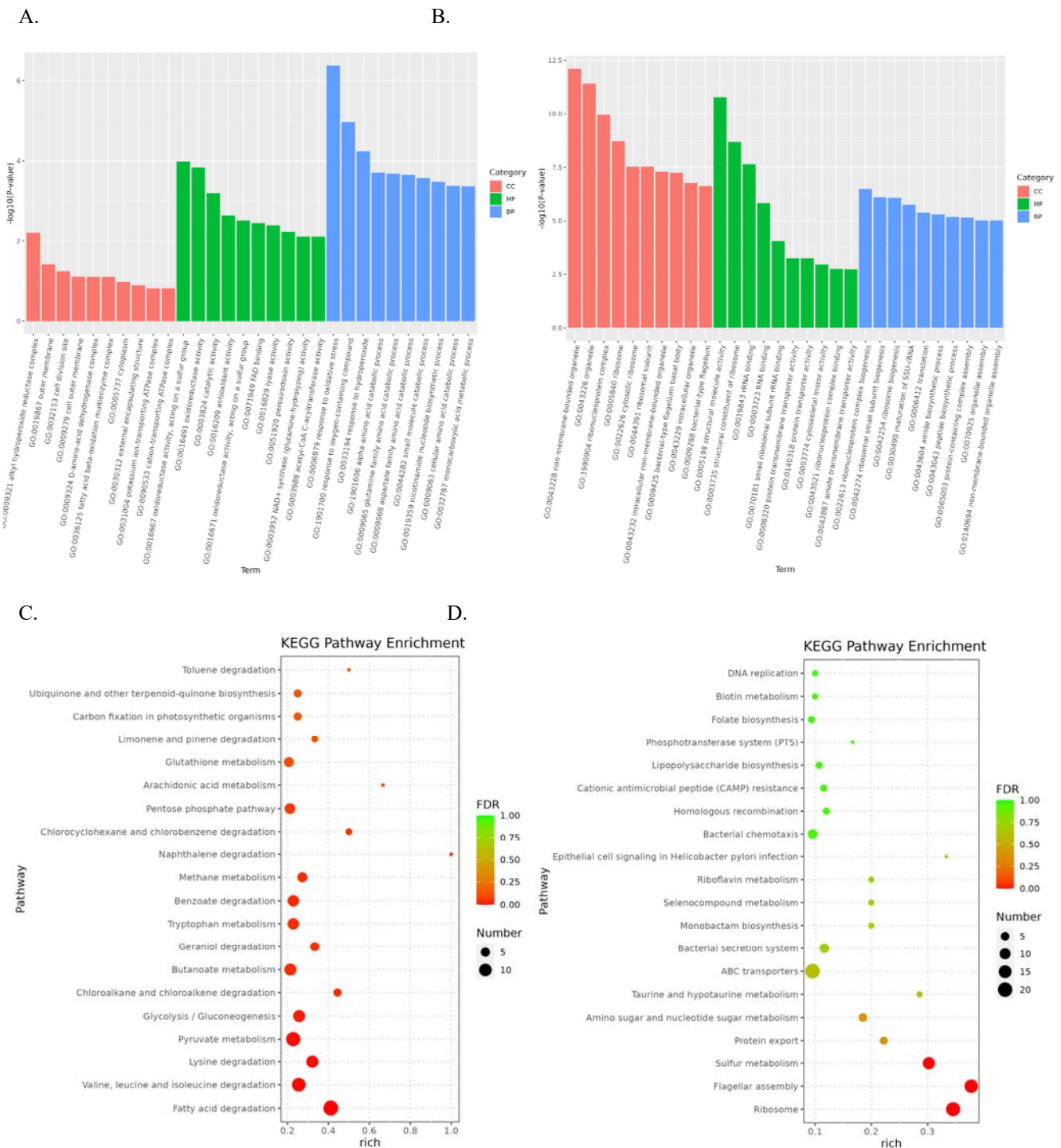


Fig. 6 Transcriptomic profiling revealed enrichment pathway and altered gene transcription patterns in *P. fluorescens* biofilms. A. GO enrichment analysis of the upregulated DEGs. B. GO enrichment analysis of the downregulated DEGs. C. KEGG pathway analysis of the upregulated DEGs. D. KEGG pathway analysis of the downregulated DEGs.

Table 1 DEGs in biofilm cells in comparisons of 4°C vs 25°C.

KEGG pathway	Gene ID	Gene name	Annotated gene function	log ₂ FC (4°C/25°C)	P - value
Fatty acid degradation	CPH89_RS19055	<i>fadA</i>	acetyl-CoA C-acyltransferase FadA	1.13	2.62E-06
	CPH89_RS19050	<i>fadB</i>	fatty acid oxidation complex subunit alpha FadB	1.86	5.33E-14
	CPH89_RS03605	<i>fadD2</i>	long-chain-fatty-acid--CoA ligase FadD2	1.29	8.84E-07
Lysine degradation	CPH89_RS11190	<i>gabD</i>	NADP-dependent succinate-semialdehyde dehydrogenase	1.58	9.50E-10
	CPH89_RS11195	<i>gabT</i>	4-aminobutyrate--2-oxoglutarate transaminase	1.39	6.84E-08
	CPH89_RS23890	<i>mdeB</i>	alpha-ketoglutarate dehydrogenase	1.26	7.30E-05
Pyruvate metabolism	CPH89_RS00705	<i>lldD</i>	FMN-dependent L-lactate dehydrogenase LldD	1.31	2.55E-07
	CPH89_RS18055	<i>viaY</i>	L-threonine dehydrogenase	1.79	1.11E-05
	CPH89_RS10775	-	acyl-CoA dehydrogenase	1.72	1.69E-08
Tryptophan metabolism	CPH89_RS02580	-	acetyl-CoA C-acetyltransferase	1.37	1.85E-07
	CPH89_RS02555	-	crotonase/enoyl-CoA hydratase family protein	1.36	5.92E-08
	CPH89_RS06865	<i>rpsE</i>	30S ribosomal protein S5	-1.06	4.13E-05
Ribosome	CPH89_RS13040	<i>rpsF</i>	30S ribosomal protein S6	-1.23	6.60E-06
	CPH89_RS06970	<i>rpsG</i>	30S ribosomal protein S7	-1.28	1.28E-06
	CPH89_RS02950	<i>flgA</i>	flagellar basal body P-ring formation chaperone FlgA	-1.29	0.00077
Flagellar assembly	CPH89_RS02925	<i>flgC</i>	flagellar basal body rod protein FlgC	-1.63	0.0025
	CPH89_RS01455	<i>flgG</i>	flagellar basal-body rod protein FlgG	-1.47	0.0060
	CPH89_RS01380	<i>fliE</i>	flagellar hook-basal body complex protein FliE	-1.81	9.28E-05
	CPH89_RS01375	<i>fliF</i>	flagellar basal-body MS-ring/collar protein FliF	-1.15	0.0033
	CPH89_RS01370	<i>fliG</i>	flagellar motor switch protein FliG	-1.46	0.0044
	CPH89_RS01365	<i>fliH</i>	flagellar assembly protein FliH	-1.66	6.28E-05
Protein export	CPH89_RS01360	<i>fliI</i>	flagellar protein export ATPase FliI	-1.56	0.00046
	CPH89_RS07015	<i>secE</i>	preprotein translocase subunit SecE	-1.06	0.00071
	CPH89_RS05675	<i>secG</i>	preprotein translocase subunit SecG	-2.68	8.07E-05
Amino sugar and nucleotide sugar metabolism	CPH89_RS06850	<i>secY</i>	preprotein translocase subunit SecY	-1.60	6.00E-10
	CPH89_RS25580	<i>arnD</i>	4-deoxy-4-formamido-L-arabinose-phosphoundecaprenol deformylase	-1.16	5.08E-05
	CPH89_RS04575	<i>nagA</i>	N-acetylglucosamine-6-phosphate deacetylase	-1.02	0.0060
	CPH89_RS28345	<i>murB</i>	UDP-N-acetylmuramate dehydrogenase	-1.53	3.13E-06

The biological functions of the DEGs were further analyzed on the basis of the KEGG pathway in Fig. 5. Compared with biofilms formed at 25°C, it was found that the up-regulated DEGs at 4°C were enriched in fatty acid degradation, lysine degradation, pyruvate metabolism and tryptophan metabolism (Fig. 6C); while down-regulated DEGs were enriched in ribosome, flagellar assembly, protein export and amino sugar and nucleotide sugar metabolism attributed to chilled conditions (Fig. 6D). The eight KEGG pathways mentioned above were the most remarkably altered in response to chilled temperature, of which major DEGs were selected (Table 1). Among these genes, *fadA*, *fadB* and *fadD2* associated with fatty acid degradation were significantly upregulated, which were also involved in fatty acid beta-oxidation multienzyme complex (GO:0036125). As the most prominent down-regulated pathway, the down-regulation of *rpsE*, *rpsF* and *rpsG*

in the ribosome pathway resulted in the regulation of ribosome (GO:0005840), implying a potential role of these genes in biofilms formation at 4°C. Similarly, *flgA*, *flgC*, *flgG*, *fliE*, *fliF*, *fliH* and *fliI* genes related flagellar assembly were down-regulated at 4°C which was a key factor acting on the down-regulation of bacterial-type flagellum basal body (GO:0009425) and bacterial-type flagellum (GO:0009288), which was suggested that flagellum-mediated motility may play a significant role in biofilm formation at chilled temperature.

3.7 RT-qPCR validation

RT-qPCR results related genes indicated that the expression trends of the relevant genes were found to be consistent with the transcriptomics results (Fig. 7). As demonstrated by Pearson's correlation, well correlation existed between the results of RT-qPCR and transcriptomic analysis ($P < 0.001$, $R^2 = 0.9104$), verifying the credibility of the omics results.

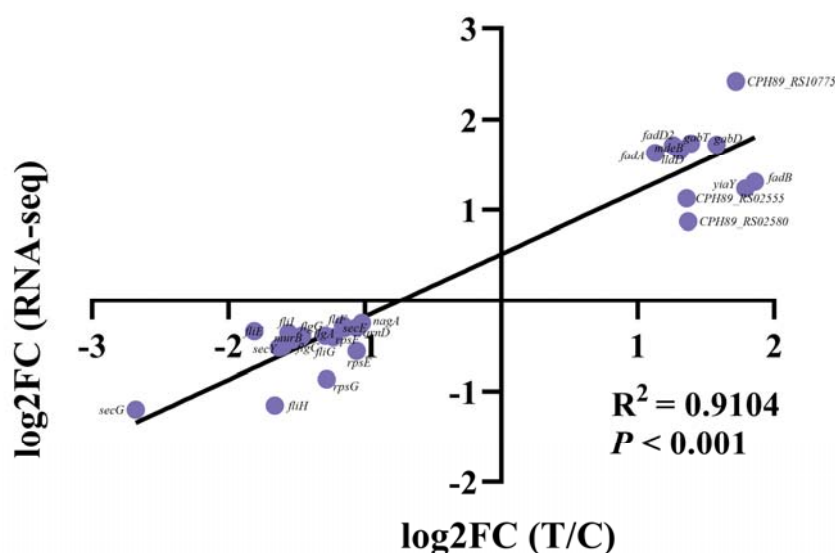


Fig. 7 The correlation analysis between RNA-seq data and RT-qPCR.

3.8 Verification of the effect of chilled temperature on motility

The swarming, swimming and twitching motility were visually presented in Fig.8. With the increase of incubation time, the motility ability at 25°C were increasingly more pronounced than those at 4°C, especially swimming ability. Specifically, the diameters of swimming were 46.39 ± 0.66 mm and 61.31 ± 0.44 mm at 25°C and increased about 41.68 mm and 55.77 mm in comparison with that at 4°C for 72 and 96 h, respectively (Fig. 8A). With regard to swarming ability, the diameter was approximately 9.11 ± 0.13 mm at 25°C when incubated to 120 h, while it was 5.98 ± 0.17 mm at 4°C (Fig. 8B). As for twitching, the diameters at 25°C for 72, 96 and 120 h increased about 2.36 mm, 2.96 mm and 3.5 mm significantly compared with those at 4°C (Fig. 8C, $P < 0.05$). In addition, for swarming, swimming and twitching motility, the increased diameter from 24 h to 120 h was only 1.72 mm, 2.87 mm and 1.31 mm at 4°C, whereas the corresponding increments were 4.18 mm, 62.54 mm and 3.91 mm at 25°C. This further demonstrated that chilled temperature inhibited *P. fluorescens* motility, which validated the results of transcriptomics.

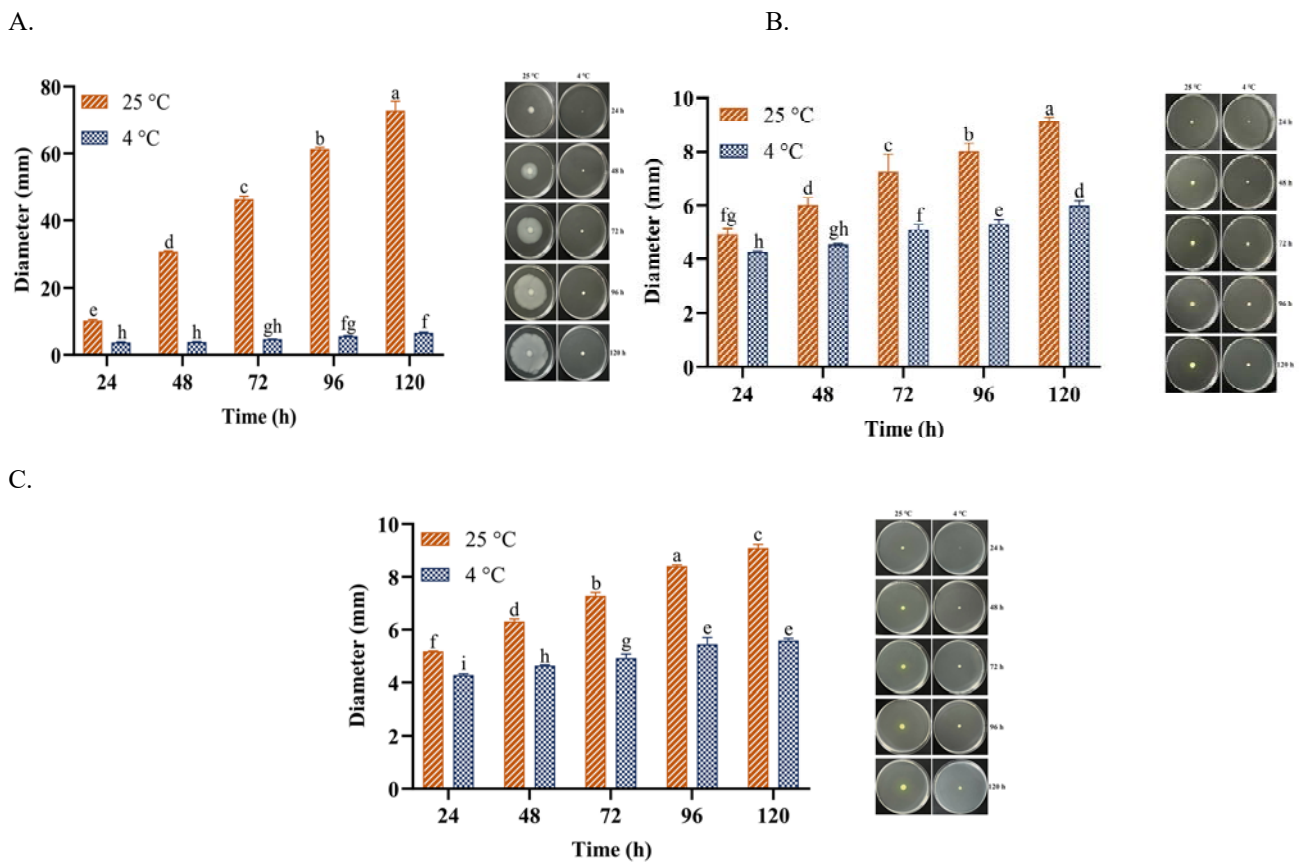


Fig. 8 The swimming (A), swarming (B) and twitching (C) ability of *P. fluorescens* at 4°C and 25°C within 120 h. a – i Means with different letters were significantly different at $P < 0.05$. The data is represented as the means $\pm$ standard errors.

3.9 Verification of the effect of chilled temperature on fatty acid profile

For validation of the omics results about fatty acid degradation pathway, particular attention was given to the changes in fatty acid composition on biofilm membrane at 96 h. The metabolome of the fatty acid profiles of biofilm cell membranes at 25°C and 4°C was presented in Table 2. The primary fatty acids of biofilm cells at 25°C were composed of C16:0 (26.55%), C16:1 (18.77%), C17:1 (15.70%), C18:0 (16.70%) and C18:1n9c (17.22%). Due to the impact of chilled temperature, the fatty acid composition altered, and the main fatty acids transformed to C16:0 (18.17%), C16:1 (40.30%), C18:0 (11.06%) and C18:1n9c (26.03%). The unsaturated fatty acids (UFAs) C16:1 and C18:1n9c emerged a rising trend, with ascent of 21.53% and 8.81% ($P < 0.05$), respectively. In particular, the lipid membrane of biofilm cells at 4°C exhibited significantly higher quantities of C16:1 and C18:1n9c fatty acids compared to those at 25°C ($P < 0.05$). Notably, the amount of UFAs in the cell membrane at 4°C represented 66.33% of the whole fatty acids, which was greatly higher than that at 25°C (51.69%) ($P < 0.05$).

Table 2 Fatty acid profile (%) of biofilm cells for 48 h to 96 h at 25°C and 4°C.

Fatty acid	Temp. (°C)	Incubation time (h)			Means	SE ^f	<i>P</i> -value		
		48	72	96			Temp. × Incubation time	Temp.	Incubation time
C12:0	25°C	1.59	1.64	1.58	0.0005		0.10	0.08	0.32
	4°C	2.19	1.49	2.31					
C14:0	25°C	0.97 ^{ab}	1.09 ^a	1.06 ^a			<0.05		

	4°C	0.91 ^b	0.58 ^d	0.75 ^c				
C15:0	25°C	1.01 ^b	1.26 ^a	1.36 ^a				
	4°C	0.85 ^{bc}	0.67 ^c	0.86 ^{bc}	0.0007	<0.05		
C16:0	25°C	21.26 ^{bc}	28.98 ^a	26.55 ^{ab}				
	4°C	17.89 ^{cd}	13.11 ^d	18.14 ^{cd}	0.02	<0.05		
C16:1	25°C	25.88 ^d	17.67 ^c	18.77 ^c				
	4°C	44.83 ^b	52.31 ^a	40.30 ^c	0.02	<0.05		
C17:0	25°C	0.64 ^c	0.78 ^b	1.06 ^a				
	4°C	0.48 ^{de}	0.40 ^c	0.55 ^{cd}	0.0004	<0.05		
C17:1	25°C	17.85	15.73	15.70	16.43 ^a			
	4°C	nd	nd	nd	nd			
	SE				0.005	0.34	<0.05	0.34
C18:0	25°C	9.05 ^{cd}	13.35 ^b	16.70 ^a				
	4°C	10.27 ^c	7.49 ^d	11.06 ^{bc}	0.0082	<0.05		
C18:1n9c	25°C	21.75 ^{bc}	19.50 ^{cd}	17.22 ^d				
	4°C	22.58 ^b	23.95 ^{ab}	26.03 ^a	0.0096	<0.05		
Saturated fatty acids	25°C	34.52 ^b	47.10 ^a	48.31 ^a				
	4°C	32.59 ^b	23.74 ^c	33.67 ^b	0.02	<0.05		
Unsaturated fatty acids	25°C	65.48 ^b	52.90 ^c	51.69 ^c				
	4°C	67.41 ^b	76.26 ^a	66.33 ^b	0.02	<0.05		

3.10 Verification of the effect of low temperature on CSH and AAG

The effect of chilled temperature on CSH and AAG were demonstrated in Fig. 9. CSH and AAG exhibited time-dependent increases, reaching the peak at 96 h with proportion in 47.11% and 45.67%, respectively, under chilled temperature condition. Meanwhile, the CSH and AAG was remarkable (31.19% and 34.05%) higher than those at 25°C ($P < 0.05$). Furthermore, the CSH at 25°C exhibited hydrophilic within 24 h, while demonstrated moderate hydrophobic for the mature period and anaphases of biofilm formation at 4°C.

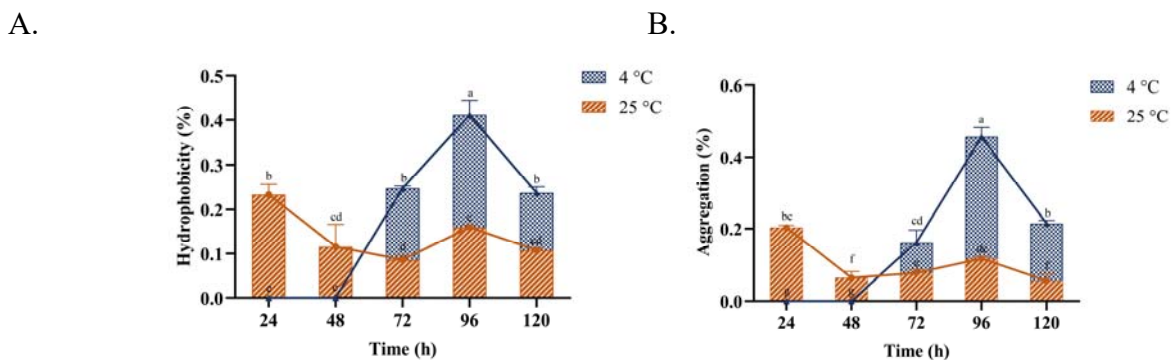


Fig. 9 Cell surface hydrophobicity (CSH, A) and auto-aggregation (AAG, B) of *P. fluorescens* biofilms at 4°C and 25°C. a – f Means with different letters were significantly different at $P < 0.05$. The data is represented as the means $\pm$ standard errors.

4. Discussion

4.1 Effect of chilled temperature on biofilm formation

Chilled temperatures represented a kind of primary environmental stress factor constrained on bacterial survival in chilled meat processing environments. *P. fluorescens* exhibits excellent cold tolerance and is capable of existing in biofilm forms at chilled temperatures^[11]. Consequently, this results in persistent

contamination by *P. fluorescens*, which is a vitally important reason for meat spoilage under chilled-temperature conditions^[12]. The biomass results provided a prerequisite for further research into the underlying mechanisms of these differences in Fig. 1. Namely, the major stages of *P. fluorescens* biofilm formation at chilled temperatures have been largely determined: reversible attachment (0 - 24 h); irreversible attachment (24 - 48 h); microcolony formation (48 - 72 h); biofilm maturation (72 - 96 h) and biofilm dispersion (96 - 120 h). The number of viable cells at 25°C during the initial period of biofilm formation was dominant (Fig. 2), which corresponded to the trend of biomass. However, combined with biomass and viable cell results in this study, it illustrated that chilled temperature delayed the adhesion in the initial stage of *P. fluorescens* biofilm formation rather than inhibited bacterial loads in the maturation stage. It can be hypothesized that *P. fluorescens* would produce more biofilms in response to the cold stimulus. However, the increased biomass was not contingent on an increase in cell number, which was assumed to determine by other predominant factors. Excessive EPS production may serve as a critical indicator of the increased total biomass observed in *P. fluorescens* biofilms at chilled temperatures (Fig. 4). During the mature biofilm phase (96 h), clustered structures were clearly observed at 4°C (Fig. 3A), which can be also attributed to EPS. The physical properties of EPS were determined by its constituents, and it was difficult for any single component to confer both “rigidity and viscosity.” Extracellular polysaccharides, as the main components of EPS, usually occurred in chilled temperature and saline environments, which were responsible for the stability and integrity of biofilms^[29]. The primary responsibility of extracellular proteins was to mitigate repulsive forces between biofilms and contacting surface, facilitating bacterial attachment and biofilms growth. While eDNA can promote biofilms expansion through twitching motility^[1, 30]. When bacteria are exposed to extreme environments, the production of EPS acts as a barrier and increased their tolerance by limiting the penetration of some antibodies, antibiotics and other immune factors, enables them to adapt to a wide range of stressful conditions^[31,32]. EPS distributed around bacteria was more abundant at 4°C (Fig.3B), which may be one of the strategies for *P. fluorescens* to adapt to chilled temperatures. Meanwhile, the trend in EPS content (Fig. 3B) aligned with the results of CV staining, offering additional evidence that coldness promoted biofilm formation, which probably contributed to the overproduction of EPS^[14]. EPS immobilized bacteria by forming a stable matrix through the substrate scaffold, promoting surface adhesion and intercellular cohesion^[33]. This could account for the persistence of *P. fluorescens* in chilled environments and the potential risk of cross-contamination.

4.2 Effect of chilled temperature on transfer ability

Cross-contamination is widely recognized as one of the major sources of foodborne diseases and can occur at various stages of food processing^[34]. That is to say, in abattoirs and meat processing plants, food contact surfaces such as knives, cutting boards, and conveyor belts are frequently the primary sources of direct microbial transfer to meat, leading to cross-contamination^[35]. However, the public seem to be more concerned about the transfer of pathogenic bacteria, probably due to the attention drawn to their harmful effects on humans^[36,37]. Nevertheless, the transfer of spoilage bacteria, particularly *P. fluorescens* with strong biofilm-

forming ability, not only is detrimental to food quality, but also can enhance biofilm formation in other genera, as confirmed by our previous laboratory findings^[15]. This was concerning, as mentioned above, *P. fluorescens* was found to be strong biofilm producers, particularly at 4°C. Microbial transfer was related to attachment strength, surface density and biofilm structures^[38], namely, favorable conditions for biofilm formation facilitated microbial translocation. Results mentioned in section 3.2, there was no significant difference in the population of biofilm cells between 25°C and 4°C during maturation phase (96 h). That was to say, the initial cell density of the biofilm was comparable during the meat transfer experiment. However, in this study, the level of biofilm cells transferred during the first coupon at 4°C was 0.4 log CFU/cm² higher than at 25°C (Fig. 5, $P < 0.05$). This phenomenon may be explained by the enhanced adhesion of cells to surfaces at chilled temperatures. After all, before transfer experiments, polystyrene plates (biofilm carriers) were rinsed to remove medium and plankton. As noted by Silagyi et al.^[39], bacteria remain firmly adhered to surfaces despite water rinsing. It was noteworthy that the population of transferred biofilm cells progressively decreased with transfer event and eventually stabilized. However, the number of biofilm cells transferred onto the beef at 4°C remained higher than at 25°C. This could be accounted for the enhanced cohesion provided by EPS, which promoted the large clusters of biofilms to transfer collectively onto the beef coupons. CLSM also revealed substantial biofilm clusters were observed at 4°C during biofilm maturation phase (Fig.3B). Taking it together, it was obvious that chilled temperature exerted a positive effect on *P. fluorescens*, in terms of biofilm formation ability and transfer ability^[14].

4.3 Formation mechanism of *P. fluorescens* biofilm at chilled temperature

For the sake of exploring the formation mechanism, this study analyzed the regulated network associated with *P. fluorescens* biofilms at 4°C. Based on transcriptome results, it was extremely significant that ribosome and flagellar assembly pathways were primarily inhibited by chilled condition (Fig. 6D). Generally speaking, the inhibition of ribosomal and some protein transport activities seemed to correlate with the reduction in the speed of most chemical reactions at 4°C^[40]. Ribosomes presented in all cells and played a vital role in protein translations^[41], and it appeared to function as temperature sensors in cells^[42]. When exposed to coldness, the initiation of ribosomal translation was immediately hindered^[43]. Nevertheless, certain cold-adapted bacteria may undergo a cold shock response, whose induction signals were cold-adapted translatable ribosomes^[43]. These translatable ribosomes were transformed by some untranslated ribosomes, which were combined with cold shock proteins at low temperature^[44]. However, this study found that inhibition of ribosomes was not accompanied by significant changes in genes or regulatory pathways associated with the cold shock response. That was to say, it was possible that *P. fluorescens* were not adapting to coldness by undergoing cold shock responses. Biofilm formation was a process wherein bacteria transition from reversible to irreversible attachment, in which the flagellum was instrumental. Based on the motility provided by flagella, bacteria can move towards the surface and overcome surface repulsion, which was advantageous for biofilm formation^[45]. The flagellum-mediated motility was required to promote bacterial adhesion in the early stages of biofilm formation, yet motility was progressively suppressed as the biofilm formed and matured, promoting the

development of biofilm complex three-dimensional structures^[46]. It was noteworthy that motility was reactivated to promote biofilms spreading in the later stages of the process^[47]. During biofilm maturation, although chilled temperatures significantly inhibited the motility dynamics of *P. fluorescens*, it facilitated biofilm development. It was possible that the effect of chilled temperatures on *P. fluorescens* was a double-edged sword: on one hand, coldness retarded the motility mediated by flagella in the early stages of biofilm formation, resulting in low levels of viable cells and biomass; on the other hand, the inhibition of bacterial motility caused by coldness coincidentally served to retard cell spreading during the mature period of biofilm formation, allowing superior accumulation of microorganisms^[48]. This was proved that the reduced motility of *P. fluorescens* at 4°C was inextricably linked to the downregulation of flagellum-related genes.

AAG, refers to as the phenomenon whereby multiple individual organisms aggregate to form microbial communities via specific molecules on living or non-living surfaces, is the first step in the formation of biofilms^[26]. On the other hand, surface wettability including hydrophobicity and hydrophilicity, can influence the rate of biofilm attachment and the resilience of the adhered biofilms in the interaction between microorganisms and contact surfaces^[49]. It appeared to be a more profound relationship between CSH and fatty acid composition^[50] Membrane fluidity was more profoundly affected by alteration in external conditions such as pH, temperature and osmotic pressure, which influenced fatty acid composition^[51]. There was an inextricable link between temperatures and the fatty acid composition of the cell membrane in *P. fluorescens*. The fatty acid composition of bacterial cell membranes at chilled temperatures was adjusted as follows: (i) changed from saturated fatty acids to UFAs; (ii) shortened acyl chain length; (iii) increased straight-chain fatty acid^[52]. In the present study, the percentage of UFAs on the cytoplasmic membrane at 4°C was higher compared to the content at 25°C (Table 2), which was similar to previous studies^[53]. When bacteria were stressed by coldness, these fatty acids had a low melting point increased, which could reduce the gel-liquid-crystal phase transition temperature and allow the cytoplasmic membrane to maintain favorable fluidity and conserve energy at chilled temperatures^[54]. The ascending percentage of UFAs promoted the nutrient and enzyme transport^[55] and improved bacterial survival at chilled temperatures, which was beneficial for biofilm formation. Furthermore, the major fatty acids of *P. fluorescens* at 4°C were C16:1 and C18:1n9c, which were similar to bacteria found in the psychrophiles from the Mingyong Glacier soil sample^[56], revealing that these two fatty acids may be key to *P. fluorescens* for cold adaptation. Similarly, the fatty acid degradation pathway was significantly up-regulated in *P. fluorescens* at 4°C during biofilm formation. The previous study had proved that *fadA*, *fadB*, *fadD1* and *fadD2* played an important role in fatty acid degradation (β -oxidation). Of these, *fadD1* was significant for long-chain fatty acids, while *fadD2* was more beneficial for short-chain fatty acids. That was to say, upregulation of the *fadD2* gene could lead to the shortening of fatty acids carbon chain, which hinted that it was responding to low temperature^[57]. Collectively, chilled temperatures promoted biofilm formation in *P. fluorescens* by modulating two key pathways: flagellum-mediated motility and fatty acid degradation^[26,45,53]. To provide a clear mechanistic perspective, it graphically summarized the impact of chilled condition on biofilm formation in Fig. 10, which highlighted the critical DEGs within these pathways.

Amino acids were favorable sources of nitrogen and carbon for microorganisms, which can influence microbial growth and development^[58]. Cadaverine and indole were metabolic intermediates of lysine and tryptophan, respectively. Cadaverine had been shown to regulate bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) levels^[59], while indole acted as a chilled-temperature affinity molecule that reduced bacterial motility^[60]. Notably, c-di-GMP itself regulates bacterial motility. Together, these findings provided an additional perspective, demonstrating that c-di-GMP may be a vital mechanism underlying chilled-temperature biofilm facilitation. The consumption of pyruvic acid under hypoxic conditions was crucial for *P. fluorescens*. Cells located in the most superficial layer of the biofilm had access to oxygen and could produce pyruvic acid, which could then pass deeper into the biofilm^[61]. As the eventual product of glycolysis, pyruvate was a crucial intermediate for many intracellular carbon metabolism (including sugar degradation, fatty acid degradation, the tricarboxylic acid cycle, gluconeogenesis), which may function as an upstream “master switch” that orchestrates the production of EPS (extracellular polysaccharides and proteins) and fatty acid degradation^[62–66]. Hence, promoting the pyruvate metabolic at chilled temperature in this study may be one of the methods by which biofilm cells maintain their activity^[61]. However, to the best of our knowledge, these pathways represent hypothetical opinions that require further experimental validation to elucidate the comprehensive mechanism of biofilm formation in *P. fluorescens* at 4°C. Meanwhile, the complexity of the mechanisms warrants further multi-dimensional investigation. Future research will focus on the functional validation of the key pathways identified herein by coupling protein-level expression analysis with targeted gene deletion studies.

5. Conclusion

This research illuminated the underlying formation mechanisms of *P. fluorescens* biofilm under low temperature (4°C) during chilled meat distribution. Biofilm formation was enhanced with improved structural integrity and increased extracellular polymeric substance production at 4 °C. Chilled temperatures also facilitated the transfer of *P. fluorescens* from food-contact surfaces to beef, elevating the risk of cross-contamination. Transcriptomic analysis revealed that flagellar assembly and fatty acid degradation pathways other than cold shock responses, played key roles in cold adaptation. Further investigation demonstrated that upon exposure to cold stress, the motility of *P. fluorescens* during mature period was suppressed, creating favorable conditions for microbial accumulation. Concurrently, the fatty acid conversion mechanism was activated, which modulated the content of UFAs to mitigate the adverse effects of environmental stress. Higher levels of UFAs enhanced cell membrane fluidity, thereby facilitating energy conservation and modulating the transport of substances across the membrane. These findings enlighten that targeting flagellar motility and fatty acid metabolism represented a promising strategy for inhibiting *P. fluorescens* biofilm formation during chilled meat distribution.

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Conflicts of interest

No conflict of interest exists in the submission of this manuscript, and this manuscript has been approved by all authors for publication.

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