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Research Article 研究报告

芽孢杆菌无细胞上清液对致腐隆德假单胞菌单/混合生物被膜的抑制活性

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WU Shiyuan, LU Zhong, SHI Honghui, ZHANG Jun, LU Haixia, ZHU Junli. Cell-free supernatants of *Bacillus* inhibit the biofilms of *Pseudomonas lundensis* cultured with and without *Acinetobacter johnsonii*[J]. Acta Microbiologica Sinica, 2024, 64(10): 3886-3900.

摘　要：【目的】假单胞菌(*Pseudomonas*)是冷鲜肉制品及加工环境中的优势腐败菌之一，在单一或混合培养下均具有较强的生物被膜形成能力。本研究评价食品来源的 3 株芽孢杆菌无细胞上清液(cell-free supernatants, CFSs)对隆德假单胞菌(*Pseudomonas lundensis*, PL)单培养及与约氏不动杆菌(*Acinetobacter johnsonii*, AJ)混合培养下的抗生物被膜作用。【方法】采用结晶紫染色、分光光度法、激光共聚焦显微镜(confocal laser scanning microscopy, CLSM)观察、qPCR 等方法分别测定生物被膜细胞活力、生物量、胞外聚合物、被膜结构及其相关基因转录变化。【结果】解淀粉芽孢杆菌 ZG08、贝莱斯芽孢杆菌 B5、枯草芽孢杆菌 YB11 的 CFSs 能有效抑制 2 种腐败菌的生物被膜形成，并且不影响其生长。经 50% CFSs 处理后，ZG08 和 B5 处理组的单种或混合生物被膜的细胞活性下降 12.73%–21.04%，显著高于 YB11 处理组(0.15%–4.38%)。3 种 50% CFSs 处理后，隆德假单胞菌单种和混合生物被膜的抑制率分别为 59.75%–79.59%和 63.62%–78.57%，其中 YB11 的 CFS 抑制效果较弱。同时，CFSs 处理显著降低胞外基质分泌，单/混合生物被膜的胞外多糖和胞外蛋白含量分别下降 53.77%–73.30%和 54.84%–62.38%，处理后的被膜变得疏松，分散贴壁黏附，被膜厚度减少 57.63%–74.49%和 60.43%–64.64%。此外，与 YB11 无清除活性相比，ZG08 和 B5 的 CFS 对 PL 和 PL+AJ 成熟生物被膜的清除率为 41.77%–69.79%。经稳定性评价，3 种 CFSs 在 4 种酶消化和加热处理后仍保持稳定的拮抗活性。与对照相比，ZG08 和 B5 的 CFSs 显著下调单/混合假单胞菌的 6 个生物被膜相关基因 *lapA*、*alg44*、*pelG*、*luxR*、*wspR* 和 *rpoS*。【结论】ZG08

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和 B5 的 CFS 对单种或混合培养下隆德假单胞菌和约氏不动杆菌均有较好的抗生物被膜活性。

关键词：芽孢杆菌；无细胞上清液；抗生物被膜；隆德假单胞菌；约氏不动杆菌；混合生物被膜

Cell-free supernatants of *Bacillus* inhibit the biofilms of *Pseudomonas lundensis* cultured with and without *Acinetobacter johnsonii*

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Abstract: [Objective] *Pseudomonas* as one of the dominant spoilage bacteria highly form biofilms in chilled meat products and processing environment when contaminating single or mixed with other species. This study aims to investigate the antibiofilm properties of the cell-free supernatants (CFSs) of three *Bacillus* species isolated from fermented food and rice seeds on *Pseudomonas lundensis* (PL) or and *Acinetobacter johnsonii* (AJ) as mono- or dual-species. **[Methods]** Biofilm biomass, extracellular polymeric substances (EPSs), and biofilm structure were measured by crystal violet staining, spectrophotometry, confocal laser scanning microscopy (CLSM), respectively, as well as transcription of biofilm-related genes determined by qPCR. **[Results]** The CFSs of *Bacillus amyloliquefaciens* ZG08, *B*. *velezensis* B5, and *B*. *subtilis* YB11 inhibited the biofilm formation of PL and AJ without affecting their growth. The treatment with 50% CFSs of ZG08 and B5 decreased the cell viability of two biofilms by 12.73%–21.04%, which was higher than that of YB11 (0.15%–4.38%). The inhibition rates of 50% CFSs of the three strains were 59.75%–79.59% against the PL biofilm and 63.62%–78.57% against the biofilm of PL+AJ, in which the CFS of YB11 had weaker activity. The content of exopolysaccharides and exoprotein in the two biofilms treated with these CFSs were reduced by 53.77%–73.30% and 54.84%–62.38%, respectively. The treatment with the three CFSs also reduced the adhesive cells, loosened biofilm structures, and thinned their thickness by 57.63%–74.49% and 60.43%–64.64%, respectively. Moreover, the CFSs of ZG08 and B5 effectively eradicated by 41.77%–69.79% against the mature biofilms of PL and PL+AJ, compared to weak activity of YB11. In addition, the antibiofilm activities of the three CFSs were stable under four enzyme digestion and heating conditions. Compared with the control, the CFSs of ZG08 and B5 significantly down-regulated the expression of six biofilm-related genes, *lapA*, *alg44*, *pelG*, *luxR*, *wspR*, and *rpoS*. **[Conclusion]** The CFSs of ZG08 and B5 have strong antibiofilm activities against PL and AJ as mono- or dual-species.

Keywords: *Bacillus* spp.; cell-free supernatant; antibiofilm; *Pseudomonas lundensis*; *Acinetobacter johnsonii*; biofilm of mixed species

Meat production, processing, storage, transportation, and sales in low-temperature cold chain effectively maintain the quality and safety of products and extend the shelf-life, by inhibiting the microbial growth and enzyme activity, thereby retarding the meat deterioration. However, some psychrotrophs attach on carcass surfaces and multiply rapidly under small fluctuation of temperature during cold chain. Psychrotrophic *Pseudomonas* species are the dominant spoilage bacteria that cause spoilage in aerobically chilled meat[1-2]. *Pseudomonas lundensis* as one of the most prevalent meat-spoilage bacteria, secrete high protease and lipase, resulting in unpleasant off-odors and slime[3]. *Acinetobacter* as psychrotrophic bacteria contaminate the surface of processing equipment, which are responsible for decreased meat quality through sulfur metabolism and protease secretion[4].

Biofilms are microbial communities formed by bacteria embedded in the auto-produced extracellular polymeric substances (EPSs) composed of polysaccharides, proteins, nucleic acids and other macromolecular substances[5]. Most biofilms on meat contact surfaces harbored the genera *Pseudomonas*, *Acinetobacter*, *Brochothrix* during meat processing, which are all typical psychrotrophic spoilage bacteria[6]. Moreover, the majority of biofilms in food are comprised of multiple bacterial species[7]. The interactions among bacterial community in the multi-species biofilms influence their biofilm forming ability of the dominant bacteria, thus altering their structure, characteristics, and resistance to external stressful environment[7]. The dual-biofilm formed by *P. lundensis* and *Listeria monocytogenes* showed stronger resistance to preservatives than single biofilm, such as cinnamon essential oil[8]. Kim et al. reported that a higher concentration of grapefruit seed extract, was required to eradicate the dual-species biofilms of *P. fluorescens* and *Bacillus cereus* than single biofilm[9]. Thus, multi-species biofilms of these spoilage bacteria pose an increasing contamination risk for processed animal-derived products.

Bacillus spp. are widely distributed in soil, human gut and fermented food. A variety of antimicrobial substances secreted by them, including polypeptides, antimicrobial proteins, lipids and organic acids, affect the survival and colonization of other bacteria[10]. Recently, several strains of *Bacillus* spp. with antagonistic activity, including *Bacillus subtilis*, *B. licheniformis*, and *B. amyloliquefaciens*, were reported no antibiotic resistance and no toxicity to animals. The biosurfactants in cell-free supernatants (CFSs) produced by *B. subtilis* VSG4 and *B. licheniformis* VS16 have antioxidant, antimicrobial, and anti-adhesive or antibiofilm potential against *Staphylococcus aureus*, *S. typhimurium* and *B. cereus*[11]. Fengycin in the CFSs of *B. subtilis* decreased the colonization of *Staphylococcus aureus* by inhibiting the Agr quorum sensing[12]. CFSs of *B. subtilis* KU43, *B. subtilis* KU201, and *B. polyfermenticus* KU3 effectively repressed biofilm-forming *L. monocytogenes*[13]. However, there are few studies on the antibiofilm activity of *Bacillus* CFS against spoilage related *Pseudomonas* as mono- or dual-species.

The aim of this study was to evaluate the antibiofilm efficacy of CFSs of *Bacillus* spp. isolated from traditional Chinese fermented food and rice seeds against meat spoilage related bacteria, *Pseudomonas lundensis*, and *Acinetobacter johnsonii* as mono- or dual-species. Subsequently, the temperature and enzyme tolerance of three CFSs of *Bacillus* were investigated, and transcription of biofilm-associated genes of *P. lundensis* treated by CFS was further performed to uncover their mechanism of antibiofilm activity.

1 Methods and Materials

1.1 Bacterial strains and growth conditions

Two meat-derived strains *Pseudomonas lundensis* (PL) and *Acinetobacter johnsonii* (AJ) were used in this study for antibiofilm assay[14].

Two strains *Bacillus amyloliquefaciens* ZG08, *B. subtilis* YB11 were isolated from fermented food, and *B. velezensis* B5 was obtained from rice seeds, which were identified and stored in our laboratory. Their 16S rRNA gene sequences of ZG08, B5, YB11 strains were GCF_030063805.1, GCF_031851705.1, OR794289.1, respectively. These stored strains were revitalized twice for 24 h in TSB at 25 ℃ for PL and AJ or for 12 h at 37 ℃ for ZG08, B5, and YB11 with 160 r/min shaking.

1.2 Preparation of CFSs of *Bacillus* strains

Overnight cultures of three *Bacillus* were centrifuged (Sigma Laborzentrifugen) at 8 000×*g* for 5 min at 4 ℃ to obtain supernatants, followed by filtration through a sterile syringe filter (Green Mall) to obtain CFS[13]. The growth and biofilm in the tested PL and AJ were measured in the broth containing no CFS (control) and different concentration of CFS.

1.3 Biofilm formation assay

Briefly, overnight activated cultures of PL and AJ were diluted at a ratio of 1:1 000, with 10^4 CFU/mL as the final cell concentration for each species. The diluted PL and AJ were inoculated separately or mixed with TSB, and CFS was also diluted with TSB, ranging from 50% to 1.56%. Diluted CFS and an equal volume of bacterial culture were loaded onto a 96-well plate. After cultivated statically for 48 h at 15 ℃, the biofilm biomass was determined by crystal violet (CV) staining, according to Djordjevic et al.[15]. The absorbance under OD_{590} was determined by microplate reader (TECAN). In addition, the mature biofilms as mono- or dual-species formed at 15 ℃ for 48 h was removed suspensions, and was added CFS ranging from 1.56% to 50%. After incubating at 15 ℃ for 24 h, the CV biomass was measured to determine eliminating rate against mature biofilms.

1.4 Cell viability assay

Cell viability of biofilm as mono- or dual-species was determined by 2,3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) method[16]. Overnight cultures of PL were diluted to 10^4 CFU/mL with TSB. The diluted CFS and an equal volume of bacterial culture were loaded onto a 96-well plate and bacterial culture without CFS was used as positive control. After incubated at 15 ℃ for 48 h, the planktonic cells in 96-well plate were removed and washed with sterile saline. According to the requirements of XTT Cell Proliferation and Cytotoxicity Detection Kit (Jiangsu KeyGEN BioTECH Corp., Ltd.), 100 μL saline and 20 μL XTT working solution were added to each well and determine OD_{450} after incubated at 37 ℃ for 2 h.

1.5 Two EPSs quantitative assay

The extraction of exopolysaccharides and exoprotein in biofilms was slightly modified according to the method of Liu et al.[17]. Briefly, the above diluted strain cultures of PL and AJ as mono- or dual-species contain 50% CFS were added to individual wells of 6-well plates. No inoculated TSB and no CFS TSB were used as negative and positive control. After incubated at 15 ℃ for 48 h, the content of exopolysaccharides was extracted and measured by phenol-sulfuric acid method[18]. The content of exoprotein was determined by Micro BCA Protein Assay Kit (Shanghai Saint-Bio Biotechnology Co., Ltd.) according to manufacturer instructions.

1.6 Confocal laser scanning microscopy observation (CLSM)

Biofilm structures of PL as mono- or dual-species treated with three CFSs were examined using CLSM with a 40× water immersion objective lens (ZEISS). Biofilms were stained with 0.3% SYTO-9 and 0.3% propidium iodide (PI) (Sigma-Aldrich) in the dark at 30 ℃ for 15 min. The maximum excitation/emission used for these stains was approximately 505/525 nm for SYTO-9 and 493/635 nm for PI. Representative CLSM images from each sample were acquired by scanning z-stacks at a scanning step size of 1 μm and were processed in Imaris 7.6 software (Bitplane AG). Three parts of each sample were randomly selected, and the average value was taken as the thickness of biofilm.

1.7 CFS stability assay

To assess the thermal stability on the

antibiofilm activity of CFS, the three CFSs was treated at 40, 60, 80, 100, 120 ℃ for 10 min, and heated at 100 ℃ for 10−60 min[19]. After cooling to room temperature, their inhibition against PL biofilm formation was determined by CV staining method, and unheated individual CFS was as control. Meanwhile, the final concentration of 5 mg/mL pepsin, 100 μg/mL proteinase K, 2 mg/mL lipase, and 10 μg/mL cellulase[20] were added to CFS, respectively, and these mixtures reacted at 37 ℃ for 1 h. After inactivated at 100 ℃ for 10 min, CV biofilm biomass of CFS treated by enzymes was measured and untreated CFS as the control.

1.8 Quantitative RT-PCR

Biofilm cells of PL treated by two CFSs of ZG08 and B5 cultured at 15 ℃ for 48 min were collected to extract RNA[17]. The total RNA of PL biofilm cells was isolated using a TRIzol Kit (QIAGEN), and then treated with RNase-Free DNase Set to remove genomic DNA contamination. The concentration of RNA was determined by NanoDrop spectrophotometer (DHS Life Science & Technology Co., Ltd.). Meanwhile, reverse transcription according to the instructions of the manufacturer of the RevertAid First Strand cDNA Synthesis Kit. Power SYBR Premix *Ex Taq*™ II Kit (ThermoFisher Scientific) and QuantStudio™ 6 Flex Real-Time PCR System (ThermoFisher Scientific) were used for qRT-PCR. Primer Premier 5.0 software was used to design primers for biofilm-related genes according to Liu et al.[17]. Five biofilm associated genes, alginate production (*alg44*), Pel production (*pelG*), adhesion (*lapA*), flagella (*flgA*), two regulatory genes, quorum sensing (*luxR*), c-di-GMP signaling pathway (*wspR*), and RNA polymerase sigma (*rpoS*) were amplified. 16S rRNA gene was used as internal reference, and the results were calculated by $2^{-\Delta\Delta C_t}$.

1.9 Statistical analysis

Three independent experiments of antibiofilm activity were performed, and the mean and standard deviation of experimental values were calculated. Data were expressed as mean±standard deviations (n=3), and means by the same lowercase letters were no significant difference (P>0.05). The figures were processed using Prism 8.0 software (GraphPad Software Inc.). Statistical significance of results was analyzed using One-Way ANOVA (SPSS Inc.), and the significance levels are expressed at a 95% confidence level (P≤0.05) throughout.

2 Results

2.1 Inhibitory activity of CFS against PL as biofilm and planktonic cells

When the concentration of CFS was 25% and 50% in mono-culture of PL, the inhibition rates of ZG08 on the cell viability of biofilm were 5.28% to 12.73%, and those of B5 were 12.69% to 17.79%, in contrast with 3.42% and 4.74% for YB11 (Figure 1A), which was significantly lower than ZG08 and B5 (P<0.05). As the dual-species of PL and AJ, the inhibition rates of 25% and 50% CFSs of ZG08 and B5 on the cell viability of biofilm were 12.69%−18.24%, and 14.05%−21.04%, respectively, in contrast with about 4.38% for 50% CFS of YB11 (Figure 1B).

The planktonic and biofilm cell population of PL treated with 50% CFS after culture were counted after cultured at 15 ℃, as presented in Figure 1C and 1D. The planktonic cell number of PL treated with CFS reached 6.84−7.07 lg CFU/mL for 24 h and 8.27−8.42 lg CFU/mL for 48 h, which showed no apparent difference between the treated groups and control group. In addition, PL formed biofilm on the surface of stainless steel, but the biofilm cell population of PL treated with CFS of ZG08 and B5 were significantly lower than that in the control group (P<0.05). Compared to the weak inhibition for 24 h, the biofilm cell numbers of PL treated with CFSs of ZG08, B5, and YB11 decreased to 7.31−7.44 lg CFU/cm^2 for 48 h (P<0.05), in contrast to 7.90 CFU/cm^2 in the PL control. According to the results, the three CFSs of *Bacillus* reduced the cell viability and adhesive population of the biofilm, especially ZG08 and B5 strains.

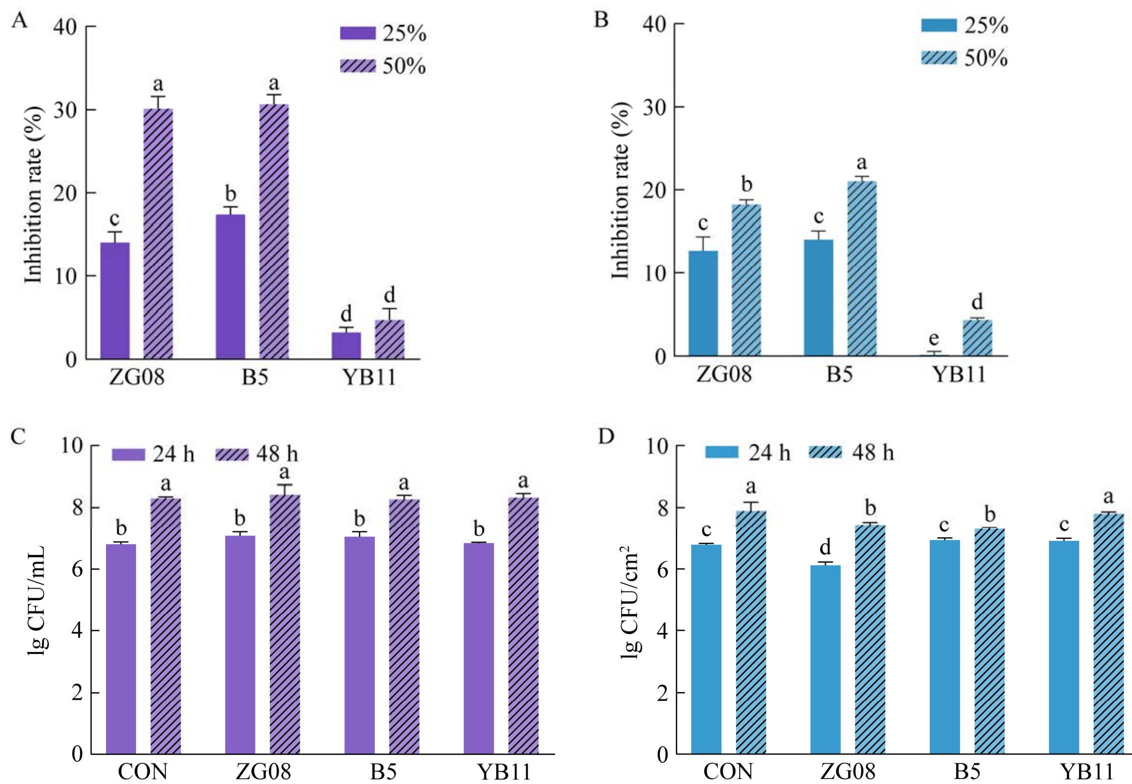

Figure 1 Inhibitory activity of three CFSs of *Bacillus* strains against PL as biofilm and planktonic cells at 15 ℃. Inhibition rate of 25% and 50% CFSs on PL and PL+AJ biofilm cell viability. A: PL. B: PL+AJ effect of 50% CFSs on populations of PL as biofilm and planktonic cells cultured for 24 h and 48 h. C: Planktonic cells. D: Biofilm. Culture without CFSs was used as control (CON). Means in the same row not followed by the same lowercase letters were significantly different (P<0.05). The same below.

2.2 Three CFSs inhibit biofilm formation

The CFSs of ZG08, B5, and YB11 significantly repressed the PL biofilm development as mono-species in the range from 1.56% to 50% (P<0.001), exhibiting the decrease of inhibitory activity with the dropping concentration (Figure 2A, 2B, 2C). When treated by CFSs of ZG08 and B5, biofilm biomass of PL reduced by 50.57%−79.29% and 49.48%−69.59%, respectively, in contrast with 27.42%−59.75% by CFS of YB11. Similarly, CFSs of three *Bacillus* inhibited effectively biofilm formation of PL and AJ as dual-species. Their CV biomass remarkably decreased by 55.02%−76.76%, 52.94%−78.57%, and 15.13%−63.62%, respectively after treated by CFSs of ZG08, B5, and YB11 (Figure 2D, 2E, 2F), showing that two CFSs of ZG08 and B5 exhibited the stronger inhibition against both two biofilms.

2.3 CFS repress EPSs production and structure of biofilms

As shown in Figure 3A and 3B, dual-biofilms produced the more exopolysaccharides and exoproteins than those of single PL biofilm after incubation at 15 ℃ for 48 h. The treatments of three CFSs significantly decreased the secretion of two EPSs in biofilms as mono- and dual-species (P<0.05), especially ZG08 and B5. For single biofilm of PL, the CFS of ZG08 and B5 decreased exopolysaccharides secretion by 53.57% and 53.77%, respectively, while they reduced exoprotein secretion by 39.76% and 60.42%, which was significantly lower than that of the individual control (24.25 μg/mL and 26.86 μg/mL) (P<0.05). Similarly, the treatment by CFSs of ZG08, B5 and

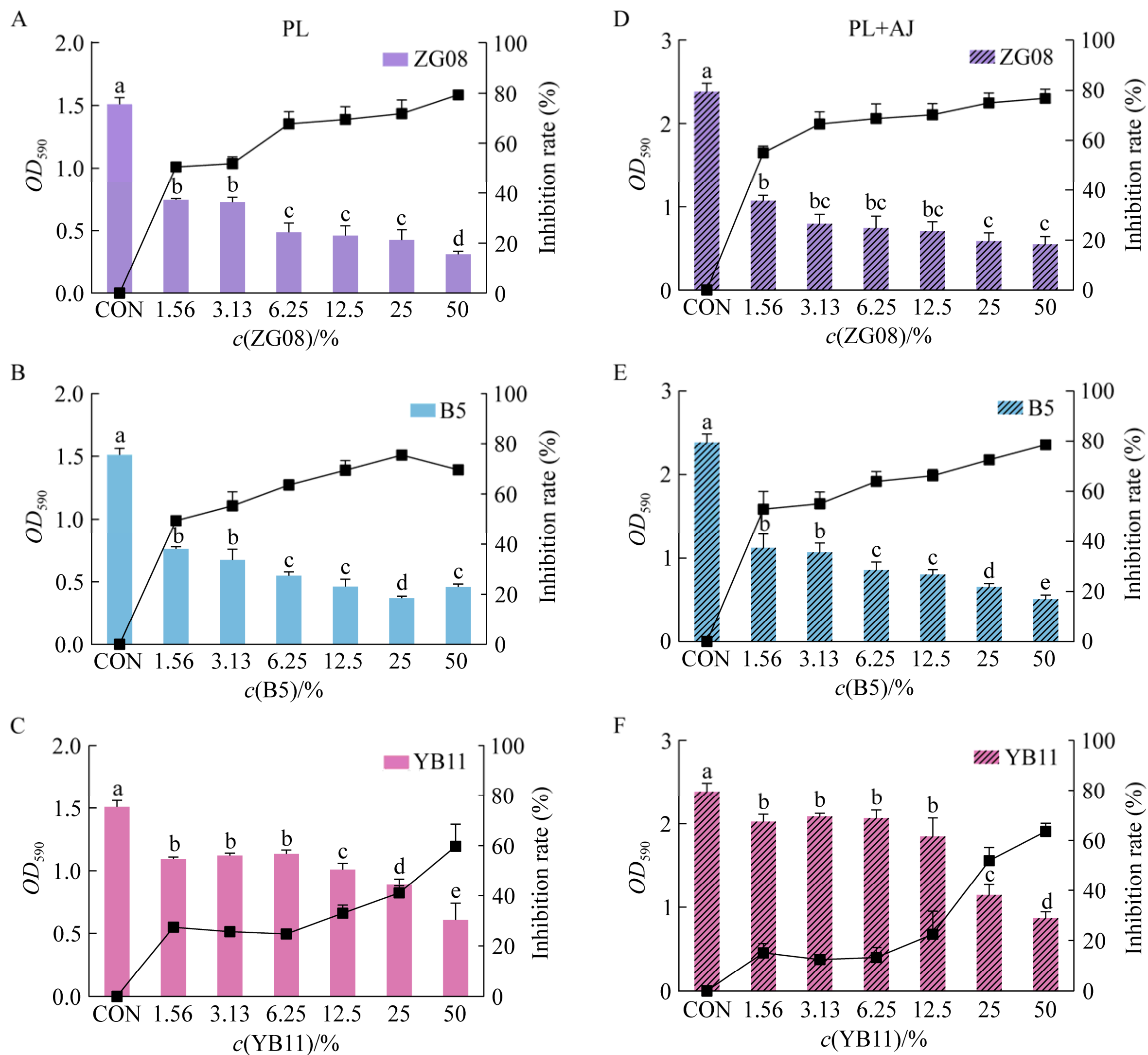

Figure 2 Inhibition rate of PL biofilm formation as mono- and dual-species biofilm treated with 1.56%−50.00% CFSs of ZG08, B5, and YB11. A–C: PL. D–F: PL+AJ.

YB11 repressed the secretion of exopolysaccharides of dual-species biofilms by 73.30%, 67.16% and 43.26%, respectively, and dropped the levels of exoprotein by 53.56%, 48.05% and 29.18%, respectively (P<0.05). These results indicated that the three CFSs of *Bacillus* could effectively inhibit the production of EPSs in the two biofilms. In addition, no pellicle was formed treated by 50% CFSs of ZG08 and B5 under either mono- or dual-species biofilms, and their pellicles of YB11 CFS became looser and easier to crack, compared with the dense and thick structure of control (Figure 3C).

As present in the CLSM images, a large number of bacteria adhered tightly to the glass surface in PL biofilm control, while the dual-species biofilms had aggregated to clusters (Figure 4), resulting in their heterogeneous structure. After treatment with CFS, the biofilm structures both of mono- or dual-species became spare and loose, as well as weak fluorescence signal, especially for ZG08 and B5 CFSs treatment. The heterogeneous structure of dual-species biofilms was apparently destroyed treated by three CFSs,

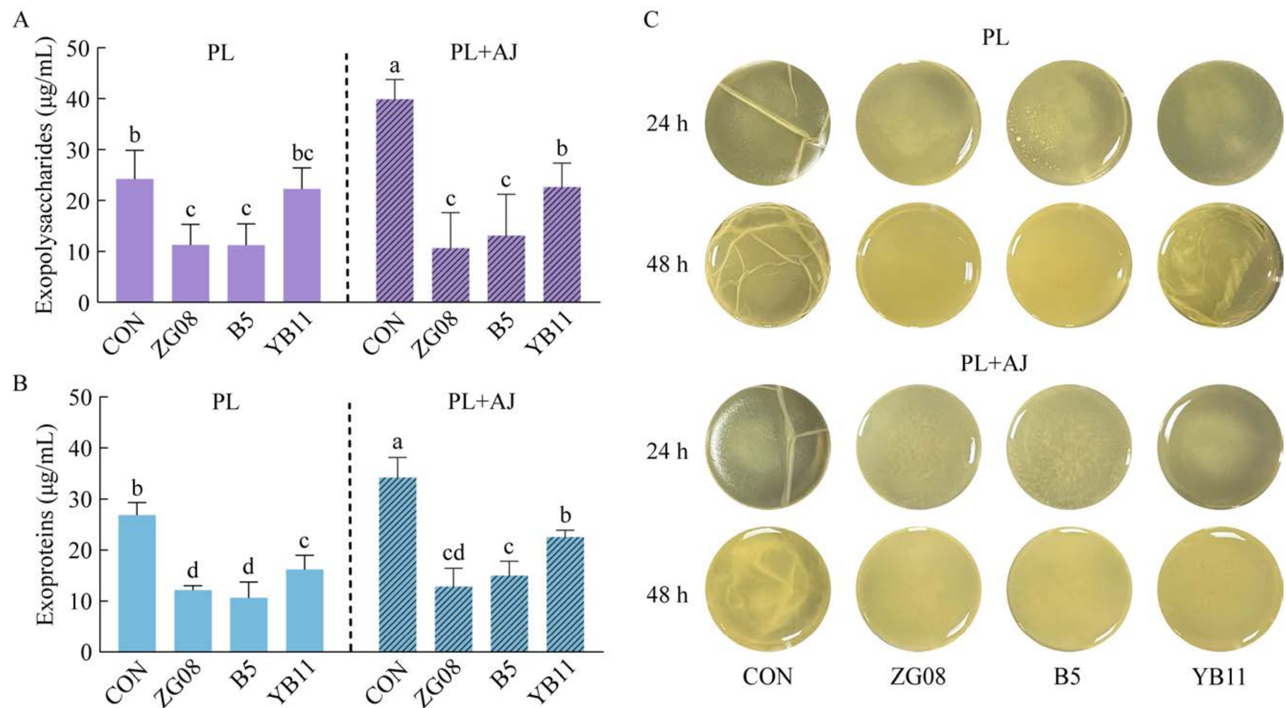

Figure 3 Changes in EPSs production and liquid-air interface pellicles formed by PL mono- and dual-species treated with 50% CFS of ZG08, B5, and YB11. A: Exopolysaccharides. B: Exoproteins. C: Pellicles.

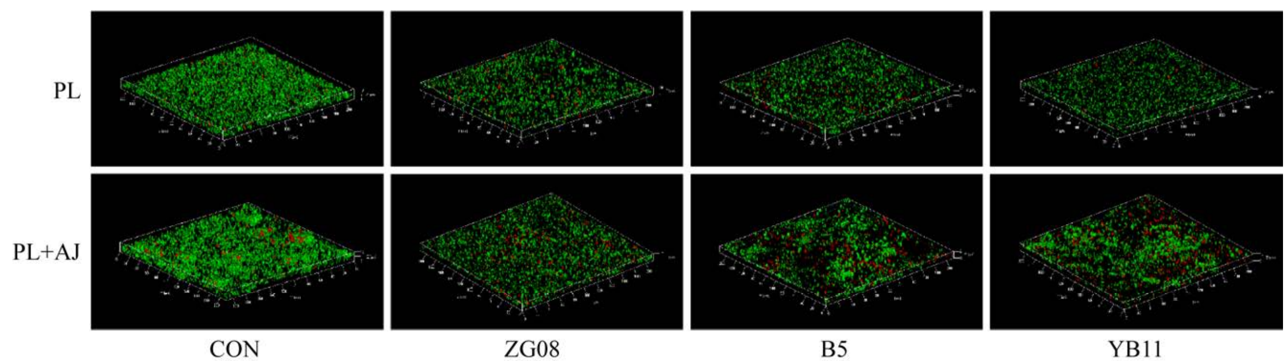

Figure 4 CLSM observation on the structure of mature biofilms of PL and PL+AJ treated with 50% CFSs of ZG08, B5, and YB11 at magnification 40×. Living cells of biofilms were stained with SYTO-9 (green) and dead cells of biofilms were stained with PI (red).

and only few bacterial cells were attached to the surface of glass treated by ZG08 and B5 CFSs. The biofilm thickness with CFSs of ZG08, B5 and YB11 decreased from 17.56 μm to 4.48−7.44 μm for mono-species PL, and dropped from 30.15 μm to 10.66−11.93 μm for dual-species, respectively (P<0.001).

2.4 CFS eradicate mature biofilms

CFSs from ZG08 and B5 significantly destroyed the mature biofilms formed by PL as mono- and dual-species (P<0.05), in contrast with weak activity of YB11 (P>0.05) (Figure 5). The treatment with 25% and 50% CFSs of ZG08 and B5 decreased the CV biomass of PL by 65.62%−70.89%

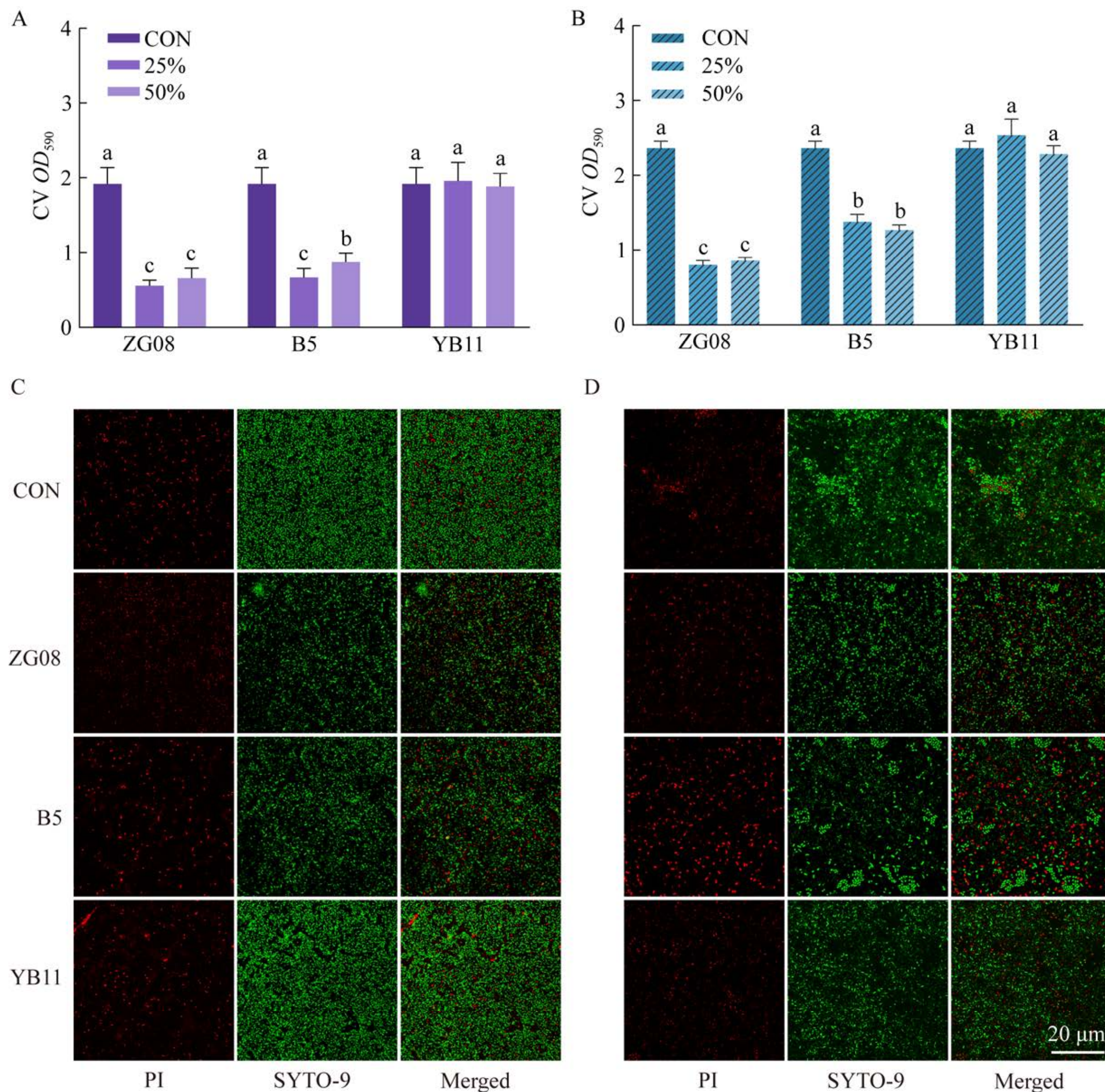

Figure 5 Biofilm eradication activity of the three CFSs on mature biofilms formed by PL as mono- and dual-species. A: CV biomass of PL. B: CV biomass of PL+AJ. CLSM observation on the biofilm structure of PL and PL+AJ treated by 50% CFS at magnification 40×. C: PL. D: PL+AJ. The scale is 20 μm. Living cells of biofilms were stained with SYTO-9 (green) and dead cells of biofilms were stained with PI (red).

and 54.31%−65.06%, respectively (Figure 5A). Similarly, the CV biomass as dual-species reduced by 63.64%−65.99% in the treatment of ZG08 CFS, and 41.75%−46.39% in the treatment of B5 CFS (Figure 5B). Furthermore, CLSM image also confirmed that 50% CFSs of ZG08 and B5 effectively eliminated the mature biofilms as mono- or dual-species, in contrast to no activity of YB11 CFS (Figure 5C, 5D). Following the two CFS treatment, viable biofilm cells with green fluorescence significant decreased, while dead cells in red fluorescence increased throughout the two biofilms. More specifically, cell aggregation in the treated biofilms apparently distributed, resulting in the spare and thin structure of biofilms.

2.5 Temperature and enzyme tolerance of CFS

The thermal stability and enzyme tolerance of the three CFSs were further evaluated. As shown in Figure 6, their antibiofilm activity gradually decreased by 84.70%−26.18% with the increasing temperature after thermal treatment for 10 min at 40−120 ℃, and finally inactivated by heated at 120 ℃. Furthermore, when CFSs of ZG08 and B5 heating at 100 ℃ for 60 min was supplemented, the biofilm biomass of mono- and dual-species still had been inhibited 64.54%−80.17% and 60.07%−87.00%, respectively, indicating the good thermal stability for the active compounds in the two CFSs. Conversely, antibiofilm of YB11 CFS was completely inactivated after heating for 20 min at 100 ℃.

In addition, the resistance of CFSs to four enzyme digestion was further evaluated, including pepsin, proteinase K, lipase and cellulase (Figure 7). Both the ZG08 and B5 CFSs showed good stability to proteinase K, lipase and cellulase, which was no difference between these treated CFSs and control group (P>0.05). However, CV biomass of PL increased from 0.62 and 0.97 to 1.95 and 1.36 (P<0.05), respectively after treatment with pepsin, indicating the decrease of antibiofilm for ZG08 and B5 CFSs. Additionally, despite the increase of biofilm biomass under proteinase K treatment (OD_{590}=1.69, P<0.05), the antibiofilm activity of YB11 CFS to pepsin, cellulase and lipase exhibited good stability (P>0.05).

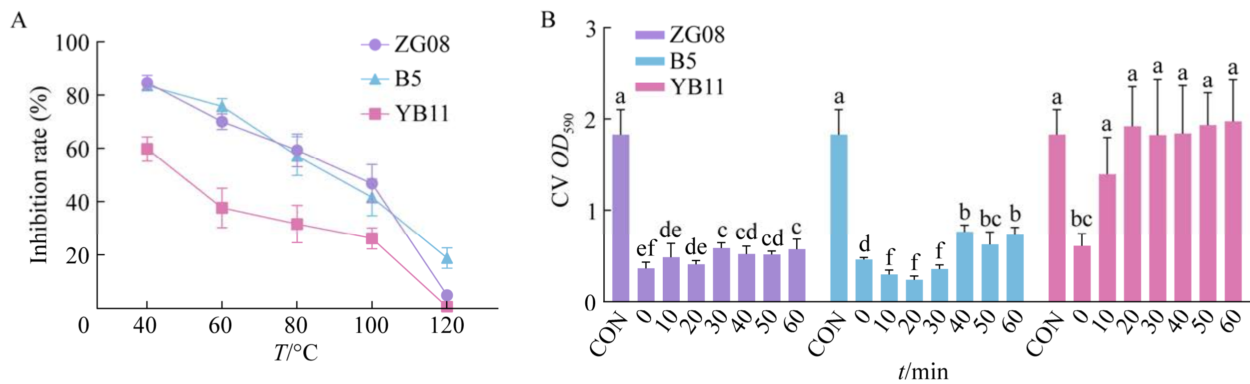

Figure 6 Thermal stability of CFSs. A: The inhibition rate of CFSs on PL biofilm varies with heating temperature. B: Effect of heating time at 100 ℃ on the anti-biofilm ability of CFSs.

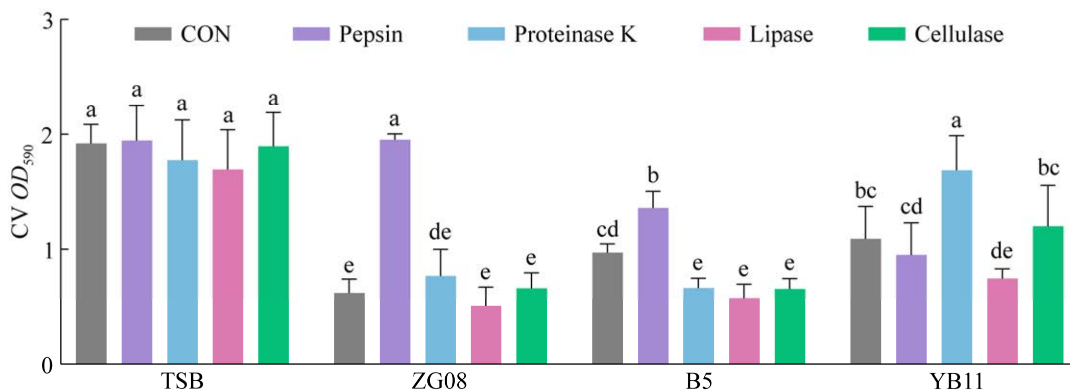

Figure 7 Enzyme tolerance of CFSs. CV biofilm biomass of the three CFSs treated with pepsin, proteinase K, lipase, and cellulase at 37 ℃ for 2 h.

2.6 CFS regulate gene transcription levels

Compared to those of the control, the transcription levels of *lapA*, *alg44*, *pelG*, *luxR*, *wspR*, and *rpoS* genes of PL were down-regulated by 0.11−0.28 folds and 0.06−0.33 folds after treatment of CFSs from ZG08 and B5, respectively (Figure 8). Similarly, when PL co-cultured with AJ, the transcription levels of the six genes were reduced by 0.15−0.41 folds after treatment of ZG08 CFS, and decreased to 0.22−0.94 folds after B5 CFS treatment. Conversely, the relative transcription of *flgA* gene of PL in mono- and dual-species biofilm was up-regulated in the two treatments.

3 Discussion

Current recent studies have examined *Pseudomonas* in co-culture with other spoilage bacteria or foodborne pathogens in muti-species biofilms[8,18], leading to the increasing resistance and persistent contamination in food processing[21]. In the present study, three CFSs of ZG08, B5, YB11 isolated from food showed strong inhibitory ability against both mono- and dual-species biofilm formed by PL and AJ. Islam et al. similarly reported that CFS of *B. subtilis* had certain inhibition against biofilm formation of pathogenic bacteria[22], such as *Klebsiella pneumoniae*, *Pseudomonas* sp., *Escherichia coli* and *Staphylococcus aureus*. *B. subtilis* KATMIRA1933 and *B. amyloliquefaciens* B-1895 were reported antibacterial and antibiofilm activity against pathogen *Acinetobacter* spp.[23]. The two strains of *Bacillus* of ZG08 and B5, greatly inhibited the cell viability, CV biomass and adhesive population of biofilms both as mono- and dual-species, even at low concentrations. Thus, the treatment of CFS have excellent inhibitory effect on dual-species biofilm formation of PL and AJ, despite the higher resistance for multi-species biofilms[24].

In the process of bacterial colonization and biofilm development, the large extracellular matrix, such as exopolysaccharide, exoprotein and eDNA, are produced to facilitate bacterial adhesion on surfaces and subsequently to maintain spatial structure stability[5,25]. Our results showed that CFSs of all three *Bacillus* strains significantly decreased the secretion of two EPSs of PL as mono- and dual-species without influencing planktonic cells population. Ray et al. reported that the CFS of *B. thuringiensis* contained substances such as squalene, which could repress the formation and dispersion of *Staphylococcus aureus* biofilm without affecting its growth[26]. Viszwapriya et al. reported that 2,4-di-*tert*-butyl-phenol (DTBP) isolated from CFS of *B. subtilis* significantly reduced the EPS secretion of *Streptococcus pyogenes*, thereby repressing its initial adhesion[27]. An exopolysaccharide EPS1-T14 produced by *B. licheneniformis* could reduce biofilm formation of *Escherichia coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, and *S. aureus* on abiotic

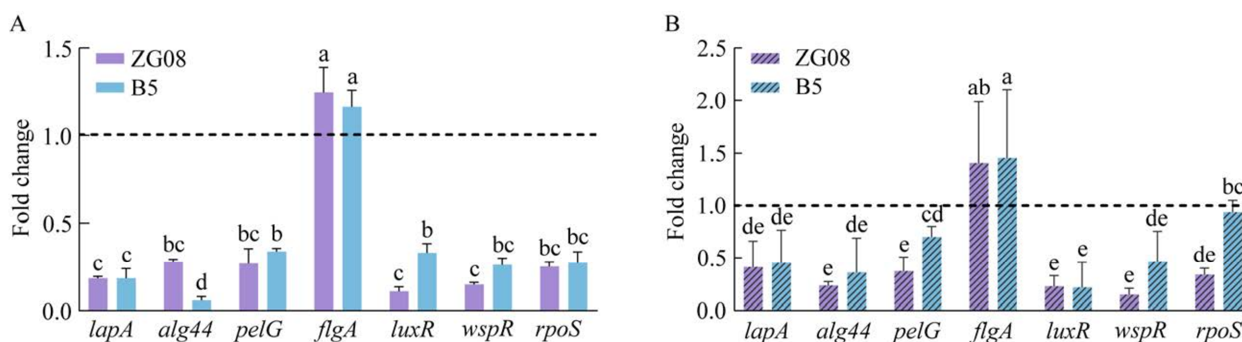

Figure 8 Expression of biofilm-related genes in PL or co-culture with AJ treated with CFSs of ZG08 and B5. A dashed line indicated the control group value of each gene was 1.0. A: PL. B: PL+AJ.

surfaces[28]. In addition, *P*. *lundensis* contained several gene clusters of exopolysaccharides, including Pel, alginate[17]. RT-qPCR revealed that the significant down-regulation of two gene *alg44* and *pelG*, and cell-surface-localized adhesin *lapA* treated by CFSs of ZG08 and B8. The adhesion gene *lapA* is closely related to biofilm formation, especially the adhesion to solid surfaces[29]. It was indicated that the treatment of CFS might effectively repress the production of alginate and Pel, and adhesin in PL, resulting in the decrease of initial adhesion, EPS matrix secretion and biofilm development.

Moreover, two CFSs of ZG08 and B8 strains effectively eliminated mature biofilms and destroy their biofilm structure as mono- or dual-species. Similarly, CFS of marine *Bacillus* strains, such as *B*. *indicus*, *B*. *nabensis* and *B*. *arenicus*, inhibited biofilm formation by *P*. *aeruginosa* PAO1, with about 70%−74% disruption of mature biofilm[30]. It was reported that *Bacillus* sp. could produce various extracellular enzymes, such as amylases, proteases, and lipases, involving in the degradation of EPS matrix[31]. According to current research, amylase and cellulase belong to polysaccharide-degrading enzymes[32], and amylase produced by *B*. *cereus* exhibited excellent antibiofilm activity against *P*. *aeruginosa* and *S*. *aureus*[33].

Furthermore, the antibiofilm active substances in the CFSs of ZG08 and B5 had great thermal stability and enzyme tolerance. Previous documents also reported that *Bacillus* spp. can produce several active substances, such as bacteriocins, peptides, enzymes, antibacterial proteins, macrolides, lipopeptides and polyketides[34-35]. Most bacteriocins have characteristics with their broad antimicrobial activity spectrum, pH- and heat-tolerance, but sensitive to digestive proteases such as pancreatic complex, host proteases, trypsin, and chymotrypsin[36]. Lahiri et al. found that α-amylase secreted by *Bacillus subtilis* almost kept 100% degradation activity at the range of 50−70 ℃[37]. Lipopeptides, such as fengycin, were thermostable and resistant to protease digestion, after heating (95 ℃, 20 min) or proteinase K digestion remain inhibitory active[12]. It was assumed that the antibiofilm compounds could be associated with lipopeptides in the CFSs of ZG08 and B5 due to the strong thermal resistance.

Generally, bacterial attachment to the surface, and subsequent development of biofilm are mediated by flagella, fimbriae, and regulated by quorum sensing and c-di-GMP signaling in *Pseudomonas*[5,38]. In the present study, *P*. *lundensis* favored swimming, and its motility remarkably increased after treatment with two CFSs of ZG08 and B5 Results no shown), related to up-regulation of flagellar genes. The stronger motility caused by CFS treatment in PL weakened the initial adhesion of planktonic bacteria, and subsequently decreased the biofilm development[39-40]. Moreover, the transcription of two regulatory genes was downregulated after treated with two CFSs, including *luxR* and *wspR* genes. In *Pseudomonas*, LuxI/LuxR mediated quorum sensing and c-di-GMP are two important signaling systems, that modulate biofilm formation of *Pseudomonas*[41-42]. The two regulators, LuxR receptor in quorum sensing, and WspR as one of diguanylate cyclases (DGCs) in c-di-GMP signaling[42], were down-regulated in the treatment of CFS from ZG08 and B5 strains, hinting that the negative impact of two key regulating factors could result in the decrease of adhesion and EPS secretion. Our results also confirmed that the treatment of two CFSs downregulated transcription of *rpoS* gene in *P*. *lundensis* both as mono- or dual-species, suggesting the decrease of its survival[43].

4 Conclusions

Overall, this study investigated the antibiofilm properties of the three CFSs from *Bacillus* strains against *P*. *lundensis* and *A*. *johnsonii* as mono- and dual-biofilms. The CFSs of *B*. *amyloliquefaciens* ZG08 and *B*. *velezensis* B5 effectively inhibited the cell viability, CV biomass, and cell adhesion of biofilm cells, as

well as the secretion of two extracellular EPSs, resulting in forming spare and loose structure of the two biofilms. Compared to YB11, CFSs of ZG08 and B5 have stronger inhibiting and eradicating efficacy against two biofilms, associated with their good thermal stability and enzyme tolerance. Moreover, the two CFSs of ZG08 and B5 significantly down-regulated the transcription of genes associated with quorum sensing and c-di-GMP in *P. lundensis*, decreasing adhesion and EPS production. Therefore, CFSs of food-derived *Bacillus* ZG08 and B5 has excellent antibiofilm efficacy against spoilage related *Pseudomonas* as mono- or dual-species, which provide a basis for the application in the controlling biofilm contamination of spoilage bacteria in meat product and processing environment.

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