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# Identification and comparison of protein composition of biofilms in response to EGCG from *Enterococcus faecalis* and *Staphylococcus lugdunensis*, which showed opposite patterns in biofilm-forming abilities

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A B S T R A C T

Bacterial biofilm is resistant to conventional antibiotic treatments, leading to complications associated with many infection-related human diseases. Epigallocatechin Gallate (EGCG), a phenolic catechin enriched in green tea, is recognized for its anti-bacterial and anti-biofilm activities. In this study, we examined the protein components of the biofilms formed in the absence or presence of EGCG using *Enterococcus faecalis* and *Staphylococcus lugdunensis,* which had shown opposing patterns in biofilm formation. A clustering heatmap revealed that the two microorganisms expressed the different protein sets in response to EGCG. Proteins that were noticeably upregulated included those associated with stress responsiveness and gluconeogenesis in *E. faecalis*, and gene modification in *S. lugdunensis*. Conversely, downregulated proteins were related to tRNA-modifying enzyme activity in *E. faecalis*, and anabolic metabolism in *S. lugdunensis*. Among the proteins identified only in EGCG-responsive biofilms, enzymes involved in de novo purine biosynthesis were enriched in *E. faecalis*, while proteins likely to cause DNA instability and pathogenicity changes were abundantly present in *S. lugdunensis*. The classification based on gene ontology (GO) terms by microorganism exhibited that metabolic process or catabolic activity was at the top rank in *E. faecalis* with more than 33 proteins, and in *S. lugdunensis*, localization or transport was highly ranked with 4 proteins. These results support the hypothesis that EGCG might cause different cellular programs in each microorganism. Finally, comparison of the proteomes between two groups that form biofilms to similar extents discovered that 2 proteins were commonly found in the weak biofilm-forming groups (*E. faecalis* and EGCG-responding *S. lugudunensis*), whereas 9 proteins were common among the strong biofilm-forming groups (*S. lugdunensis* and EGCG-responding *E. faecalis*). It was suggested that these proteins could serve as potential indicators to detect the presence and predict the extent of biofilm formation by multiple microorganisms. Taken all together, proteomics data and analyses performed in this study provided useful and new information on the proteins embedded in the biofilms formed at the specific conditions, which can aid in diagnosis and the development of tailored treatment strategies.

## 1. Introduction

Bacteria typically exist either as a free-floating planktonic cell or as a surface-attached biofilm [1]. Bacterial biofilms are primarily composed of microbial cells and self-produced extracellular polymeric substance (EPS) including polysaccharides, proteins, extracellular DNA, and lipids [2]. The transition from planktonic cell mode to biofilm formation occurs by arrays of cellular factors depending on environmental conditions [3] and involves the following processes; attachment of planktonic cells to a surface, microcolony formation, EPS production, and biofilm maturation [4]. Once established, the biofilm releases individual bacterial cells that can form new biofilms in other areas [5]. Until now, various biological mechanisms including quorum sensing (QS), outer membrane structure, stress responses, etc. were known to regulate biofilm formation [6].

While planktonic cells freely disperse and escape from harmful conditions, statically fixed biofilms provide protection to the bacterial cells from external damages, enabling them to reproduce and survive [7–9]. In that way, biofilms can evade the host immune system and become resistant to conventional antibiotic treatments [8], leading to complications in numerous infection-related human diseases [10–14]. In particular, biofilms are considered a serious problem in periprosthetic infections [12–14]. In addition to surgical implants and catheters [15], biofilms can also form on the surfaces of water pipe walls [16], so issues

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related to biofilm formation are an important research subject not only in medical aspects but also in our daily lives. Therefore, a better understanding of biofilm formation in diverse microorganisms is essential to overcome the challenges posed by biofilms.

Epigallocatechin gallate (EGCG) is a phytochemical found in green tea extract. Accumulating evidence has shown that EGCG has beneficial effects on various aspects of human health, including anti-inflammatory, anti-cancer, and antioxidant activities [17–19]. EGCG is also known for its strong anti-bacterial activities against both gram-negative and gram-positive bacteria [20,21]. Various mechanisms for the anti-bacterial activity of EGCG have been revealed, including damaging bacterial cell membranes, disrupting the bacterial membrane transporters, inhibiting bacterial cell binding to host cells, modulating bacterial enzymes, etc. [21–25]. Furthermore, the most prominent anti-bacterial activity of EGCG is associated with the biofilm formation of pathogenic bacteria, as a number of previous studies suggested that EGCG inhibited biofilm formation through inhibiting QS, impairing amyloid curli fiber assembly, downregulating the biofilm regulator, etc. [26–28].

Interestingly, in our previous study examining pathogenic microorganisms frequently found in periprosthetic infections, we found that EGCG did not exhibit anti-biofilm activity against all microorganisms tested, resulting in different responses in biofilm formation depending on the microorganism [29]. Some microorganisms formed higher levels of biofilm in the presence of EGCG, while other microorganisms showed reduced biofilm formation by EGCG. Notably, the two microbes *Staphylococcus lugdunensis* and *Enterococcus faecalis* were of particular interest. Coagulase-negative staphylococci, such as *Staphylococcus lugdunensis,* are recognized for causing numerous periprosthetic joint infections [30], and *Enterococcus* species are also reported as notable pathogens associated with medical implants [31]. On the given surfaces, *S. lugdunensis*, noted for having the highest biofilm-forming ability, and *E. faecalis*, showing the lowest biofilm-forming ability, exhibited opposite trends of biofilm formation in the presence of EGCG [29]. In other words, the degree of biofilm formation of *S. lugdunensis*, initially potent, was significantly reduced by EGCG, whereas the degree of biofilm formation of *E. faecalis*, originally the lowest, was significantly increased by EGCG.

In this study, proteomics using LC-MS/MS was performed and analyzed with bioinformatics tools to elucidate unique molecular characteristics of biofilms formed in the absence or presence of EGCG and to identify key proteomic factors that distinguished the differences in the biofilm-forming abilities and trends. The results showed that the biofilms of the two microbes possessed distinct protein profiles, suggesting that EGCG could switch on different cellular programs to reverse the biofilm-forming ability of each microorganism. Finally, we discovered several common proteins from the groups with the same biofilm-formation trends, which might be used for useful indicators to detect the presence and predict the extent of biofilm formation by multiple microorganisms.

## 2. Materials and methods

### 2.1. Bacteria culture

*Enterococcus faecalis* and *Staphylococcus lugdunensis* were obtained from National Culture Collection for Pathogens (NCCP, Korea) (https://nccp.kdca.go.kr/main.do). *Enterococcus faecalis* (NCCP 15611) and *Staphylococcus lugdunensis* (NCCP 15630) are described as isolated from pus and blood, respectively. Unless otherwise specified, tryptic soy agar (TSA, Difco) or tryptic soy broth (TSB, Difco) were used to culture the bacteria. Bacteria stocks were maintained on TSA plates. A primary bacterial culture was prepared by inoculating one single colony on the agar plates into 5 ml of TSB media in a 14-mL round-bottom tube (40114; SPL Life Sciences, Korea) that was sterilized by gamma irradiation and incubating overnight at 37 °C with shaking at 120 rpm.

### 2.2. Biofilm formation

All experimental procedures or materials, devices, and equipment were aseptically handled or maintained; a lack of cross-contamination was confirmed using empty plates. The primary bacterial culture was diluted with fresh broth media to achieve an optical density (OD) at 600 nm (OD600) value of 0.9–1.0 (DeNovix DS-C Spectrophotometer) and then, 1 mL of the diluted bacterial suspension was dispensed in 2–5 14-mL round-bottom tubes (40114; SPL Life Sciences, Korea), followed by incubation at 37 °C with shaking at 50 rpm. After 72 h, the culture supernatant containing non-adherent planktonic cells was removed, and the culture tubes were added with glass beads with a diameter of 2 mm diameter in 5 ml PBS, and vigorously vortexed to dislodge the biofilm from the tube surfaces in the liquid phase. This detachment process was repeated 3 times, after which the collected biofilm was centrifuged, washed with PBS, and the resulting pellets resuspended in RIPA buffer. Proteins were obtained from the supernatant following centrifugation, and protein amount in each sample was determined by BCA assay according to the manufacturer's protocol (Pierce™, 23227).

### 2.3. LC-MS/MS proteomics

The quantified protein samples were delivered to a proteomics service company (ebiogen, Seoul, Korea) and proceeded for proteome analysis according to the following procedures: filter-aided sample preparation (FASP) digestion, desalting, and LC-MS/MS analysis. Protein samples were first reduced by incubation with 5 mM tris(2-carboxyethyl)phosphine (TCEP) at 37 °C for 30 min before being alkylated with 50 mM Iodoacetamide (IAA) in the dark at 25 °C for 1 h. Subsequently, 8 M urea was added for 15 min. Trypsin in 50 mM ammonium bicarbonate (ABC) was then added and the mixture was incubated at 37 °C for 18 h, followed by stopping the reaction by adding formic acid (pH 2). Desalting was carried out with a C18 micro spin column prepared with 100 % methanol, 0.1 % formic acid, and 80 % acetonitrile (ACN), followed by speed-vac drying. Samples were stored at −20 °C until analysis. Finally, the samples were subjected to LC-MS/MS analysis using ultra performance liquid chromatography (UPLC)/Q-Exactive. The parameters and conditions for the LC-MS/MS analysis are as follows.

| Parameters | Conditions | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Trapping column | $C_{18}$, 3 μm, 100 Å, 75 μm × 2 cm | | | | | | | |
| Analytical Column | PepMap™ RSLC $C_{18}$<br>2 μm, 100 Å, 75 μm × 50 cm | | | | | | | |
| Mobile phase | A: Water with 0.1 % formic acid<br>B: 80 % ACN with 0.1 % formic acid | | | | | | | |
| Gradient | Time (min) | 0 | 14 | 120 | 120.1 | 130 | 130.1 | 180 |
| Solvent B(%) | 4 | 4 | 40 | 96 | 96 | 4 | 4 | |
| Column flow rate | 300 nL/min | | | | | | | |
| Mass Range | 400–2000 *m/z* | | | | | | | |

The peptides of each sample isolated by LC-MS/MS were identified through Proteome discoverer using Uniprot *Enterococcus faecalis* or *Staphylococcus lugdunensis* databases (https://www.uniprot.org). Protein abundances were normalized based on BCA protein assay. Experiments were repeated twice, and each experiment contained 3 independently prepared samples (n = 2–5).

### 2.4. Data analysis and processing

From all identified peptides or proteins, only those consistently identified across the repeated experiments were selected, and overlapping items or uncharacterized proteins were excluded from further analysis. Also, the same protein for different gene names was unified. The selected protein lists were organized based on gene names. Data

analysis and processing displayed as Venn diagram, cluster heatmap, scatter plot, etc. was primarily performed using the following web-based tools; https://www.bioinformatics.com.cn/srplot and http://bioinformatics.sdstate.edu/go/. For gene ontology (GO) analysis, protein-protein network, and functional annotation analyses, the following web was also used; https://string-db.org. Statistical analysis between protein abundances of biofilm with or without EGCG group was conducted using (un)paired t-tests or ANOVA, and $P < 0.05$ was considered to indicate a statistically significant difference.

## 3. Results

### 3.1. Protein identification in biofilms formed in the absence or presence of EGCG

In our previous study [29], a significant difference was observed in the biofilm-forming ability of *E. faecalis* and *S. lugdunensis*; without EGCG, *E. faecalis* was the relatively weak, and *S. lugdunensis* was relatively strong in biofilm formation. However, the biofilm formation pattern was completely reversed in the presence of EGCG, that is, the biofilm-forming ability of *E. faecalis* increased in contrast to that of *S. lugdunensis* decreased by EGCG. In this study, we aimed to find protein molecules that could indicate strong or increased biofilm formation. To this end, proteins were extracted from the biofilms of the two microorganisms formed in the absence and presence of EGCG, and subjected to proteomics analysis. Through LC-MS/MS, a total of 1036 or 1149 proteins were identified from the biofilms with or without EGCG of *E. faecalis* or *S. lugdunensis*, respectively (Supplementary table 1~4).

As the two species have different genomes, among all identified proteins in each group, only proteins that are shared in both species were aligned along with abundances based on their genetic names for a clustering heatmap (Fig. 1A). The result revealed the tendencies that the two microorganisms have their distinct and unique proteomes, and also expressed the differential protein sets when treated with the same EGCG. In particular, most of the expressed proteins varied significantly between the two groups with similar biofilm formation phenotypes; namely, between the low biofilm-forming groups, *E. faecalis* with no EGCG (*E*_B) and *S. lugdunensis* with EGCG (*S*_EB), or between the strong biofilm-forming groups, *E. faecalis* with EGCG (*E*_EB) and *S. lugdunensis* with no EGCG (*S*_B). This finding implied that distinct molecular mechanisms were involved in the regulation of biofilm formation in each microorganism, despite their similarities in the extent of biofilm-forming phenotypes.

We then analyzed the proteins identified in biofilms from the two groups of each microbe. Among 1036 proteins of *E. faecalis*, 864 proteins were consistently present in both biofilms (*E*_Biofilm and *E*_EGCG + Biofilm), while 113 proteins were only identified in *E*_Biofilm, and 59 proteins were solely found in *E*_EGCG + Biofilm (Fig. 1B–a). In the case of *S. lugdunensis*, out of 1149 proteins, 1044 proteins were common across both biofilms (*S*_Biofilm and *S*_EGCG + Biofilm), and the other 89 or 16 proteins were unique to *S*_Biofilm or *S*_EGCG + Biofilm, respectively (Fig. 1B–b).

### 3.2. Analysis of expression regulation according to the abundance of commonly identified proteins in biofilms with and without EGCG

The proteins commonly identified in both B and EB groups of each microbe were further analyzed for changes in expression levels either in the absence or presence of EGCG (Fig. 2). Among the 864 proteins of *E. faecalis*, EGCG induced 121 proteins to be up-regulated and 96 proteins to be down-regulated, while 647 proteins were not significantly affected (Fig. 2a). In the case of *S. lugdunensis*, 170 proteins out of 1044 proteins were found to be up-regulated, 434 proteins were down-regulated by EGCG, and 960 proteins were in the range of no change (Fig. 2b).

The proteins commonly identified in the biofilms formed with or without EGCG were assessed for up- or down-regulation in biofilms by dividing the abundances in the biofilms formed with EGCG by the abundances in the biofilms without EGCG (EB/B). Based on the EB/B ratios, the 10 proteins with the highest and lowest EB/B ratios are listed in Table 1.

In *E. faecalis*, proteins with the higher EB/B ratio include bifunctional protein GlmU (*glmU*), 50S ribosomal protein L9 (*rplI*), nitroreductase family protein, putative (*EF_0655*), 30S ribosomal protein S20 (*rpsT*) and pyruvate, phosphate dikinase (*ppdK*). Conversely, peptidyl-tRNA hydrolase (*pth*), formate acetyltransferase (*pflB*), aminoacyltransferase FemA (*EF_2150*), phosphate-binding protein (*EF_1759*) and 50S ribosomal protein L36 (*rpmJ*) showed the lower EB/B ratio, indicating that they were more abundant in the biofilm formed without stimuli. The upregulation of stress-responsive proteins such as bifunctional protein GlmU and nitroreductase family protein, putative, and gluconeogenesis-related enzymes such as pyruvate, phosphate dikinase, and the downregulation of proteins involved in tRNA-modifying enzyme activity such as peptidyl-tRNA hydrolase and aminoacyltransferase FemA, and anaerobic glucose metabolism-related formate acetyltransferase reflected the dynamics of the microorganism, which switch cellular programs from routine maintenance toward survival or adaptation to the external stimuli.

In the case of *S. lugdunensis*, tRNA-specific adenosine deaminase (*tadA*), ATP-dependent helicase/deoxyribonuclease subunit B (*addB*), NYN domain-containing protein (*EQ (812)_08325*), UPF0291 protein (*EQ (812)_07465*), glycosyltransferase family 2 protein (*EQ (812)_08890*), etc., were revealed to be more abundant in the biofilm formed with EGCG (EB) than in the biofilm formed with no stimuli (B). On the other hand, dihydrolipoyl dehydrogenase family protein (fragment) (*HMPREF3225_01403*), anthranilate synthase component I family protein (*EQ (812)_09225*), TspO/MBP family protein (*HMPREF3225_00031*), GTP pyrophosphokinase (*EQ (812)_10775*), DNA-binding helix-turn-helix protein (*EQ (812)_00955*), etc., appeared to be relatively higher in the biofilm with no stimuli. The finding that tRNA-specific adenosine deaminase, ATP-dependent helicase/deoxyribonuclease subunit B, and NYN domain-containing protein were upregulated could indicate that EGCG might induce genetic modifications leading to de novo protein synthesis. On the contrary, the downregulation of dihydrolipoyl dehydrogenase family protein (fragment) associated with energy metabolism and anthranilate synthase component I family protein involved in tryptophan biosynthesis suggested the reduction of anabolic metabolism by the stimuli.

Although the difference between E and EB groups of each microorganism was statistically significant ($p = 0.0385$ for *E. faecalis* and 0.0001 for *S. lugdunensis*), the proteins commonly identified in the biofilms formed with and without EGCG were excluded from further analyses to focus on new components incorporated into the biofilm composition by EGCG.

### 3.3. Comparison of the proteins identified only in EGCG-responsive biofilms

To examine which proteins were newly and highly enriched in the biofilms upon EGCG stimulation, the top 10 proteins exclusively identified in EGCG-responsive biofilms were listed in order of their abundance (Table 2). In the case of *E. faecalis*, proteins such as helix-turn-helix protein, iron-dependent repressor family (*EF_0578*), phosphoribosylaminoimidazole-succinocarboxamide synthase (*purC*), protease synthase and sporulation negative regulatory protein pai1 (*EF_3001*), etc., were found to be most abundant in the EGCG-responsive biofilm. The enrichment of helix-turn-helix protein, iron-dependent repressor family in the biofilm formed with EGCG might be related to the iron-chelating activity of EGCG [32]. Phosphoribosylaminoimidazole-succinocarboxamide synthase is an enzyme involved in de novo purine biosynthesis that had been reported for the relation with biofilm formation [33], and thus its presence in the

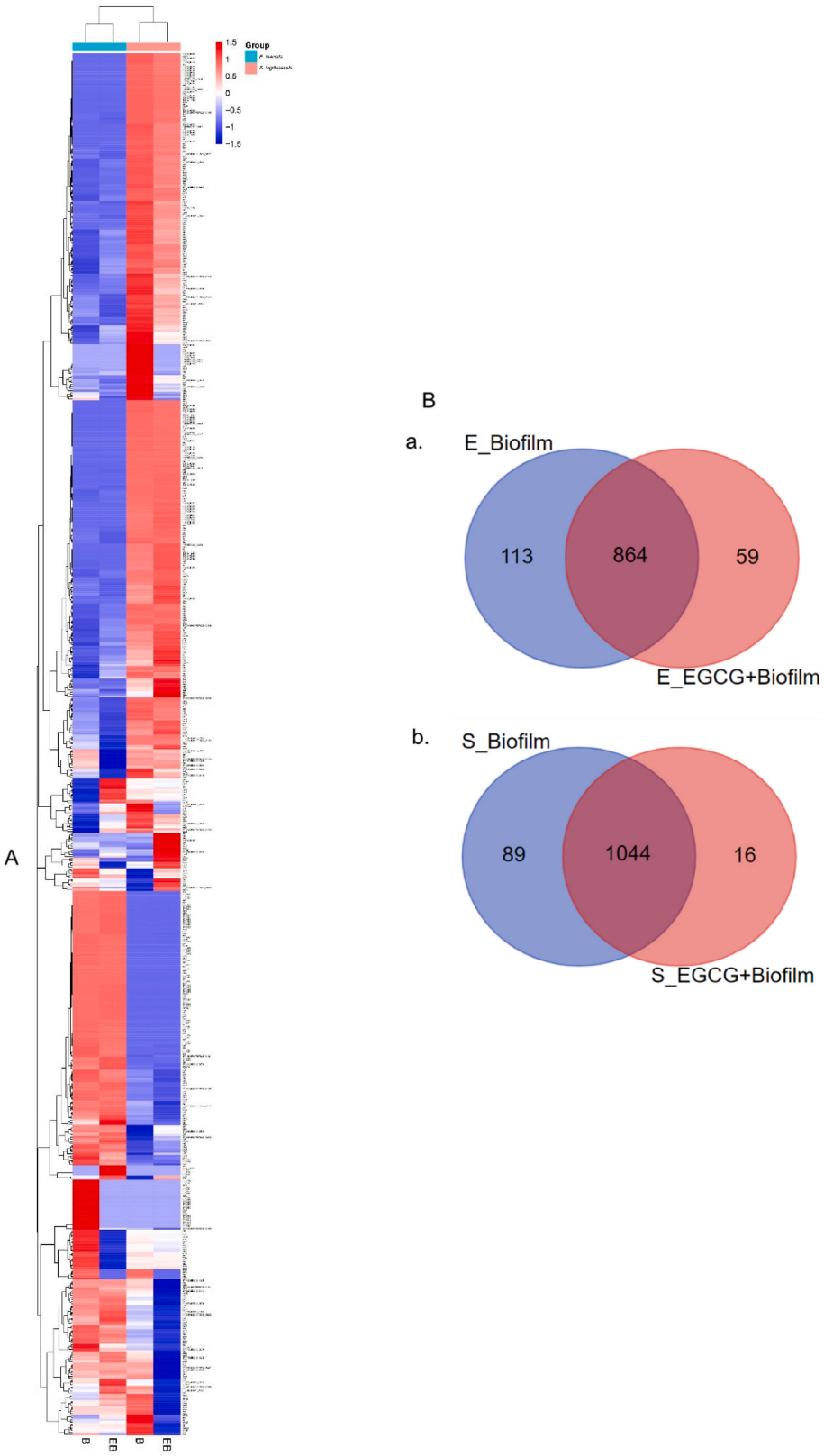


**Fig. 1.** Clustered heat map and Venn diagram for the proteins identified from 4 experimental groups. A. Clustered heat map for the relative abundance of proteins identified in the indicated sample. All proteins in the biofilms formed either without (B) or with EGCG (EB) of *E. faecalis* or *S. lugdunensis* were aligned and relatively compared at the abundance basis. The color scale [blue (lower levels) to red (higher levels)] represents the abundance of each protein across the different samples. B. Venn diagram for the proteins identified in biofilm formed without (Biofilm) or with EGCG (EGCG + Biofilm) of *E. faecalis* (a) and *S. lugdunensis* (b). The left panel (in blue) indicates the number of proteins exclusively identified in the biofilm formed without EGCG (E_Biofilm or S_biofilm), and the right panel (in red) represents the number of proteins only identified only in the biofilm formed with EGCG (E_EGCG + Biofilm or S_EGCG + biofilm). The number of the proteins identified in both groups of biofilms were displayed in the middle panel (in violet). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

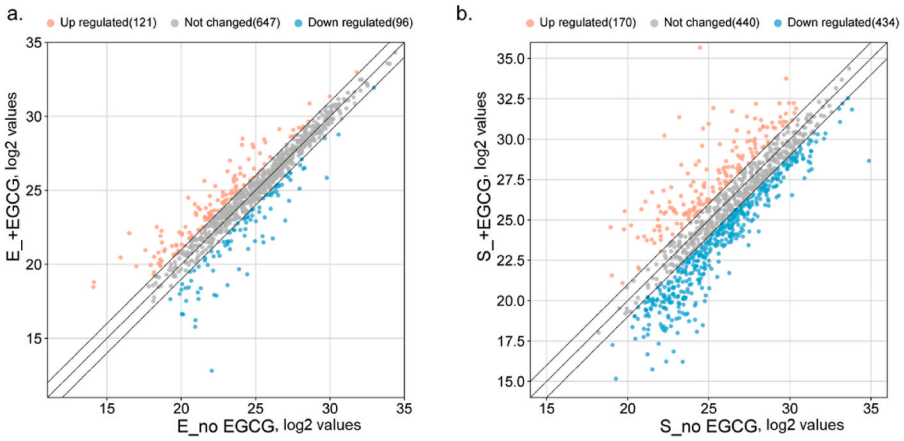


**Fig. 2.** Scatter plots for the proteins identified in common in the biofilms without or with EGCG of *E. faecalis* and *S. lugdunensis*. The x- or y-axis scales represent the log2 values of the protein abundances from each indicated group, where the fold change cutoff is 2. **a.** Out of a total of 864 proteins of *E. faecalis*, 121 or 96 proteins were relatively more abundant in the biofilm formed with EGCG (E_+EGCG) or without EGCG (E_no EGCG), respectively, and 647 proteins were at no changed level. **b.** From 1044 proteins of *S. lugdunensis*, 170 or 434 proteins were relatively more abundant in the biofilm formed with EGCG (S_+EGCG) or without EGCG (S_no EGCG), respectively, and 440 proteins were at a similar level in both biofilms.

**Table 1**
A list of the top 10 proteins that are relatively more abundant in either the biofilms formed with EGCG (EB) (*white panel*) or without EGCG (B) (*gray panel*) among those commonly identified in both groups of *E. faecalis* or *S. lugdunensis*.

| ID | Accession | Gene name | Description | Abundance [log2] | | Ratio |
|---|---|---|---|---|---|---|
| | | | | Biofilm (B) | EGCG + Biofilm (EB) | (EB/B) |
| *E. faecalis* | Q839U1 | *glmU* | Bifunctional protein GlmU | 16.50 | 22.10 | 1.34 |
| | Q839Y6 | *rplI* | 50S ribosomal protein L9 | 14.12 | 18.80 | 1.33 |
| | Q838B7 | *EF_0655* | Nitroreductase family protein, putative | 14.09 | 18.48 | 1.31 |
| | Q831Q7 | *rpsT* | 30S ribosomal protein S20 | 15.91 | 20.46 | 1.29 |
| | Q836T3 | *ppdK* | Pyruvate, phosphate dikinase | 18.35 | 22.52 | 1.23 |
| | Q834R9 | *EF_1191* | DegV family protein | 20.47 | 24.50 | 1.20 |
| | Q82ZC9 | *uxuA* | Mannonate dehydratase | 18.67 | 22.26 | 1.19 |
| | Q835Q8 | *nagA-1* | N-acetylglucosamine-6-phosphate deacetylase | 17.58 | 20.96 | 1.19 |
| | Q832M2 | *EF_2203* | Transcriptional regulator, TetR family | 21.56 | 25.63 | 1.19 |
| | Q82ZZ6 | *EF_2901* | D-isomer specific 2-hydroxyacid dehydrogenase family protein | 24.12 | 28.49 | 1.18 |
| | Q839C2 | *EF_0253* | Aldehyde dehydrogenase | 20.04 | 16.65 | 0.83 |
| | Q82ZR6 | *EF_2433* | Phosphoglycerate mutase family protein | 20.12 | 16.62 | 0.83 |
| | Q834K0 | *topA* | DNA topoisomerase 1 | 23.88 | 19.67 | 0.82 |
| | Q831P0 | *EF_2461* | Inositol monophosphatase protein family | 22.21 | 17.94 | 0.81 |
| | Q833B0 | *EF_2057* | Heptaprenyl diphosphate synthase, component II, putative | 23.11 | 18.59 | 0.80 |
| | Q839E1 | *rpmJ* | 50S ribosomal protein L36 | 22.37 | 17.89 | 0.80 |
| | Q834B0 | *EF_1759* | Phosphate-binding protein | 24.43 | 19.32 | 0.79 |
| | Q832R8 | *EF_2150* | Aminoacyltransferase FemA | 20.95 | 16.23 | 0.77 |
| | Q834N1 | *pflB* | Formate acetyltransferase | 20.93 | 15.78 | 0.75 |
| | Q839C0 | *pth* | Peptidyl-tRNA hydrolase | 22.05 | 12.81 | 0.58 |
| *S. lugdunensis* | A0A133PZU0 | *tadA* | tRNA-specific adenosine deaminase | 24.45 | 35.67 | 1.46 |
| | A0A4Q9WD10 | *addB* | ATP-dependent helicase/deoxyribonuclease subunit B | 22.26 | 30.22 | 1.36 |
| | A0A133PZW5 | *EQ (812)_08325* | NYN domain-containing protein | 23.17 | 31.36 | 1.35 |
| | A0A133Q3I3 | *EQ (812)_07465* | UPF0291 protein EQ (812)_07465 | 18.95 | 24.55 | 1.30 |
| | A0A4Q9W9P8 | *EQ (812)_08890* | Glycosyltransferase family 2 protein | 19.82 | 25.54 | 1.29 |
| | A0A4Q9WB32 | *EQ (812)_01820* | YSIRK signal domain/LPXTG anchor domain surface protein | 25.28 | 31.93 | 1.26 |
| | A0A133Q5V6 | *EQ (812)_01540* | ATP-binding cassette domain-containing protein | 24.64 | 30.66 | 1.24 |
| | A0A2Z2GAA4 | *EQ (812)_01305* | Amino acid ABC transporter ATP-binding protein | 21.51 | 26.71 | 1.24 |
| | Q0KKP0 | *tanA* | Esterase | 21.09 | 26.14 | 1.24 |
| | A0A4Q9WD50 | *EQ (812)_00625* | DUF1641 domain-containing protein | 24.99 | 30.95 | 1.24 |
| | A0A4Q9W838 | *EQ (812)_12050* | Lysine decarboxylase | 19.27 | 15.16 | 0.79 |
| | A0A4Q9VZY2 | *EQ (812)_13945* | Histidine kinase (Fragment) | 21.22 | 16.68 | 0.79 |
| | A0A4Q9WB69 | *brnQ* | Branched-chain amino acid transport system carrier protein | 23.07 | 18.00 | 0.78 |
| | A0A133Q5K0 | *HMPREF3225 _01325* | Putative antiholin-like protein LrgA | 24.15 | 18.82 | 0.78 |
| | A0A4Q9W0S0 | *EQ (812)_13520* | ABC transporter ATP-binding protein (Fragment) | 24.63 | 18.47 | 0.75 |
| | A0A133QAF5 | *EQ (812)_00955* | DNA-binding helix-turn-helix protein | 24.26 | 18.07 | 0.74 |
| | A0A4Q9W935 | *EQ (812)_10775* | GTP pyrophosphokinase | 22.83 | 16.84 | 0.74 |
| | A0A133QCA4 | *HMPREF3225 _00031* | TspO/MBR family protein | 21.52 | 15.74 | 0.73 |
| | A0A133Q1C6 | *EQ (812)_09225* | Anthranilate synthase component I family protein | 22.37 | 16.23 | 0.73 |
| | A0A133Q519 | *HMPREF3225 _01403* | Dihydrolipoyl dehydrogenase family protein (Fragment) | 23.39 | 16.21 | 0.69 |

**Table 2**
A list of the top 10 proteins identified only in the biofilms formed with EGCG, which were not found in the biofilms formed without EGCG.

| ID | Accession | Gene name | Description | Abundance [log2] |
|---|---|---|---|---|
| *E. faecalis* | H7C6W4 | *EF_0578* | Helix-turn-helix protein, iron-dependent repressor family | 30.57 |
| | Q833Y6 | *purC* | Phosphoribosylaminoimidazole-succinocarboxamide synthase | 29.33 |
| | Q82ZP9 | *EF_3001* | Protease synthase and sporulation negative regulatory protein pai 1 | 28.67 |
| | P0A0C2 | *aacA-aphD* | Bifunctional AAC/APH | 28.42 |
| | Q830R3 | *EF_2709* | Beta-galactosidase | 27.68 |
| | Q839Q9 | *sdhA-1* | L-serine dehydratase | 27.64 |
| | P27543 | *mtlD* | Mannitol-1-phosphate 5-dehydrogenase | 27.41 |
| | Q820V2 | *copY* | Transcriptional repressor CopY | 27.06 |
| | Q82YZ3 | *EF_0458* | Phosphosugar-binding transcriptional regulator, putative | 26.53 |
| | Q831A0 | *atpE* | ATP synthase subunit c | 26.47 |
| *S. lugdunensis* | A0A133Q3A6 | *HMPREF3225_01840* | DNA-binding protein HU-beta | 30.12 |
| | A0A4Q9WE07 | *EQ (812)_02425* | YSIRK-type signal peptide-containing protein | 27.24 |
| | A0A133QBY1 | *HMPREF3225_00123* | Iron-regulated surface determinant protein C | 25.19 |
| | A0A4Q9W4A3 | *argF* | Ornithine carbamoyltransferase (Fragment) | 24.98 |
| | A0A133QBY0 | *EQ (812)_05930* | Heme oxygenase (staphylobilin-producing) | 21.97 |
| | A0A4Q9VZ05 | *EQ (812)_14510* | DNA helicase (Fragment) | 21.96 |
| | A0A4Q9W856 | *EQ (812)_11590* | DNA starvation/stationary phase protection protein | 21.90 |
| | A0A133Q8Y2 | *EQ (812)_03315* | DUF5011 domain-containing protein | 21.37 |
| | A0A4Q9W0X3 | *EQ (812)_13685* | Homoserine dehydrogenase (Fragment) | 20.95 |
| | A0A133Q8D0 | *argH* | Argininosuccinate lyase | 20.71 |

biofilm formed with EGCG might support the finding that biofilm formation was strongly induced by the stimuli. As biofilm formation and sporulation are the two main survival mechanisms [34], the enrichment of protease synthase and sporulation negative regulatory protein pai1 supported the observed phenotype that EGCG enhanced biofilm formation. Taken together, as predicted from the increase in biofilm formation ability by EGCG, it was indicated that EGCG might strengthen the cellular program of *E. faecalis* toward enhancing biofilm formation.

In contrast, in the EGCG-responsive biofilm of *S. lugdunensis*, proteins such as DNA-binding protein HU-beta (*HMPREF3225_01840*), YSIRK-type signal peptide-containing protein (*EQ (812)_02425*), iron-regulated surface determinant protein C (*HMPREF3225_00123*), etc., were shown to be abundantly present. The enrichment of DNA-binding protein HU-beta is involved in DNA recombination and repair [35], and YSIRK-type signal peptide-containing protein, which is associated with translocation of cell wall anchoring effectors [36], indicated that EGCG might cause changes in genome and pathogenicity. Similarly to the helix-turn-helix protein, iron-dependent repressor family in *E. faecalis*, the abundant presence of iron-regulated surface determinant protein C might be attributed to the iron-chelating activity of EGCG [32], possibly as a survival mechanism. Collectively, in *S. lugdunensis*, EGCG was presumed to act as a signal for microbial pathogenic variation rather than as a signal for harm or threat, while reducing biofilm formation.

When comparing the proteins identified only in biofilms formed in the presence of EGCG across the two microorganisms, no overlapping proteins were found between the two groups. These data implied that EGCG regulated distinct molecular programs in each microorganism, as evidenced in the contrasting patterns of biofilm formation mediated by EGCG [29].

### 3.4. Further analysis of the proteins identified only in EGCG-responsive biofilms

We then performed a functional analysis of the proteins identified only in the EGCG-responsive biofilms of each microbe utilizing STRING-db (https://string-db.org) (Fig. 3). When 59 proteins of *E. faecalis* were applied to the database, 57 proteins were annotated, excluding RepS protein, putative (*EF_C0019*), bifunctional AAC/APH (*aacA-aphD*). On the other hand, among the 16 proteins of *S. lugdunensis*, 9 proteins were annotated, excluding ornithine carbamoyltransferase (Fragment) (*argF*), YSIRK-type signal peptide-containing protein (*EQ (812)_02425*), pathogenicity island protein (*EQ (812)_05185*), DNA starvation/stationary phase protection protein (*EQ (812)_11590*), homoserine dehydrogenase, Fragment (*EQ (812)_13685*), DNA helicase (Fragment) (*EQ (812)_14510*), ATPase/histidine kinase/DNA gyrase B/HSP90 domain protein (*HMPREF3225_01689*). The excluded proteins were not part of the STRING database and therefore could not be included in the analysis.

For both microorganisms, the results of known or predicted protein-protein interactions for the annotated proteins are depicted in Fig. 3A. The analytical tool explained the results as follows; the network had significantly more interactions than expected, implying that the proteins have more interactions among themselves than what would be expected for a random set of proteins of the same size and degree distribution drawn from the genome, and such an enrichment indicates that the proteins are at least partially biologically connected, as a group. It was expected that the interaction between purC (Phosphoribosylaminoimidazole-succinocarboxamide synthase)/purD (Phosphoribosylamine–glycine ligase)/purE (N5-carboxyaminoimidazole ribonucleotide mutase)/purS(Phosphoribosylformylglycinamidine synthase subunit)/purM (Phosphoribosylformylglycinamidine cyclo-ligase)/purH (Bifunctional purine biosynthesis protein) and sdhA-1(L-serine dehydratase), or the interaction between ARJ28483.1 (EQ (812)_05930, Heme oxygenase) and ARJ28479.1 (HMPREF3225_00121, High-affinity heme uptake system protein IsdE) and ARJ30084.1 (EQ (812)_03315, DUF5011 domain-containing protein) would be contributed to biofilm formation and function in the presence of EGCG in *E. faecalis* or *S. lugdunensis*, respectively. The functional enrichments in the networks are summarized in Table 3.

Among the functional annotations, further analysis was conducted for the classification based on gene ontology (GO) terms (biological process, molecular function, cellular component) (Fig. 3B). The results demonstrated that terms like “cellular process”, “binding” and “cellular anatomical entity” were highly designated in both species. For each microorganism, distinctions were observed; in *E. faecalis,* “(organic substance/primary) metabolic process” or “catabolic activity” featured with more than 33 proteins, while in *S. lugdunensis*, “(establishment of) localization” or “transport” was highly ranked with 4 proteins. The GO terms analyzed distinctly in each microorganism supported the findings from identified protein sets that EGCG triggered different cellular programs in each microorganism, possibly encouraging metabolism in *E. faecalis* and transfer activities in *S lugdunensis*.

As expected, the two microorganisms, having relatively contrasting properties in biofilm formation, obviously exhibited a distinct, rather than similar, trends in the proteomes and functional annotations of the biofilms formed in response to EGCG. Finally, we compared the

proteomes of two groups that form biofilms to similar extents to find out whether there were any similarities; the weak biofilm-forming *E. faecalis* without EGCG (*E*_B) and *S. lugdunensis* with EGCG (*S*_EB), or the strong biofilm-forming *E. faecalis* with EGCG (*E*_EB) and *S. lugdunensis* without EGCG (*S*_B). For this purpose, among the commonly identified proteins in the biofilms formed with or without EGCG, proteins with a B/EB or EB/B ratio of more than 1.1 were selected for analysis, as along with the proteins identified only in either B or EB group (Supplementary Table 5). Among the 145 proteins from *E*_B and the 79 proteins from *S*_EB, 2 proteins were commonly discovered. On the other hand between the 103 proteins from *E*_EB and the 242 proteins from *S*_B, 9 proteins were shared (Fig. 4).

The two proteins commonly identified in the weak biofilm-forming groups were ribonuclease M5 and holliday junction ATP-dependent DNA helicase RuvA, whereas the nine proteins found in common from the strong biofilm-forming groups were S1 RNA binding domain protein, tRNA 5-hydroxyuridine methyltransferase, UDP-N-acetylenolpyruvoylglucosamine reductase, elongation factor Tu, GTPase HflX, HD domain protein, L-serine dehydratase, N5-carboxyaminoimidazole ribonucleotide mutase and 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase (Table 4). From this result, it was suggested that these proteins could serve as indicators for detecting the presence and predicting the extent of biofilm formation by multiple microorganisms.

## 4. Discussion

Biofilm-associated infections in medical fields are considered as a major problem to be solved due to the possibility of causing serious complications. The two microorganisms explored in this study are among representative species previously reported for their relationship with biofilm-related pathology. *E. faecalis* is the most prevalent enterococcal species in biofilm-associated infections [37], and the ability of biofilm formation by *S. lugdunensis* is implicated as an important pathogenic factor for prosthetic joint infections (PJIs) [38]. To properly handle the challenges posed by biofilms, a better understanding of the biological properties and characteristics of biofilms is still needed.

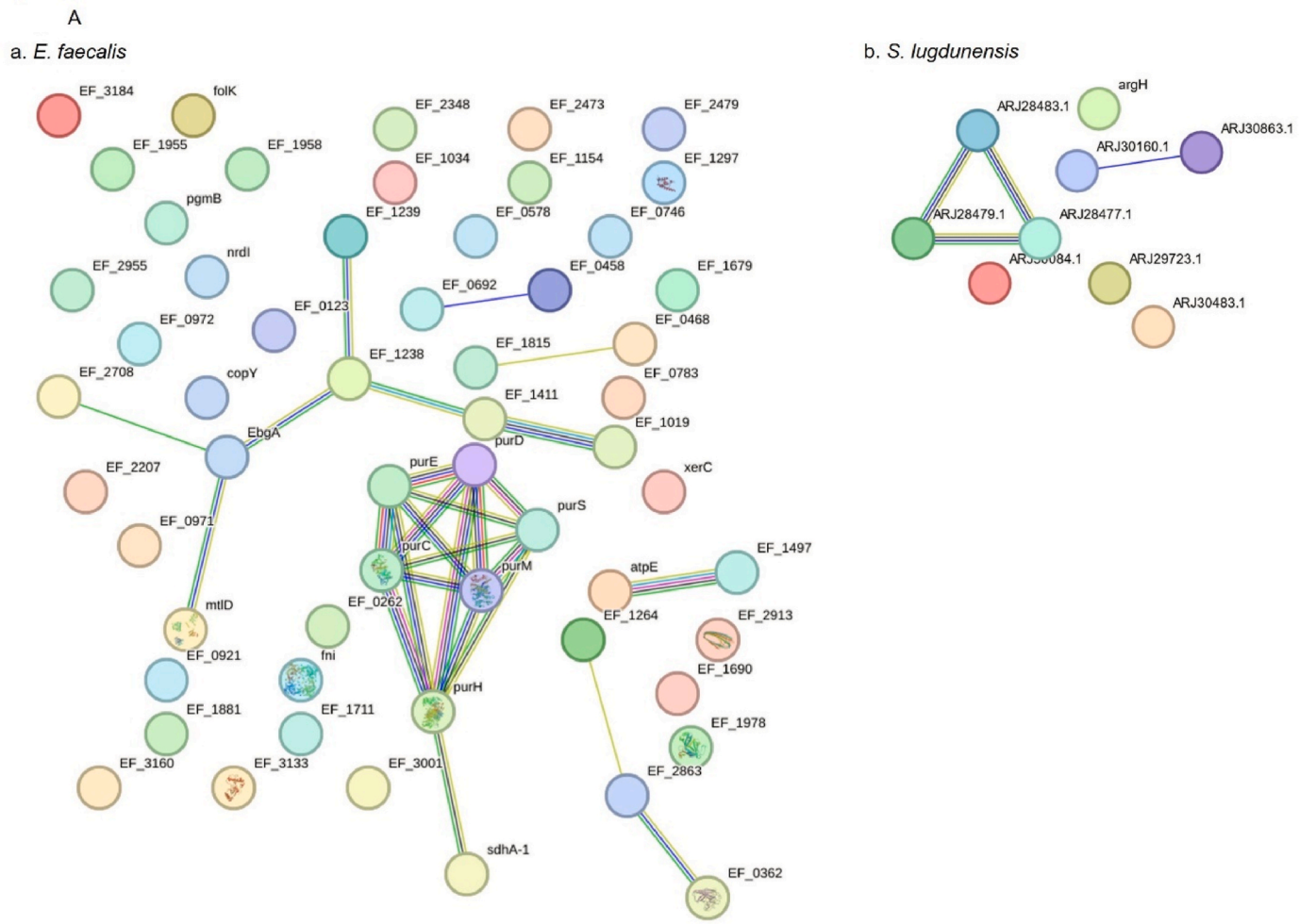


**Fig. 3.** Protein interaction networks and functional analysis of the proteins identified only in EGCG-responsive biofilms of *E. faecalis* and *S. lugdunensis*.
A. Known and expected protein-protein interactions obtained through STRING-db. Proteins are illustrated as nodes. Colored or white nodes denotes query proteins and the first shell of interactions, or second shell of interactions, respectively. Light blue or pink lines indicate known interactions from curated databases or experimentally determined, respectively. Some proteins are labeled with their preferred names on the website as follows; EbgA = EF_2709, ARJ29723.1 = HMPREF3225_01840, ARJ28477.1 = HMPREF3225_00123, ARJ28483.1 = EQ (812)_05930, ARJ30084.1 = EQ (812)_03315, ARJ30863.1 = HMPREF3225_00356, ARJ30483.1 = HMPREF3225_02201, ARJ28479.1 = HMPREF3225_00121, ARJ30160.1 = EQ (812)_03480.
B. Gene ontology (GO) classification. Annotated proteins were classified into three main GO categories: biological process, molecular function, and cellular component. The X-axis displays each GO term, and the Y-axis represents the number of proteins annotated under each GO term. Graphs only include GO terms annotated with >10 proteins for *E. faecalis* or 2 proteins for *S. lugdunensis*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

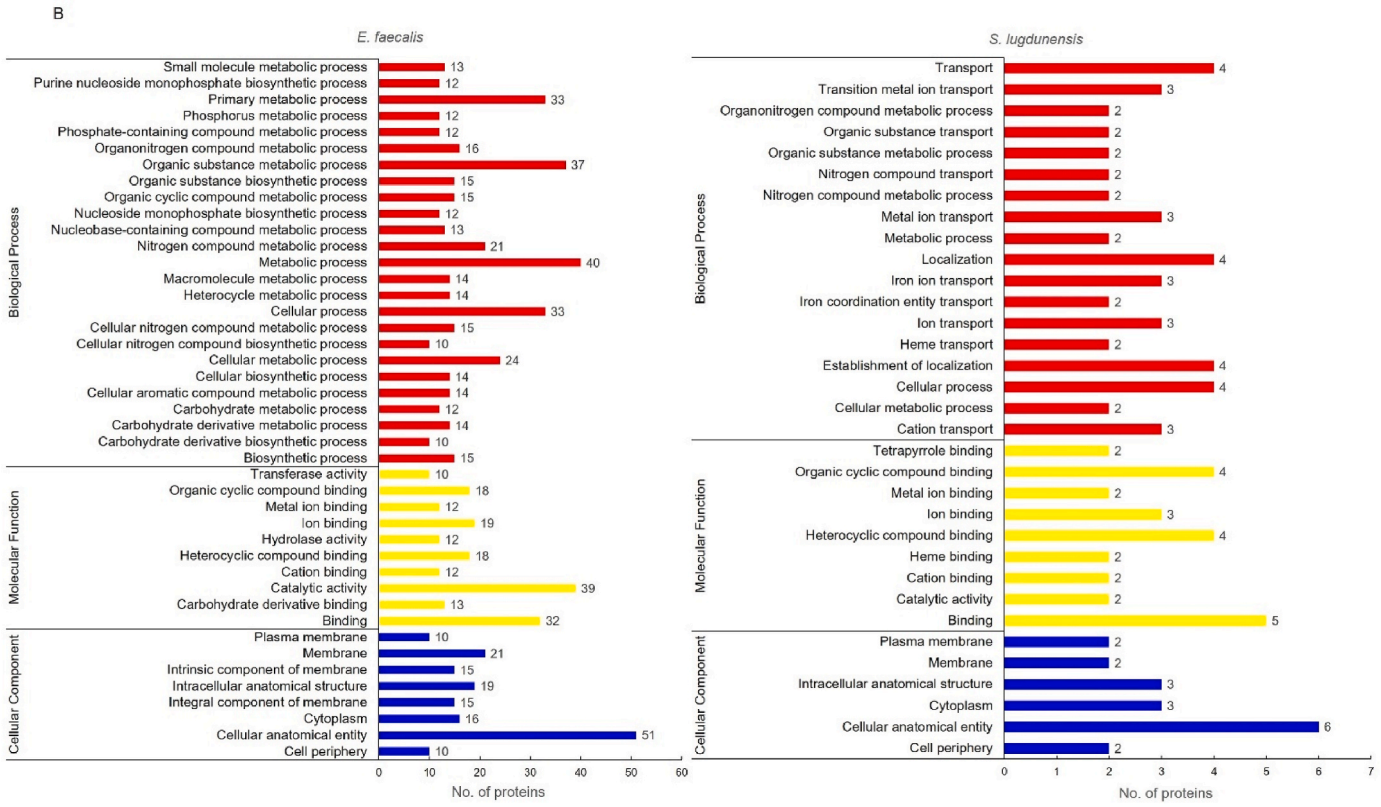


**Fig. 3.** (*continued*).

Interestingly, our previous study showed that the microorganisms had relatively opposite abilities for biofilm formation, which were reversed upon EGCG stimulation [29]. Although EGCG is widely recognized for its antibacterial and antibiofilm effects [20,21,26–28], it was unexpected that some bacteria could increase biofilm formation in response to the stimulus. Such a finding contrasts with a previous study published by others in the case of *E. faecalis* [39], and highlights the infrequent research on the effects of EGCG on *S. lugdunensis*. In this study, we examined how the stimulus altered the protein compositions of each microbial biofilm.

The clustered heatmap of all the proteins identified in the biofilms formed with or without EGCG from both microorganisms revealed no overall similarity between the groups. Although expected from the contrasting trends in their biofilm-forming abilities, it was still interesting that proteins were differentially enriched in each microorganism by the same stimulation. The result led us to predict that EGCG induced distinct molecular programs in each microorganism, which generated biofilms with different properties. As expected, the proteomic profiles exclusive in the biofilms formed with EGCG presented unique protein combinations in each microbe, with no overlapping proteins between the groups. Furthermore, the gene annotation and functional analyses also exhibited markedly different features in each microorganism. For instance, in terms of biological process among GO terms, *E. faecalis* was mainly represented with metabolism, while *S. lugdunensis* was notably represented with localization or transport. In other words, it was implicated that EGCG redirected cellular programs in opposite directions, where *E. faecalis* might use cellular energy more for producing molecules for biofilm formation, while *S. lugdunensis* might shift their focus to maintenance.

Although the total number of proteins identified in the proteome of the biofilms was higher for *S. lugdunensis* than for *E. faecalis*, the quantity of proteins exclusively present in the biofilms formed with EGCG was considerably lower for *S. lugdunensis*, and moreover, the number of annotated proteins was also very limited, likely due to the lack of researches related to biofilm formation in this particular species, as mentioned above. In this regard, this study provides academically valuable insights into biofilm formation-related protein profiles of *S. lugdunensis*. Especially, this appears to be the first time that protein composition changed by EGCG has been discovered in this species.

The observation that biofilm formation can be increased by EGCG could lead to caution in its use under certain conditions, such as where *E. faecalis* dominates. Fortunately, the proteomics results from this study did not present direct evidence for increasing the production of other virulence factors besides biofilm formation in the microbe. On the other hand, EGCG could be a good choice for handling situations where *S. lugdunensis* is dominant. The proteomics analysis presented in this study supported that EGCG reduced the biofilm-forming ability of this microbe by altering cellular activities other than synthesis or metabolism. It is expected that the protein profiles identified in this study could assist in choosing an appropriate pharmacological interventions to handle a given situation.

The comparison between two groups with similar trends in biofilm formation revealed that several proteins were commonly found within either the weak or strong biofilm-forming groups. One of the two proteins commonly found in the weak biofilm-forming groups is ribonuclease M5, which is an enzyme responsible for the maturation of 5s rRNA [40]. The other protein commonly found in the weak biofilm-forming groups is holliday junction ATP-dependent DNA helicase RuvA, which is involved in the cellular response to DNA damage [41]. Both proteins impact on nucleic acids, suggesting a potential role in the nucleic acid production for the extracellular polymer substances that make up biofilms. However, at this time, it is not clear how both proteins are related to biofilm formation and regulation, needing further studies.

Among the nine proteins found in common from the strong biofilm-forming groups, S1 RNA binding domain protein is an RNA-associated protein that plays a role in translation [42]. A previously published study in *Streptococcus pneumoniae* has demonstrated that the absence of CvfD, an S1 RNA binding domain protein, caused virulence attenuation,

**Table 3**
Functional enrichments analyzed from the protein-protein networks generated through STRING database.

| ID | term ID | #category | term description | observed gene count | matching proteins |
|---|---|---|---|---|---|
| *E. Faecalis* | GO:0006189 | GO Process | De novo IMP biosynthetic process | 6 | purD, purH, purM, purS, purC, purE |
| | GO:0008152 | GO Process | Metabolic process | 40 | sdhA-1, EF_0123, EF_0262, EF_0362, mtlD, EF_0458, EF_0468, nrdI, EF_0692, EF_0783, fni, pgmB, EF_0972, EF_1034, EF_1154, EF_1238, EF_1239, EF_1264, EF_1411, xerC, EF_1679, EF_1690, EF_1711, purD, purH, purM, purS, purC, purE, EF_1955, EF_1958, EF_1978, EF_2207, EF_2473, EF_2479, atpE, EbgA, EF_2863, EF_2955, folk |
| | GO:0009152 | GO Process | Purine ribonucleotide biosynthetic process | 7 | purD, purH, purM, purS, purC, purE, atpE |
| | GO:1901135 | GO Process | Carbohydrate derivative metabolic process | 14 | EF_0362, EF_0458, EF_0692, EF_0783, EF_0972, EF_1264, purD, purH, purM, purS, purC, purE, EF_1958, atpE |
| | GO:0006163 | GO Process | Purine nucleotide metabolic process | 8 | purD, purH, purM, purS, purC, purE, EF_1958, atpE |
| | GO:0071704 | GO Process | Organic substance metabolic process | 37 | sdhA-1, EF_0123, EF_0262, EF_0362, mtlD, EF_0458, EF_0468, nrdI, EF_0692, EF_0783, fni, pgmB, EF_0972, EF_1154, EF_1238, EF_1239, EF_1264, EF_1411, xerC, EF_1679, EF_1690, purD, purH, purM, purS, purC, purE, EF_1958, EF_1978, EF_2207, EF_2473, EF_2479, atpE, EbgA, EF_2863, EF_2955, folK |
| | GO:0005975 | GO Process | Carbohydrate metabolic process | 12 | sdhA-1, EF_0123, EF_0362, mtlD, EF_0783, pgmB, EF_0972, EF_1238, EF_1239, EF_1411, EbgA, EF_2863 |
| | GO:0044238 | GO Process | Primary metabolic process | 33 | sdhA-1, EF_0123, EF_0262, EF_0362, mtlD, EF_0468, nrdI, EF_0783, fni, pgmB, EF_0972, EF_1154, EF_1238, EF_1239, EF_1411, xerC, EF_1679, EF_1690, purD, purH, purM, purS, purC, purE, EF_1958, EF_1978, EF_2207, EF_2473, EF_2479, atpE, EbgA, EF_2863, EF_2955 |
| | GO:1901137 | GO Process | Carbohydrate derivative biosynthetic process | 10 | EF_0783, EF_0972, EF_1264, purD, purH, purM, purS, purC, purE, atpE |
| | CL:1174 | STRING clusters | De novo IMP biosynthetic process | 6 | purD, purH, purM, purS, purC, purE |
| | CL:1169 | STRING clusters | Ribonucleoside monophosphate biosynthetic process, and One-carbon metabolic process | 7 | EF_1711, purD, purH, purM, purS, purC, purE |
| | CL:1165 | STRING clusters | Mixed, incl. Nucleoside monophosphate biosynthetic process, and Pyrimidine metabolism | 8 | nrdI, EF_1711, purD, purH, purM, purS, purC, purE |
| | CL:1177 | STRING clusters | De novo IMP biosynthetic process | 3 | purD, purH, purM |
| | Efa00230 | KEGG | Purine metabolism | 7 | purD, purH, purM, purS, purC, purE, EF_1958 |
| | KW-0658 | UniProt Keywords | Purine biosynthesis | 6 | purD, purH, purM, purS, purC, purE |
| *S. lugdunensis* | CL:3385 | STRING clusters | Mixed, incl. NEAT domain, and Iron import into cell | 3 | HMPREF3225_00123, HMPREF3225_00121, EQ (812)_05930 |

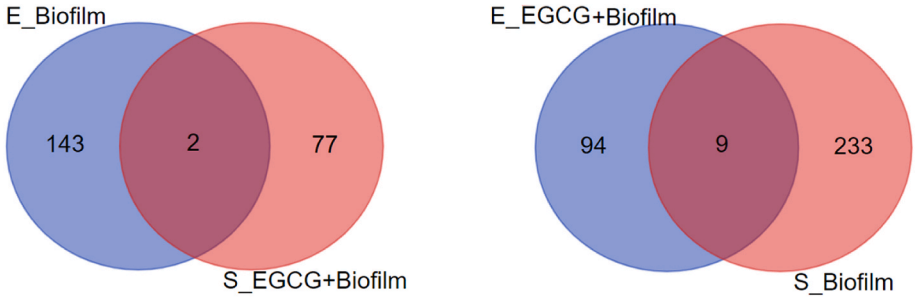


**Fig. 4. Venn diagram for discovering proteins found in common from two groups that have similar levels of biofilm formation ability. a.** When the relatively or absolutely abundant proteins from biofilms of *E. faecalis* without EGCG (*E*_Biofilm) and *S. lugdunensis* with EGCG (*S*_EGCG + Biofilm) were compared, 2 proteins were found in common between the weak biofilm-forming groups. On the other hand, the comparison of the relatively or absolutely abundant proteins from biofilms of *E. faecalis* with EGCG (*E*_EGCG + Biofilm) and *S. lugdunensis* without EGCG (*S*_Biofilm) revealed that 9 proteins were in common between the strong biofilm-forming groups.

and Cvf homolog Ygs was implicated in stress adaptation and required for biofilm-associated infections in a mouse catheter model [43]. tRNA 5-hydroxyuridine methyltransferase is an enzyme that can enhance translational fidelity by modifying the wobble position in bacterial tRNA [44]. GTPase HflX is a ribosome splitting factor, which facilitates translation. Along with elongation factor Tu, those proteins might contribute to produce protein components for biofilm composition. UDP-N-acetylenolpyruvoylglucosamine reductase is an enzyme that catalyze the final steps of the UDP-N-acetylmuramic acid (UDPMurNAc) formation during peptidoglycan synthesis [45] and thus can certainly contribute to biofilm formation. L-serine dehydratase catalyzes the conversion of L-serine to pyruvate and ammonia [46]. In a study using *Proteus mirabilis* published by others, it was shown that disrupting its activity was involved in decreased biofilm formation [47]. N5-carboxyaminoimidazole ribonucleotide mutase is a key enzyme in purine biosynthesis [48], and thus its association with nucleic acid synthesis or modification for biofilm formation can be predicted. 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase is an

**Table 4**
A list of the proteins found in common between the groups with similar levels of biofilm formation (weak or strong).

| Groups | Accession | | Gene name | | |
|---|---|---|---|---|---|
| | E. faecalis | S. lugdunesis | E. faecalis | S. lugdunesis | |
| Weak biofilm | Q837A8 | A0A133PZM0 | *rnmV* | | Ribonuclease M5 |
| | Q839T6 | A0A292DE87 | ruvA | | Holliday junction ATP-dependent DNA helicase RuvA |
| Strong biofilm | Q835R3 | A0A133Q8C0 | *EF_1312* | *EQ (812)_03780* | S1 RNA binding domain protein |
| | Q830N8 | A0A292DE51 | *trmR* | | tRNA 5-hydroxyuridine methyltransferase |
| | Q830P3 | A0A133Q1I3 | *murB* | | UDP-N-acetylenolpyruvoylglucosamine reductase |
| | Q839G8 | A0A133PZU5 | *tuf* | | Elongation factor Tu |
| | Q832Q8 | A0A133Q3P9 | *hflX* | | GTPase HflX |
| | Q836G9 | A0A133Q747 | *EF_2413* | *HMPREF3225_01109* | HD domain protein |
| | Q839Q9 | A0A133Q657 | *sdhA-1* | *sdaAA* | L-serine dehydratase |
| | Q833Y4 | A0A133Q8V3 | *purE* | | N5-carboxyaminoimidazole ribonucleotide mutase |
| | Q82Z11 | A0A133PZL4 | *folK* | | 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine diphosphokinase |

enzyme that participates in folic acid biosynthesis [49], consistent with the previous report using *Helicobacter pylori*, which showed a significant increase in folic acid synthesis in high biofilm-formers [50].

When EGCG was present, bifunctional protein GlmU (*glmU*) was the most upregulated in the biofilms of *E. faecalis*. This observation is consistent with a previously published study by others, which demonstrated that down-regulation of GlmU decreased the capabilities of *Mycobacterium smegmatis* to produce biofilm, while its overexpression increased biofilm formation [51]. Another protein upregulated by EGCG in *E. faecalis* biofilms was nitroreductase family protein, putative. Given that nitroreductase activates the antimicrobial by reducing the nitro-group [52], its upregulation by EGCG in the biofilm of *E. faecalis* might be associated with bacterial resistance to external environment. In contrast, Peptidyl-tRNA hydrolase was more abundant in the biofilm formed without EGCG in *E. faecalis*. Peptidyl-tRNA Hydrolase is a highly conserved, essential enzyme in bacteria, that participates in translation [53]. Accordingly, its downregulation by EGCG might reflect the shift in cellular energy utilization from synthesis towards other processes, possibly for survival or defense.

In the case of *S. lugdunensis*, TadA was identified as the most upregulated protein by EGCG. TadA is a tRNA-specific adenosine deaminase that is known to be essential for viability [54], and therefore it could be inferred that EGCG induced the cellular program in the direction of strengthening survival and viability. On the contrary, TspO/MBR family protein, and dihydrolipoyl dehydrogenase family protein (fragment) were downregulated in the biofilm by EGCG. Since Translocator Protein (TSPO) and dihydrolipoyl dehydrogenase (DLD) are mitochondrial proteins in mammalians, it was assumed that their downregulation by EGCG in the bacteria might also be associated with alteration or conversion of energy metabolism.

Given that *Streptococcus* is taxonomically close to the two genera to which the tested species belong, it may be of interest to examine the impact of EGCG on controlling oral infections, where *Streptococcus* plays a major role. Numerous reports have shown that EGCG and also other green tea extracts affected oral biofilm formation [55,56]. However, as reviewed by others, the anti-biofilm effects of these compounds on dental biofilms remains ambiguous because strong evidence of heterogeneity was observed [57], which is consistent with our previous study. Thus, it may be critical to consider which bacteria dominates in a pathologic site or situation for proper anti-biofilm effects.

Taken all together, the two microorganisms with relatively opposite biofilm-forming abilities responded to EGCG, resulting in a completely reversed biofilm-forming tendency. These tendencies were clearly reflected in the proteomics results obtained in this study. Although some of the proteins showed relevance to biofilm formation or consistency with findings from previous studies by others, most of the proteins identified in this study require more detailed investigations in molecular and biochemical aspects to clarify their specific roles in the test microbes.

## CRediT authorship contribution statement

**Jung-Ah Cho:** Writing – original draft, Project administration, Investigation, Funding acquisition, Formal analysis. **Sangsoo Jeon:** Formal analysis, Data curation. **Youngmin Kwon:** Formal analysis. **Yoo Jin Roh:** Data curation. **Sukjin Shin:** Writing – review & editing. **Chang-Hun Lee:** Writing – review & editing, Investigation. **Sung Jae Kim:** Writing – review & editing, Supervision, Funding acquisition.

## Declaration of competing interest

There are no relevant financial or non-financial competing interests to report.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2024.100232.

## Data availability

Data will be made available on request.

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