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Secretome analysis and virulence assessment in *Abiotrophia defectiva*

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ABSTRACT

Background: Abiotrophia defectiva, although infrequently occurring, is a notable cause of culture-negative infective endocarditis with limited research on its virulence. Associated with oral infections such as dental caries, exploring its secretome may provide insights into virulence mechanisms. Our study aimed to analyze and characterize the secretome of A. defectiva strain CCUG 27639.

Methods: Secretome of A. defectiva was prepared from broth cultures and subjected to mass spectrometry and proteomics for protein identification. Inflammatory potential of the secretome was assessed by ELISA.

Results: Eighty-four proteins were identified, with diverse subcellular localizations predicted by PSORTb. Notably, 20 were cytoplasmic, 12 cytoplasmic membrane, 5 extracellular, and 9 cell wall-anchored proteins. Bioinformatics tools revealed 54 proteins secreted via the 'Sec' pathway and 8 via a non-classical pathway. Moonlighting functions were found in 23 proteins, with over 20 exhibiting potential virulence properties, including peroxiredoxin and oligopeptide ABC transporter substrate-binding protein. Gene Ontology and KEGG analyses categorized protein sequences in various pathways. STRING analysis revealed functional protein association networks. Cytokine profiling demonstrated significant proinflammatory cytokine release (IL-8, IL-1β, and CCL5) from human PBMCs.

Conclusions: Our study provides a comprehensive understanding of A. defectiva's secretome, laying the foundation for insights into its pathogenicity.

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Abiotrophia defectiva; infective endocarditis; secretome; proteomics; oral infections

Introduction

Abiotrophia defectiva, is part of the normal human oral microbiota, and urogenital &intestinal tracts [1]. Although a commensal, the pathogenic potential of *A. defectiva* has been reported [2]. It is a species of clinical significance, contributing to infective endocarditis with potentially devastating consequences albeit occurring infrequently [3–6] and has also been involved in various disease processes such as cerebral abscess, pancreatic abscess, corneal ulcer, sinusitis, osteomyelitis, scrotal abscess [1] and aortitis [7]. Its pathogenic role in oral diseases also, like caries [8,9] and orthodontic infections [10,11] cannot be overlooked. This relatively unknown bacterium is frequently associated with infections resulting from dental procedures [3]. It is pleomorphic on Gram stain [12], difficult to isolate, and characterized by its growth requiring complex media enriched with pyridoxal (vitamin B6) or cysteine or as small satellite colonies supported by helper bacteria [3]. These characteristics of *A. defectiva* contribute to some cases of culture-negative endocarditis and underestimate its pathogenic role [13].

Protein secretion is an essential mechanism utilized by bacteria for their growth and survival. It also serves multiple functions in enhancing its virulence, including facilitating attachment to host cells, acquiring resources from the environment, and directly disrupting the functions of the target cells. The pathogens employ dedicated protein secretion systems for the extracellular transfer of their cytosolic virulence factors into the host cell or host environment. Due to distinct cell wall structures, Gram-positive and Gram-negative bacteria possess different mechanisms for extracellular protein secretion. Gram-positive bacteria mainly employ both, the general secretory system (Sec) and the Twin-arginine transport (Tat) pathways to transport proteins across the cytoplasmic membrane while Gram-negative bacteria use more complex secretory systems (Type I to Type VIII). However, in many cases, these transport mechanisms alone are insufficient to deliver proteins to their final destinations. Consequently, pathogens employ conserved mechanisms of protein secretion to transport important virulence factors extracellularly during an infection [14].

The protein secretion mechanisms of *Abiotrophia* species are not well understood. However, a closely related species, *Granulicatella elegans* has been reported to secrete arginine deiminase, which inhibits the proliferation of human peripheral blood mononuclear cells (PBMCs) *in vitro* through citrullination [15] and impede the surface attachment of certain dental plaque bacteria

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in biofilms [16], suggesting a potential association of *G. elegans* with the pathogenesis of dental and certain systemic diseases. It is a well-established fact that the bacteria causing endocarditis interact with proteins of the extracellular matrix (ECM) during adhesion and colonization of damaged heart valves [17,18]. *In vitro* studies have investigated the ability of *Abiotrophia* and *Granulicatella* isolates to bind to certain ECM proteins, such as, fibrinogen, fibronectin, collagen and laminin, aiming to explore if variations in binding abilities could explain differences in clinical behavior of these strains [19]. In our recently published similar kind of investigation, the secretome of *G. adiacens* was analyzed and functionally characterized in addition to obtaining a preliminary information on the induction of immunoinflammatory response [20]. The findings have generated interest in exploring similar secretome analyses in close phylogenetic relative like *A. defectiva* to understand the possible pathogenic mechanisms in such bacteria.

Here, we investigated and functionally characterized the secretome of extracellularly released proteins from *A. defectiva*. Further, we examined the cytokine production and immune-stimulatory effect of these extracellular proteins on human peripheral blood mononuclear cells (PBMCs).

Methods

The procedures employed in this study for the methods described under sections 2.1, 2.2, 2.3, 2.4, 2.7, 2.9, 2.11, and Gene Ontology Analysis in 2.10, adhered closely to the protocols outlined in the prior work [20]. An overview of these methods is presented here.

Bacterial culture

A. defectiva CCUG 27,639 was cultured on chocolate blood agar for 48 hours at 37°C with 5% CO_2 in the air. The medium was supplemented with 0.001% pyridoxal hydrochloride [21].

Extracellular protein (ECP) release

A. defectiva grown on chocolate blood agar was inoculated into 5 ml of brucella broth supplemented with 0.001% pyridoxal hydrochloride and incubated at 37°C with 5% CO_2 in air, alongside a no-bacteria control. After 24 hours of incubation, the broth culture was centrifuged (5000 ×g, 5 min), separating ECP-containing supernatant which was subsequently filtered through a sterile 0.2 µm filter to remove residual bacterial cells.

Preparation of the secretome

The ECP (secretome) obtained in broth culture supernatant were isolated by tri-chloroacetic acid (TCA) precipitation method as described earlier [22]. Briefly, 100% w/v TCA was mixed with the secretome-containing supernatant in 1:4 ratio and incubated at −20°C for 30 minutes. Ultracentrifugation at 14,000 ×g for 20 minutes at 4°C yielded the secretome pellet which was subjected to two acetone washes (0.5 ml each) and air-drying in a fume hood. The protein samples were then diluted to 0.5 ml with lysis buffer for desalting, washed thrice by ultrafiltration (14,000 ×g for 15 minutes at 4°C), and finally eluted by centrifugation at 1000 ×g for 2 min at 4°C.

Preparation of the whole cell protein (WCP)

A. defectiva colonies from chocolate blood agar plates were PBS-washed and centrifuged at 5000 ×g for 5 minutes. The resulting pellet was resuspended in lysis buffer (1 mg/ml lysozyme and 1 mM phenyl methyl sulfonyl fluoride) and incubated for 4 h at 4–8°C. Subsequently, the sample was subjected to eight cycles of sonication at a pulse rate 40 in Omni Ruptor, followed by centrifugation at 10,000 ×g for 10 minutes at 4°C. This WCP of *A. defectiva* was used as a comparative control in western blot analysis against the ECP extract.

SDS-PAGE and gel image analysis

Samples for SDS-PAGE were prepared by denaturing proteins in 2× loading buffer at 100°C for 5 min, followed by centrifugation at 5000 ×g for 5 min. Five µg protein samples loaded in each well of the gel were separated on 12% SDS-PAGE at a constant 120 V and detected by both, coomassie blue and silver staining (Pierce Silver Stain Kit). Gel pictures were captured on Syngene G: Box Imaging System. Protein banding patterns and molecular weights of the bands were determined using GeneSys tools software. Duplicate SDS-PAGE gel was prepared in parallel for western blot analysis.

Western blot analysis

To validate that *A. defectiva* cells were not lysed and that the cellular proteins were not released in the secretome (ECP) preparations, we conducted western blot analysis on both ECP and WCP preparations. Briefly, the protein transfer from the gel to a PVDF membrane was achieved using Trans-Blot® Turbo™ transfer system (Bio-Rad). Subsequently, the membrane was blocked overnight at 4°C with 5% skimmed milk to prevent any non-specific binding. The primary antibody targeting the cytoplasmic marker protein, Ftsz (Filamenting temperature sensitive mutant z) (Agrisera AB, Sweden), was employed followed by enzyme conjugated secondary antibody and chemiluminescent substrate. The images were captured using the G:Box Imaging System (Syngene).

Protein analyses & identification

Extracellular protein samples were lyophilized, and proteomics analysis was done by using a nanoLC-ESI-MS/MS mass spectrometer (Proteome Factory AG, Germany). MS/MS ion search of the Mascot search engine (Matrix Science, England) and NCBI-nr (National Centre for Biotechnology Information, USA) protein database were used for identification of proteins.

Nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (nanoLC-ESI-MS/MS)

The composition of proteins in the *A. defectiva* secretome was determined using nanoLC-ESI-MS/MS by Proteome Factory (Proteome Factory AG, Berlin, Germany). With an Agilent 1100 nanoHPLC system (Agilent, Waldbronn, Germany) interfaced to an Orbitrap Velos (Thermo Scientific, Bremen, Germany) via a nanoelectrospray ion source. After pooling replicate samples from preparations, proteins were reduced, alkylated and digested by trypsin (Promega, Mannheim, Germany). Then, 400 ng of the resulting peptides were subjected to the nanoLC-ESI-MS/MS. 1% acetonitrile/0.5% formic acid was used as eluent for 5 minutes to trap and desalt the peptides on the enrichment column (Zorbax 300SB-C18, 0.3 × 5 mm, Agilent). A water/acetonitrile (both supplemented with 0.1% formic acid) gradient from 5% to 40% acetonitrile was then used within 120 minutes to separate the peptides on a Zorbax 300SB-C18, 75 µm x 150 mm column (Agilent). The mass spectrometer automatically recorded mass spectra, and tandem mass spectra were data-dependently acquired for multiply charged ions. Protein identification was made using the Mascot search engine (Matrix Science, London, England) against the bacterial subset of the RefSeq protein database (National Center for Biotechnology Information), (downloaded on NaN Invalid Date , 49867978 entries, NCBI, Bethesda, USA) and a database with common protein contaminants. For MS/MS spectra where assignment of the precursor ion's charge state was missing, search parameters for ions from ESI-MS/MS data acquisition was set to '2+, 3+ or 4+' according to the instrument's and method's standard charge state distribution. The search parameters were: Fixed modifications: Carbamidomethyl (C); variable modifications: Deamidated (NQ), Oxidation (M); Peptide Mass Tolerance: ±3 ppm; Fragment Mass Tolerance: ±0.6 Da; Missed Cleavages: 2. The inclusion criterion was: peptides that match with a score of 20 or above. Mass spectrometry data has been submitted to 'PRIDE Archive' (https://www.ebi.ac.uk/pride/archive/) repository with the project accession number **PXD015631**. The data files can be accessed with the username **reviewer86875@ebi.ac.uk** and password: **gCB24pty**.

Bioinformatics analyses of the secreted proteins

Protein sequences of the secreted proteins obtained from Liquid Chromatography-Mass Spectrometer (LC-MS) analysis of the *A. defectiva* secretome were analyzed by an *in silico* 2-dimensional electrophoresis (2-DE) tool. For this, a theoretical (2-DE) image was obtained by using the software JVirGel, version 2.0 (http://www.jvirgel.de/index.html) [22]. The software creates and visualizes virtual two-dimensional (2D) protein gels based on the migration behavior of proteins in dependence of their theoretical molecular weights in combination with their calculated isoelectric points.

The signal peptides, transmembrane topology and subcellular localization of the secreted proteins were determined by using SignalP version 5.0 (http://www.cbs.dtu.dk/services/SignalP/) [23], Phobius (http://phobius.sbc.su.se/) [24], and PSORTb version 3.0.2 (http://www.psort.org/psortb/) [25]. Interpretation of results was done based on a most-votes approach. SignalP was utilized to predict proteins secreted via the general *Sec*retion route (Sec-pathway) [26]. Proteins secreted via Twin-arginine translocation (Tat) pathway were predicted by TatP (http://www.cbs.dtu.dk/services/TatP/) [27] and TatFind (http://signalfind.org/tatfind.html) [28] tools. To identify proteins secreted via non-classical secretory system, SecretomeP 2.0 was used (http://www.cbs.dtu.dk/services/SecretomeP/) [29] and Proteins that were positive for signal peptides were not taken into account. To identify lipoproteins, lipoboxes were searched using the prediction tools LipoP (http://www.cbs.dtu.dk/services/LipoP/) and PRED-LIPO (http://bioinformatics.biol.uoa.gr/PRED-LIPO/input.jsp) [30]. Transmembrane alpha helices were predicted combining the tools TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) [31], SVMtm (http://ccb.imb.uq.edu.au/svmtm/) [32], and SOSUI (http://harrier.nagahama-i-bio.ac.jp/sosui/sosuiG/sosuigsubmit.html) [33]. MultitaskProtDB (http://wallace.uab.es/multitask) and Moonprot (http://www.moonlightingproteins.org/) were used for identifying 'moonlighting proteins' in the *A. defectiva* secretome [34]. Proteins identified as hypothetical by nr protein database (National Center for Biotechnology Information, USA) were further analysed by using NCBI Conserved Domains Database (CDD) [35].

Functional annotation of the secreted proteins were checked by assigning Gene Ontology (GO) IDs and protein names using 'WEGO' (http://wego.genomics.org.cn/) tool [36] and the Blast2GO program. KEGG Pathway Database (http://www.genome.jp/kegg/pathway.html) and KEGG Mapper (http://www.genome.jp/kegg/mapper.html) were used to

predict the proteins with enzymatic functions that are possibly linked to metabolic pathways. Potential virulence proteins were predicted using 'VirulentPred' software (http://203.92.44.117/virulent/) [37], and the Virulece Factor DataBase (VFDB) (http://www.mgc.ac.cn/VFs/) [38]. Further, the functional protein association networks were used to group the secreted proteins through STRING (https://string-db.org/) [39].

Stimulation of PBMCs with extracellular (ECPs) and whole cell proteins (WCPs)

PBMCs were isolated using Ficoll-Paque density gradient centrifugation method [40]. Isolated PBMCs were stimulated with different concentrations (10, 25, 50, and 100 µg/ml) of extracellular and whole-cell proteins of *A. defectiva* for 24 h. For this, 0.45 ml of PBMCs (10^6 mL^{-1}) in RPMI medium were added to 0.05 ml extracellular/whole cell proteins of *A. defectiva* in each well and incubated for 24 h at 37°C in 5% CO_2 in air for stimulation. Well containing PBS without protein samples and with PBMCs in RPMI medium was used as negative control.

ELISA based quantification of selected cytokines

Proinflammatory cytokines Human IL-8, IL-1β/1F2, CCL5/RANTES were quantified in extracellular and whole-cell protein stimulated PBMC samples using cytokine specific ELISA immunoassay kits (Quantikine® ELISA R&D systems). A cytokine specific monoclonal antibody pre-coated ELISA plate employing the quantitative sandwich enzyme immunosorbent assay (ELISA) technique was used. Standards, samples and controls were pipetted into the wells of ELISA plate and to allow the specific cytokine in the sample to be bound by the specific immobilized antibody on the bottom of the well, the plate was incubated at room temperature for 2 hours. Wells were washed with wash buffer to remove unbound substances (ImmunoWash™ 1575 microplate washer) and an enzyme-linked polyclonal antibody specific for cytokine of interest was added to the wells. Washing steps were followed to remove any unbound antibody-enzyme reagent and then a substrate solution was added to the wells. The color development was stopped by terminating the enzyme-substrate reaction using stop solution and the intensity of the color developed in proportion to the amount of bound cytokine of interest was measured in microplate reader (iMark™, Biorad).

Statistics

Data from cytokine quantification was tested for normal distribution by Skewness and Kurtosis values, Shapiro Wilkins *p* values and histograms. Student t-test was used to compare the means between the groups. SPSS vs 23 for Windows used for the analysis.

Results

Analysis of the secretome of A. defectiva

By subjecting the secretome of *A. defectiva* to analysis by LC-MS/MS and conducting a database search in NCBI-nr, a total of 84 proteins were identified (Figure 1a, Table S1). Theoretical mapping of these secretome proteins onto a 2DE map shown in Figure 1b indicated a molecular weight distribution ranging from 11.04 kDa to 105.08 kDa, with clustering based on predicted isoelectric points (pI). Most proteins were clustered within a pI range of 3.94 to 9.44. To ascertain that the identified proteins in the secretome preparation did not originate from bacterial cell lysis, a western blot analysis was performed, wherein Fts-Z protein was utilized as a cytoplasmic lysis marker. The results, as depicted in Figure 1c, clearly demonstrated the presence of Fts-Z protein in the whole-cell protein (WCP) and not in the secretome/extracellular protein (ECP). Additionally, bacterial viability was confirmed by plating a 24-hour broth culture during the experiment. The prediction tools, SignalIP and TatP were employed to get insights into the protein secretion pathways. The SignalIP predicted that 54 out of 84 secreted proteins possessed signal sequence, indicating the 'Sec' pathway for their secretion while TatP predicted three sequences with TatP signal sequences, suggesting alternative secretion mechanisms for those proteins. However, no lipoproteins were detected (Pred-Lipo, LipoP) in the secretome.

Using SecretomeP 2.0 as another prediction tool, initially, 59 sequences were predicted to utilize non-classical pathway for their secretion. However, upon further analysis, as 51 of them possessed signal sequences, suggesting secretion via Sec pathway, only eight proteins (9.5%) were finally considered to be utilizing non-classical pathway for their secretion. According to the PSORTb subcellular localization prediction tool analysis, *A.defectiva* secretome of 84 secreted proteins detected with LC-MS/MS was predicted to contain 20 cytoplasmic proteins (23.8%), 12 cytoplasmic membrane proteins (14.3%), 5 extracellular proteins (5.95%), 9 cell wall anchored proteins (10.71%), whereas the localization of 38 proteins (45.24%) could not be predicted.

For predicting the presence of transmembrane alpha helices TMHMM, SOUSIG and SVMtm tools were used. SOSUIG analyzed that out of total 84 proteins, 21 were membrane proteins (25%) and rest 63 were soluble proteins (75%). TMHMM tool analysis predicted transmembrane alpha helices in 18 proteins. Two of the 18 protein sequences that were predicted to have at least 2 transmembrane domains were considered as putatively membrane attached and therefore not included in

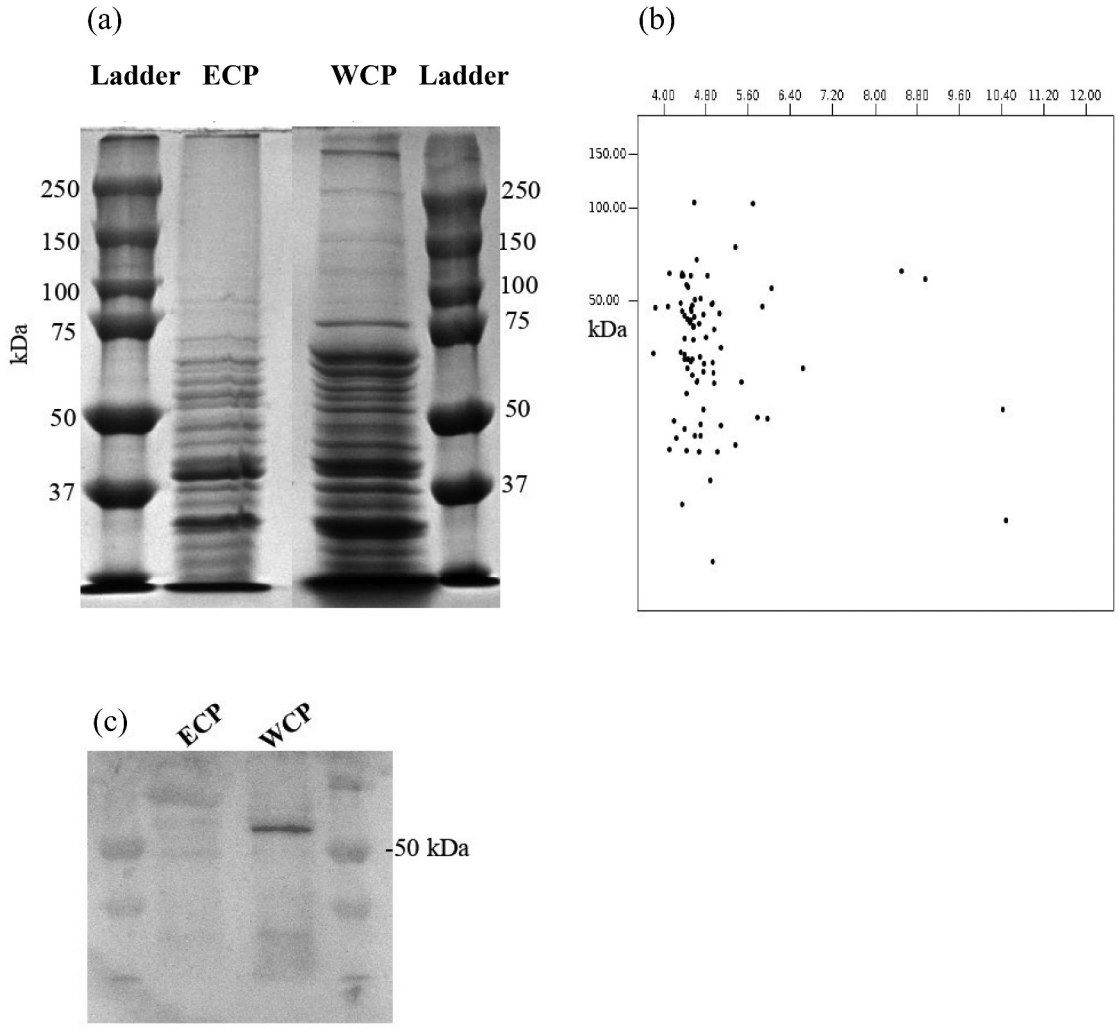

Figure 1. Analysis of the proteome of *A. defectiva* secretome. (a) SDS-PAGE gel showing protein bands from secretome/extracellular proteins (ECP) and whole cell protein preparations (WCP). (b) Protein sequences from LC-MS analysis of the secretome were analyzed by an *in silico* 2DE tool and (c) Western blot analysis showing the absence of the cytoplasmic marker protein FtsZ in the secretome and whole cell protein preparations.

further analyses. Multitask Prot DB tool for detecting moonlighting proteins identified 23 proteins with moonlighting functions in the secretome of *A. defectiva* (Table 1). Four hypothetical proteins (>gi|554771335|gb|ESK65778.1| hypothetical protein GCWU000182_00843 [*Abiotrophia defectiva* ATCC 49,176]; >gi|736332351|ref|WP_034358940.1| hypothetical protein [*Herbaspirillum rubrisubalbicans*]; >gi|949021501|gb|KRO30945.1| hypothetical protein ABR60_00340 [Actinobacteria bacterium BACL2 MAG-120802-bin41]; >gi|736332376|ref|WP_034358964.1| hypothetical protein [*Herbaspirillum rubrisubalbicans*]) were observed to have conserved domains.

Gene ontology analysis for predicting functional annotation of secreted proteins

The amino acid FASTA sequences of *A. defectiva* secretome were subjected to Gene Ontology (GO) analysis using Blast2GO and WEGO tools. For WEGO, the InterPro analysis XML file was utilized (Figure 2). Overall, 41 of the 84 sequences were assigned as annotated genes. The proteins were grouped based on GO terms: 31 in 'biological process', 15 in 'cellular component', and 31 in 'molecular function' groups.

KEGG pathway analysis for predicting enzymatic functions involved in metabolic pathways

All the detected protein sequences from the *A. defectiva* secretome were subjected to KEGG pathway annotation and analysis using KEGG mapper software tool (Figure 3). Total 28 of the 84 protein sequences were annotated and detected to be involved in various biological pathways. Proteins involved in environmental information processing were found to be predominant (11 proteins/39%) followed by carbohydrate metabolism (5 proteins/18%) and protein families involved in signaling and cellular processing (4 proteins/14%). Three proteins (11%) were found to be involved in genetic information processing while other two in Protein families: Genetic information processing (7%). Proteins categorized in energy metabolism, cellular processes and protein families: metabolism were found to be one each (3.6%).

Table 1. List of *A. defectiva* secretome proteins with a predicted moonlighting function.

S.no.	GI #	Name
1	gi|557369311	peptide ABC transporter substrate-binding protein
2	gi|554771431	ABC transporter, solute-binding protein
3	gi|557369709	maltodextrin-binding protein MdxE/extracellular solute-binding protein
4	gi|554770501	putative fumarate reductase flavoprotein subunit
5	gi|557369146	ABC transporter solute-binding protein
6	gi|557370056	peptidase S41/PDZ domain-containing protein
7	gi|557369427	D-methionine-binding lipoprotein MetQ
8	gi|557368903	type I glyceraldehyde-3-phosphate dehydrogenase
9	gi|557370233	ErfK/YbiS/YcfS/YnhG
10	gi|557368618	ABC transporter substrate-binding protein
11	gi|557369034	LysM domain-containing protein
12	gi|557369704	peptidase
13	gi|557370108	serine protease do-like protein/PDZ domain-containing protein
14	gi|554772095	LysM domain protein
15	gi|557368864	glycerol dehydrogenase
16	gi|488760879	Adenosyl homo cysteinase
17	gi|554770293	fructose-1,6-bisphosphate aldolase, class II
18	gi|554771016	LysM domain protein
19	gi|553304592	ATP-dependent Clp protease ATP-binding subunit ClpX
20	gi|554770463	peroxiredoxin, Ohr subfamily
21	gi|557370264	ABC transporter solute-binding protein
22	gi|557368899	enolase/MULTISPECIES: phosphopyruvate hydratase
23	gi|1027884671	ShlB/FhaC/HecB family hemolysin secretion/activation protein

Potential virulence proteins in A. defectiva *secretome*

A total of 20 proteins were predicted to be virulent in *A. defectiva* secretome based on *in silico* prediction using the online tools 'VirulentPred' and 'VFDB' (Virulence Factor DataBase). The results were further supported by the evidence from the literature (Table 2). Manual search for investigating the associations with virulence activities in other species helped in assessing the virulence potential of the *A. defectiva* secretome. The major proteins evidenced having virulence properties in other bacterial species were NlpC/P60 family protein, SEC10/PgrA surface exclusion domain-containing protein, DUF1002 domain-containing protein, ErfK/YbiS/YcfS/YnhG, Tat pathway signal sequence domain protein, ubiquitin-ribosomal protein fusion S27a, W×G100family type VII secretion target ubiquitin, peroxiredoxin, Ohr subfamily, signal peptide protein-YSIRK family, LysM domain protein, oligopeptide ABC transporter substrate-binding protein, PDZ

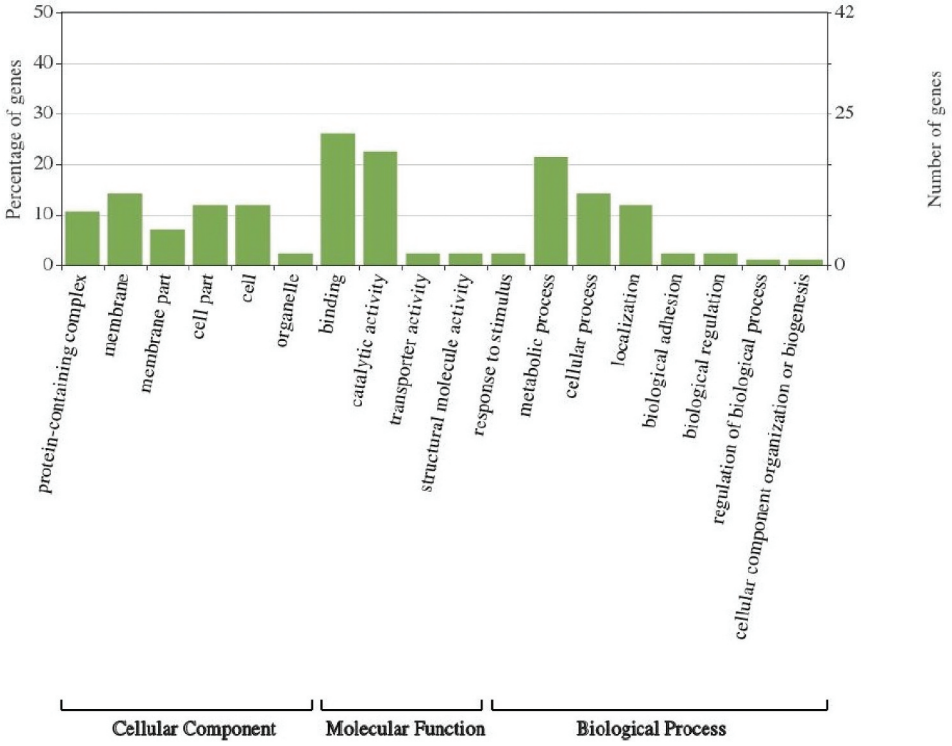

Figure 2. Gene ontology analysis of *A. defectiva* secreted proteins. Gene ontology annotation was achieved using Blast2GO and an online software 'WEGO'. Protein sequences were grouped into 3 categories based on their properties and functions.

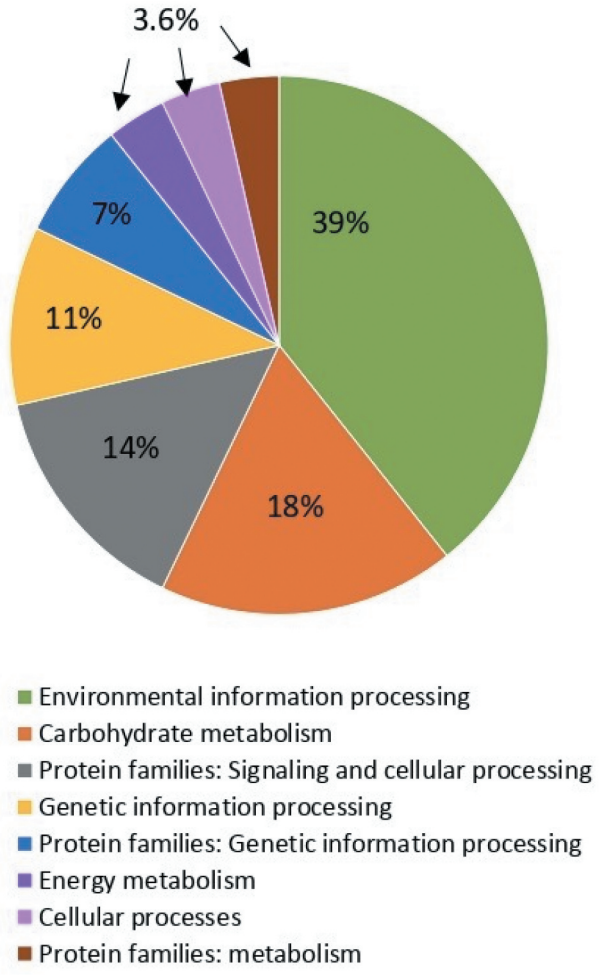

Figure 3. KEGG pathway analysis of the *A. defectiva* secretome. all protein sequences from the secretome were analyzed by KEGG pathway tool.

domain-containing protein, MULTISPECIES: 50S ribosomal protein L7/L12, D-alanyl-D-alanine carboxypeptidase, DNA starvation/stationary phase protection protein, and LysM peptidoglycan-binding domain-containing protein.

STRING for functional protein association network analysis

The functional protein association network analysis of *A. defectiva* (Figure 4) was demonstrated using tool STRING. As evident in Figure 4, secretome proteins formed three distinct protein groups based on molecular action, i.e, carbohydrate metabolism proteins, ribosomal proteins, and ATP synthase proteins. Proteins network involved in carbohydrate metabolism were Pyruvate kinase, ATP-dependent 6-phosphofructokinase, glucose-6-phosphate isomerase, Pyruvate, Enolase, Fructose-1,6-bisphosphate aldolase, 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, Triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase. The ribosomal protein group was mainly consisted of secreted 50S ribosomal proteins (L4, L5, L6, L7/12, L10, L11, L13, L14, L19 and L20). The third protein network of extracellular ATPases (or ATP synthases) involved in ATP synthesis and/or hydrolysis were ATP synthase subunits alpha, a, b, gamma chain and epsilon chain.

ELISA quantification of selected cytokines produced from stimulated human PBMCs with A. defectiva secretome (ECPs) and whole cell proteins (WCPs)

Various concentrations (10, 25, 50, and 100 µg/ml) of *A. defectiva* secretome triggered the production of proinflammatory cytokines IL-8, IL-1β and CCL 5 from PBMCs as compared to the controls (0 µg/ml). The

Table 2. Putative virulence factors identified in *A. defectiva* secretome.

S.No.	GI Number	Protein	*In silico* prediction	Literature evidence
1	gi|557370330|	NlpC/P60 family protein		[1,2]
2	gi|557369880|	SEC10/PgrA surface exclusion domain-containing protein		[41]
3	gi|557368815|	DUF1002 domain-containing protein		[42,43]
4	gi|557370233|	ErfK/YbiS/YcfS/YnhG		[44,45]
5	gi|554770642|	Tat pathway signal sequence domain protein		[46–48]
6	gi|68481104|	ubiquitin-ribosomal protein fusion S27a		[49,50]
7	gi|557369257|	WXG100 family type VII secretion target		[51–53]
8	gi|315447814|	ubiquitin		[54,55]
9	gi|554770463|	peroxiredoxin, Ohr subfamily		[56]
10	gi|554771681|	signal peptide protein, YSIRK family		[57,58]
11	gi|554772095|	LysM domain protein		[59]
12	gi|554771016|	LysM domain protein		[59]
13	gi|554771335|	hypothetical protein		[60]
14	gi|557368619|	oligopeptide ABC transporter substrate-binding protein		[61–63]
15	gi|557370108|	PDZ domain-containing protein		[64–66]
16	gi|557370394|	MULTISPECIES: 50S ribosomal protein L7/L12		[67,68]
17	gi|949021501|	hypothetical protein ABR60_00340		[69]
18	gi|557369052|	D-alanyl-D-alanine carboxypeptidase		[70,71]
19	gi|557369594|	DNA starvation/stationary phase protection protein		[72,73]
20	gi|557369034|	LysM peptidoglycan-binding domain-containing protein		[74]
21	gi|557369429|	MULTISPECIES: MetQ/NlpA family ABC transporter substrate-binding protein		[75,76]
22	gi|553304592|	ATP-dependent Clp protease ATP-binding subunit ClpX		[77]
23	gi|557368688|	MULTISPECIES: HU family DNA-binding protein		[78,79]
24	gi|505265440|	F0F1 ATP synthase subunit alpha		[80,81]
25	gi|557369704|	peptidase		[82–84]
26	gi|557370108|	PDZ domain-containing protein		
27	gi|557370264|	extracellular solute-binding protein		[85–87]

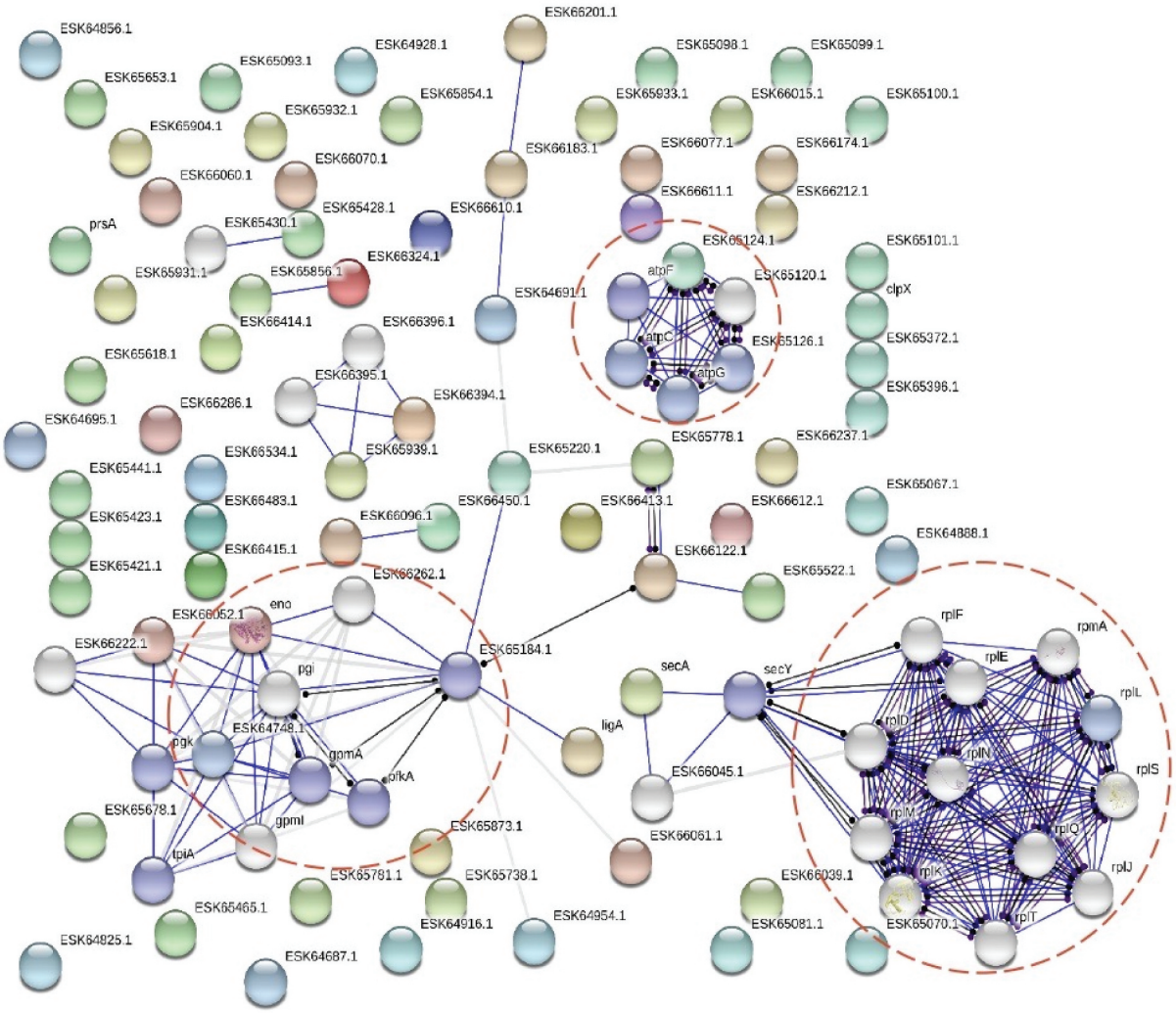

Figure 4. Functional protein association networks of *A. defectiva* secretome. The online tool STRING was used for grouping the secreted proteins on the basis of functional networks. Minimum interaction scores were set at a strong confidence level of 0.7. The three major network groups formed are shown in dotted circles. Seven different colored link a number of nodes and represent seven types of evidence used in predicting associations. A red line indicates the presence of fusion evidence; a green line represents neighborhood evidence; a blue line represents co-occurrence evidence; a purple line represents experimental evidence; a yellow line represents text mining evidence; a light blue line represents database evidence and a black line represents co-expression evidence.

secretome induced significantly higher levels of IL-1β production ($p < 0.05$) compared to WCPs of *A. defectiva* while IL-8 levels were significantly higher from the WCPs. In the case of CCL5, large amounts of upto 5000 pg/ml were also induced at 0 mg/ml protein concentration (Figure 5).

Discussion

Understanding the ability of bacteria to secrete extracellular proteins is crucial for comprehending their role in biofilm formation and pathogenesis, which aid the bacteria in developing defensive strategies against host and mount infection. Recent discoveries in protein secretome in several other oral bacteria have prompted us to consolidate the information on the secretome of *A. defectiva* [15,20,88,89]. The secretion of extracellular proteins is a fundamental aspect of bacterial physiology, with a remarkable clinical importance. Bacterial pathogens transport various virulence-associated proteins to the extracellular space, strategically deploying them in host locations to facilitate colonization and subsequent infection. In the case of oral bacteria, these secreted virulence proteins may promote bacterial proliferation at specific oral sites, potentially contributing to dysbiosis.

In the current investigation, a broad proteomics-based analysis of the A. defectiva secretome was performed and found a large number of putative virulence factors. We accomplished the characterization of most of the secretome proteins '*in silico*' by using various bioinformatics tools. Proteins belonging to several secretion systems, like the sec-dependent Tat pathway and a non-classical secretory system, were found to be part of the secretome. The possibility of contamination of the secretome with subcellular proteins was ruled out by using an established cytoplasmic marker protein, Fts-Z [90], which remained absent in all *A.*

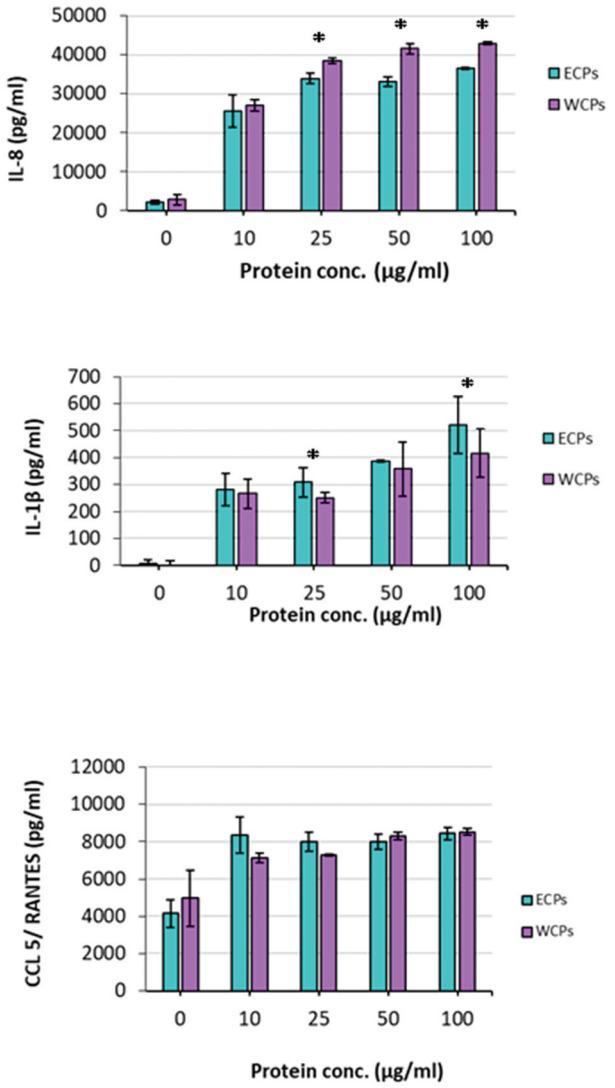

Figure 5. Cytokine induction from human PBMCs by *A. defectiva* secretome preparation.

Fractionated human PBMCs were stimulated by *A. defectiva* secretome (ECPs) and whole-cell preparation (WCPs) for 24 h (10, 25, 50, and 100 µg/ml). The cytokines produced were quantified using specific ELISA immunoassay kits (Quantikine® ELISA R&D systems). The data presented are mean (SD).

defectiva secretome preparations but was detected in whole-cell protein lysate.

We found more than 20 possible virulence-associated proteins in the A. defectiva secretome using in silico analysis. These results were backed up by experiments and published research. Virulence factors have been found in the secretomes of other oral bacteria that have been studied a lot, like *Aggregatibacter actinomycetemcomitans* [88], *Porphyromonas gingivalis* [89] and less-studied *G. adiacens* [20]. Several well-known virulence factors in other bacteria (such as NlpC/P60 family protein, SEC10/PgrA, ErfK, ubiquitin-ribosomal protein fusion S27a, peroxiredoxin, signal peptide protein-YSIRK family, LysM domain protein, 50S ribosomal protein L7/L12, DNA starvation/stationary phase protection protein, and LysM peptidoglycan-binding domain-containing protein) were also detected in the *A. defectiva* secretome. Such virulence factors are believed to enhance bacterial colonization, promote inflammation, disrupt host cellular homeostasis, and modulate host immune responses [91].

Several proteins in the *A. defectiva* secretome were categorized under the multifunctional group 'moonlighting proteins' [91,92], which have a known function inside the bacterial cell but also participate in different biological processes in the extracellular medium after their secretion. Because of their well-known function in bacterial pathogenicity, the moonlighting proteins in the secretome of *A. defectiva* are of great significance [93,94]. Secretion of 50S ribosomal proteins was noticed in the secretome of *A. defectiva*. Interestingly, ribosomal protein L7/L12 is highly antigenic and immunogenic [95,96] in other bacteria. Some of the glycolytic enzymes possess moonlighting properties, e.g. they function as adhesins [97], receptors for transferrin [98], neutrophil evasion proteins [99], immunomodulators [100] and participate in extracellular polysaccharide synthesis [101]. The versatile functionality of moonlighting proteins may facilitate the growth of *A. defectiva* in both its native habitats and sterile anatomical regions.

While predicting the functional annotation of secreted proteins, more than 75 A. *defectiva* secretome proteins were grouped into biological process, cellular component, and molecular function ontology groups.

Predicting enzymatic functions involved in metabolic pathways by KEGG, 28 of the total protein sequences were annotated and detected to be involved in various biological pathways. Proteins involved in environmental information processing were found to be predominant, followed by carbohydrate metabolism and protein families involved in signaling and cellular processing. Others were found to be involved in genetic information processing and energy metabolism. The secretome proteins are aligned with various KEGG pathways.

Several secretome proteins aligned with various KEGG pathways, notably the antibiotic biosynthesis and glycolysis pathways. The antibiotic biosynthesis pathway consisted of key enzymes, including hydratase, dehydrogenase, and aldolase, known for their essential roles in antibiotic biosynthesis [102,103]. Other significant pathways were cysteine & methionine metabolism, methane metabolism, and purine metabolism. These findings indicate that extracellular proteins of *A. defectiva* with metabolic functions might assist the bacterium in utilizing the available extracellular nutrients [104]. The functional protein association network analysis (STRING) of *A. defectiva* demonstrated three distinct protein groups based on molecular action, i.e. carbohydrate metabolism, ribosomal proteins, and ATP synthase proteins. The protein network involved in carbohydrate metabolism consisted of pyruvate kinase, phosphofructokinase, isomerases, pyruvate, enolase, aldolase,

phosphoglycerate mutase, and a dehydrogenase. Several glycolytic enzymes seemed to have overlapping functions, i.e. they were also involved in antibiotic biosynthesis, as predicted by KEGG. The ribosomal protein group mainly consisted of secreted 50S ribosomal proteins L4, L5, L6, etc. The third protein network of extracellular ATPases (or ATP synthases) involved in ATP synthesis and/or hydrolysis were the ATP synthase subunits alpha, gamma chain and epsilon chain. Living organisms generate ATP through respiration and subsequently utilize ATP to carry out cellular functions that are necessary for their survival, growth and replication. In addition to its intracellular roles in storing and supplying energy in metabolism and enzymatic reactions, ATP also has signaling functions. ATPases (or ATP synthases) are membrane-bound enzyme complexes or ion transporters that combine ATP synthesis and/or hydrolysis with the transport of protons across a membrane. In previous studies, *Escherichia coli* and *Salmonella* were shown to actively deplete the extracellular ATP and an ATP supplement in culture media, enhancing their own stationary survival. Recently, ATP was also shown to regulate virulence gene *mgtC* in *Salmonella* [105]. Extracellular ATP was also observed in a variety of other bacterial species including human pathogens such as *Acinetobacter*, *Pseudomonas*, *Klebsiella* and *Staphylococcus* [106]. Extracellular ATP interact with P2 receptors to modulate immune response by stimulating cell migration and cytokine secretion [104,107]. These findings suggest ATP as a more versatile molecule than a mere source of energy.

The secretome of *A. defectiva* was examined to assess its ability to stimulate cytokines and explore the immune response of the host. Stimulation of human PBMCs with various concentrations of *A. defectiva* secretome and whole-cell proteins triggered the production of proinflammatory cytokines IL-8, IL-1β, and CCL 5. Significant levels of IL-1β and IL-8 ($p < 0.05$) were induced by *A. defectiva* secretome. Previous studies have demonstrated that several bacterial species possess secreted components that can induce an inflammatory response in host cells [108–111]. In our previous study of cytokine induction in human PBMCs upon stimulation with biofilms and biofilm supernatants of *Granulicatella* and *Abiotrophia* spp., supernatants from *A. defectiva* biofilms were observed to be the strongest inducing among *Granulicatella elegans*, *Granulicatella adiacens*, and *A. defectiva* [112].

This study has certain limitations. We did not validate the proteomic *in silico* detection of proteins through laboratory methods. Additionally, the mass spectrometry approach we utilized offers only qualitative information about the proteins and obtaining quantitative data on the secreted proteins could provide more profound insights into their significance. The secretion of proteins into the extracellular space is influenced by numerous factors, such as growth conditions, including medium composition, and atmospheric conditions. In a prior investigation of the periodontitis-associated bacterium *A. actinomycetecomitans*, when specific conditions mimicking those encountered in the host were replicated, significant differences in protein secretion were observed in this species [113]. Likewise, exploring the secretome of *A. defectiva* under the *in vivo* conditions that the species may encounter would be of considerable interest.

In conclusion, elucidation of the secretome has revealed the role of various secreted proteins of *A. defectiva*, an oral bacterium well-documented in infective endocarditis and shown to be involved in oral infections. Putative virulence factors and several multifunctional 'moonlighting' proteins, which in other species are known to function as virulence factors, were detected [114,115]. Hence, the output of the present study is a foundation stone for investigating the role of secreted proteins of *A. defectiva* at deeper levels in oral infections as well as in infective endocarditis.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The raw data supporting the conclusions of this manuscript has been submitted to 'PRIDE Archive' (https://www.ebi.ac.uk/pride/archive/) repository with the project accession number **PXD015631**. The data files can be accessed with the username **reviewer86875@ebi.ac.uk** and password: **gCB24pty**.

Ethics statement

This study was carried out in accordance with the recommendations of the Ethical Committee of Health Sciences Center (HSC), Kuwait University. Written informed consent was obtained from the human volunteer and the consent was in accordance with the Declaration of Helsinki. The protocol was approved by the Ethical Committee of the HSC, Kuwait University.

Author contribution statement

MK: Conceived and designed the study, bioinformatics analyses

RGB, MEK, MK: Performed the laboratory experiments, bioinformatics analyses

MK, RGB, MEK: Wrote the manuscript

References

[1] Chowdhury S, German ML. Rare but not infrequent: infective endocarditis caused by Abiotrophia defectiva. Case Rep Infect Dis. 2018;2018:5186520. doi: 10.1155/2018/5186520

[2] Cerceo E, Christie JD, Nachamkin I, et al. Central nervous system infections due to Abiotrophia and granulicatella species: an emerging challenge? Diagn Microbiol Infect Dis. 2004;48(3):161–5. doi: 10.1016/j.diagmicrobio.2003.10.009

[3] Escarcega E, Trovato C, Idahosa O, et al. Abiotrophia defectiva Endocarditis: An Easy Miss. Clin Pract Cases Emerg Med. 2017;1(3):229–231. doi: 10.5811/cpcem.2017.3.33126

[4] Birlutiu V, Birlutiu RM. Endocarditis due to abiotrophia defectiva, a biofilm-related infection associated with the presence of fixed braces: a case report. Medicine (Baltimore). 2017;96(46):e8756. doi: 10.1097/MD.0000000000008756

[5] Rhodes HM, Hirigoyen D, Shabnam L, et al. Infective endocarditis due to abiotrophia defectiva and granulicatella spp. complicated by infectious intracranial cerebral aneurysms: a report of three cases and review of the literature. J Med Microbiol. 2016;65(6):493–9. doi: 10.1099/jmm.0.000260

[6] Lin CH, Hsu RB. Infective endocarditis caused by nutritionally variant streptococci. Am J Med Sci. 2007;334(4):235–9. doi: 10.1097/MAJ.0b013e3180a6eeab

[7] Nygren D, Alverbrandt M, Sunnerhagen T, et al. Aortitis caused by Abiotrophia defectiva: description of two cases. Infect Dis Rep. 2018;10(3):7746. doi: 10.4081/idr.2018.7746

[8] ElSalhy M, Soderling E, Honkala E, et al. Salivary microbiota and caries occurrence in Mutans Streptococci-positive school children. Eur J Paediatr Dent. 2016;17(3):188–92.

[9] Corby PM, Bretz WA, Hart TC, et al. Heritability of oral microbial species in caries-active and caries-free twins. Twin Res Hum Genet. 2007;10(6):821–8. doi: 10.1375/twin.10.6.821

[10] Rego RO, Oliveira CA, dos Santos-Pinto A, et al. Clinical and microbiological studies of children and adolescents receiving orthodontic treatment. Am J Dent. 2010;23(6):317–23.

[11] Birlutiu V, Birlutiu RM, Costache VS. Viridans streptococcal infective endocarditis associated with fixed orthodontic appliance managed surgically by mitral valve plasty: a case report. Medicine (Baltimore). 2018;97(27):e11260. doi: 10.1097/MD.0000000000011260

[12] Karched M, Bhardwaj RG, Inbamani A, et al. Quantitation of biofilm and planktonic life forms of coexisting periodontal species. Anaerobe. 2015;35(Pt A):13–20. doi: 10.1016/j.anaerobe.2015.04.013

[13] Hung WC, Tseng SP, Chen HJ, et al. Use of groESL as a target for identification of Abiotrophia, Granulicatella, and gemella species. J Clin Microbiol. 2010;48(10):3532–8. doi: 10.1128/JCM.00787-10

[14] Green ER, Mecsas J, Kudva IT. Bacterial secretion systems: an overview. Microbiol Spectr. 2016;4(1). doi: 10.1128/microbiolspec.VMBF-0012-2015

[15] Kanamoto T, Sato S, Nakashima H, et al. Proliferation of mitogen-stimulated human peripheral blood mononuclear cells is inhibited by extracellular arginine deiminase of Granulicatella elegans isolated from the human mouth. J Infect Chemother. 2007;13(5):353–355. doi: 10.1007/s10156-007-0546-3

[16] Abdullah SN, Farmer EA, Spargo L, et al. Porphyromonas gingivalis peptidylarginine deiminase substrate specificity. Anaerobe. 2013;23:102–8. doi: 10.1016/j.anaerobe.2013.07.001

[17] Patti JM, Hook M. Microbial adhesins recognizing extracellular matrix macromolecules. Curr Opin Cell Biol. 1994;6(5):752–8. doi: 10.1016/0955-0674(94)90104-X

[18] Okada Y, Kitada K, Takagaki M, et al. Inoue M. Endocardiac infectivity and binding to extracellular matrix proteins of oral abiotrophia species. FEMS Immunol Med Microbiol. 2000;27(3):257–61. doi: 10.1111/j.1574-695X.2000.tb01438.x

[19] Senn L, Entenza JM, Prod'hom G. Adherence of abiotrophia defectiva and granulicatella species to fibronectin: is there a link with endovascular infections? FEMS Immunol Med Microbiol. 2006;48(2):215–7. doi: 10.1111/j.1574-695X.2006.00142.x

[20] Karched M, Bhardwaj RG, Tiss A, et al. Proteomic analysis and virulence assessment of granulicatella adiacens secretome. Front Cell Infect Microbiol. 2019;9:104. doi: 10.3389/fcimb.2019.00104

[21] Karched M, Bhardwaj RG, Asikainen SE. Coaggregation and biofilm growth of granulicatella spp. With fusobacterium nucleatum and aggregatibacter actinomycetemcomitans. BMC Microbiol. 2015;15(1):114. doi: 10.1186/s12866-015-0439-z

[22] Hiller K, Schobert M, Hundertmark C, et al. JVirGel: calculation of virtual two-dimensional protein gels. Nucleic Acids Res. 2003;31(13):3862–3865. doi: 10.1093/nar/gkg536

[23] Bendtsen JD, Nielsen H, von Heijne G, et al. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol. 2004;340(4):783–95. doi: 10.1016/j.jmb.2004.05.028

[24] Kall L, Krogh A, Sonnhammer EL. Advantages of combined transmembrane topology and signal peptide prediction–the Phobius web server. Nucleic Acids Res. 2007;35(Web Server issue):W429–32. doi: 10.1093/nar/gkm256

[25] Yu NY, Wagner JR, Laird MR, et al. Psortb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics. 2010;26(13):1608–15. doi: 10.1093/bioinformatics/btq249

[26] Almagro Armenteros JJ, Tsirigos KD, Sonderby CK, et al. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nature Biotechnol. 2019;37(4):420–3. doi: 10.1038/s41587-019-0036-z

[27] Bendtsen JD, Nielsen H, Widdick D, et al. Prediction of twin-arginine signal peptides. BMC Bioinf. 2005;6(1):167. doi: 10.1186/1471-2105-6-167

[28] Rose RW, Bruser T, Kissinger JC, et al. Adaptation of protein secretion to extremely high-salt conditions by extensive use of the twin-arginine translocation pathway. Mol Microbiol. 2002;45(4):943–50. doi: 10.1046/j.1365-2958.2002.03090.x

[29] Bendtsen JD, Kiemer L, Fausboll A, et al. Non-classical protein secretion in bacteria. BMC Microbiol. 2005;5(1):58. doi: 10.1186/1471-2180-5-58

[30] Bagos PG, Tsirigos KD, Liakopoulos TD, et al. Prediction of lipoprotein signal peptides in Gram-positive bacteria with a hidden Markov Model. J Proteome Res. 2008;7(12):5082–93. doi: 10.1021/pr800162c

[31] Krogh A, Larsson B, von Heijne G, et al. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 2001;305(3):567–580. doi: 10.1006/jmbi.2000.4315

[32] Yuan Z, Mattick JS, Teasdale RD. SVMtm: support vector machines to predict transmembrane segments. J Comput Chem. 2004;25(5):632–6. doi: 10.1002/jcc.10411

[33] Hirokawa T, Boon-Chieng S, Mitaku S. SOSUI: classification and secondary structure prediction system for membrane proteins. Bioinformatics. 1998;14(4):378–9. doi: 10.1093/bioinformatics/14.4.378

[34] Hernandez S, Ferragut G, Amela I, et al. MultitaskProtDB: a database of multitasking proteins. Nucleic Acids Res. 2014;42(Database issue):D517–20. doi: 10.1093/nar/gkt1153

[35] Marchler-Bauer A, Bo Y, Han L, et al. CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. Nucleic Acids Res. 2017;45(D1):D200–D3. doi: 10.1093/nar/gkw1129

[36] Ye J, Zhang Y, Cui H, et al. WEGO 2.0: a web tool for analyzing and plotting GO annotations, 2018 update. Nucleic Acids Res. 2018;46(W1):W71–W75. doi: 10.1093/nar/gky400

[37] Garg A, Gupta D. VirulentPred: a SVM based prediction method for virulent proteins in bacterial pathogens. BMC Bioinf. 2008;9(1):62. doi: 10.1186/1471-2105-9-62

[38] Chen L, Zheng D, Liu B, et al. VFDB 2016: hierarchical and refined dataset for big data analysis–10 years on. Nucleic Acids Res. 2016;44(D1):D694–7. doi: 10.1093/nar/gkv1239

[39] von Mering C, Jensen LJ, Snel B, et al. STRING: known and predicted protein-protein associations, integrated and transferred across organisms. Nucleic Acids Res. 2005;33(Database issue):D433–7. doi: 10.1093/nar/gki005

[40] Fuss IJ, Kanof ME, Smith PD, et al. Isolation of whole mononuclear cells from peripheral blood and cord blood. Curr Protoc Immunol. 2009;Chapter 7:Unit7 1. doi: 10.1002/0471142735.im0701s85

[41] Kim YS, Yoon NK, Karisa N, et al. Identification of novel immunogenic proteins against Streptococcus parauberis in a zebrafish model by reverse vaccinology. Microb Pathog. 2019;127:56–9. doi: 10.1016/j.micpath.2018.11.053

[42] Manna S, Waring A, Papanicolaou A, et al. The transcriptomic response of streptococcus pneumoniae following exposure to cigarette smoke extract. Sci Rep. 2018;8(1):15716. doi: 10.1038/s41598-018-34103-5

[43] Noar RD, Daub ME. Transcriptome sequencing of mycosphaerella fijiensis during association with Musa acuminata reveals candidate pathogenicity genes. BMC Genomics. 2016;17(1):690. doi: 10.1186/s12864-016-3031-5

[44] Kim HS, Im HN, An DR, et al. The cell shape-determining Csd6 protein from Helicobacter pylori constitutes a new family of L,D-Carboxypeptidase. J Biol Chem. 2015;290(41):25103–17. doi: 10.1074/jbc.M115.658781

[45] Bohle LA, Riaz T, Egge-Jacobsen W, et al. Identification of surface proteins in Enterococcus faecalis V583. BMC Genomics. 2011;12:135. doi: 10.1186/1471-2164-12-135

[46] Pradel N, Ye C, Livrelli V, et al. Contribution of the twin arginine translocation system to the virulence of enterohemorrhagic Escherichia coli O157: H7. Infect Immun. 2003;71(9):4908–16. doi: 10.1128/IAI.71.9.4908-4916.2003

[47] Lavander M, Ericsson SK, Broms JE, et al. The twin arginine translocation system is essential for virulence of Yersinia pseudotuberculosis. Infect Immun. 2006;74(3):1768–1776. doi: 10.1128/IAI.74.3.1768-1776.2006

[48] Lammertyn E, Anne J. Protein secretion in Legionella pneumophila and its relation to virulence. FEMS Microbiol Lett. 2004;238(2):273–279. doi: 10.1111/j.1574-6968.2004.tb09767.x

[49] Steen BR, Zuyderduyn S, Toffaletti DL, et al. Cryptococcus neoformans gene expression during experimental cryptococcal meningitis. Eukaryot Cell. 2003;2(6):1336–49. doi: 10.1128/EC.2.6.1336-1349.2003

[50] Chen Q, Li Y, Wang J, et al. cpubi4 Is Essential for Development and Virulence in Chestnut Blight Fungus. Front Microbiol. 2018;9:1286. doi: 10.3389/fmicb.2018.01286

[51] Houben EN, Korotkov KV, Bitter W. Take five - type VII secretion systems of mycobacteria. Biochim Biophys Acta. 2014;1843(8):1707–1716. doi: 10.1016/j.bbamcr.2013.11.003

[52] Warne B, Harkins CP, Harris SR, et al. The Ess/type VII secretion system of staphylococcus aureus shows unexpected genetic diversity. BMC Genomics. 2016;17(1):222. doi: 10.1186/s12864-016-2426-7

[53] Bottai D, Groschel MI, Brosch R. Type VII secretion systems in gram-positive bacteria. Curr Top Microbiol Immunol. 2017;404:235–265.

[54] Li P, Jiang W, Yu Q, et al. Ubiquitination and degradation of GBPs by a Shigella effector to suppress host defence. Nature. 2017;551(7680):378–83. doi: 10.1038/nature24467

[55] Akhter Y, Thakur S. Targets of ubiquitin like system in mycobacteria and related actinobacterial species. Microbiol Res. 2017;204:9–29. doi: 10.1016/j.micres.2017.07.002

[56] Kaihami GH, Almeida JR, Santos SS, et al. Involvement of a 1-Cys peroxiredoxin in bacterial virulence. PLOS Pathog. 2014;10(10):e1004442. doi: 10.1371/journal.ppat.1004442

[57] Carrera M, Bohme K, Gallardo JM, et al. Characterization of foodborne strains of staphylococcus aureus by shotgun proteomics: functional networks, virulence factors and species-specific peptide biomarkers. Front Microbiol. 2017;8:2458. doi: 10.3389/fmicb.2017.02458

[58] Cavanagh JP, Pain M, Askarian F, et al. Comparative exoproteome profiling of an invasive and a commensal Staphylococcus haemolyticus isolate. J Proteomics. 2019;197:106–14. doi: 10.1016/j.jprot.2018.11.013

[59] Wu Z, Shao J, Ren H, et al. A Streptococcus suis LysM domain surface protein contributes to bacterial virulence. Vet Microbiol. 2016;187:64–9. doi: 10.1016/j.vetmic.2016.03.017

[60] Liang X, Chen YY, Ruiz T, et al. New cell surface protein involved in biofilm formation by

streptococcus parasanguinis. Infect Immun. 2011;79 (8):3239–3248. doi: 10.1128/IAI.00029-11

[61] Murphy TF, Brauer AL, Johnson A, et al. ATP-Binding cassette (ABC) transporters of the human respiratory tract pathogen, moraxella catarrhalis: role in virulence. PloS One. 2016;11(7):e0158689. doi: 10.1371/journal.pone.0158689

[62] Zhou B, Yang Y, Chen T, et al. The oligopeptide ABC transporter OppA4 negatively regulates the virulence factor OspC production of the lyme disease pathogen. Ticks Tick Borne Dis. 2018;9(5):1343–1349. doi: 10.1016/j.ttbdis.2018.06.006

[63] Maqbool A, Horler RS, Muller A, et al. The substrate-binding protein in bacterial ABC transporters: dissecting roles in the evolution of substrate specificity. Biochem Soc Trans. 2015;43(5):1011–1017. doi: 10.1042/BST20150135

[64] Deng CY, Deng AH, Sun ST, et al. The periplasmic PDZ domain-containing protein Prc modulates full virulence, envelops stress responses, and directly interacts with dipeptidyl peptidase of xanthomonas oryzae pv. oryzae. Mol Plant Microbe Interact. 2014;27(2):101–112. doi: 10.1094/MPMI-08-13-0234-R

[65] Lee C, Laimins LA. Role of the PDZ domain-binding motif of the oncoprotein E6 in the pathogenesis of human papillomavirus type 31. J Virol. 2004;78 (22):12366–77. doi: 10.1128/JVI.78.22.12366-12377.2004

[66] Tonikian R, Zhang Y, Sazinsky SL, et al. A specificity map for the PDZ domain family. PLoS Biol. 2008;6 (9):e239. doi: 10.1371/journal.pbio.0060239

[67] Zhang J, Ding SG, Zhong LJ, et al. Difference analysis on proteome of Helicobacter pylori in patients with peptic ulcer, gastritis, and gastric cancer. Zhonghua Yi Xue Za Zhi. 2006;86(38):2690–2694.

[68] Lanotte P, Perivier M, Haguenoer E, et al. Proteomic biomarkers associated with streptococcus agalactiae invasive genogroups. PloS One. 2013;8(1):e54393. doi: 10.1371/journal.pone.0054393

[69] Poonam Yennamalli RM, Bisht GS, Shrivastava R. Ribosomal maturation factor (RimP) is essential for survival of nontuberculous mycobacteria Mycobacterium fortuitum under in vitro acidic stress conditions. 3 Biotech. 2019;9(4):127. doi: 10.1007/s13205-019-1659-y

[70] Kikuchi H, Kim S, Watanabe K, et al. Brucella abortusd-alanyl-D-alanine carboxypeptidase contributes to its intracellular replication and resistance against nitric oxide. FEMS Microbiol Lett. 2006;259(1):120–5. doi: 10.1111/j.1574-6968.2006.00253.x

[71] Spidlova P, Senitkova I, Link M, et al. Identification of two substrates of FTS_1067 protein - an essential virulence factor of Francisella tularensis. Acta Microbiol Immunol Hung. 2017;64(1):37–49. doi: 10.1556/030.63.2016.013

[72] Ye Y, Li H, Ling N, et al. Identification of potential virulence factors of Cronobacter sakazakii isolates by comparative proteomic analysis. Int J Food Microbiol. 2016;217:182–8. doi: 10.1016/j.ijfoodmicro.2015.08.025

[73] Cho Y, Park S, Barate AK, et al. Proteomic analysis of outer membrane proteins in Salmonella enterica enteritidis. J Microbiol Biotechnol. 2015;25(2):288–95. doi: 10.4014/jmb.1410.10052

[74] Mesnage S, Dellarole M, Baxter NJ, et al. Molecular basis for bacterial peptidoglycan recognition by LysM domains. Nat Commun. 2014;5(1):4269. doi: 10.1038/ncomms5269

[75] Garmory HS, Titball RW. ATP-binding cassette transporters are targets for the development of antibacterial vaccines and therapies. Infect Immun. 2004;72(12):6757–63. doi: 10.1128/IAI.72.12.6757-6763.2004

[76] Reffuveille F, Leneveu C, Chevalier S, et al. Lipoproteins of Enterococcus faecalis: bioinformatic identification, expression analysis and relation to virulence. Microbiology. 2011;157(Pt 11):3001–3013. doi: 10.1099/mic.0.053314-0

[77] Cohn MT, Ingmer H, Mulholland F, et al. Contribution of conserved ATP-dependent proteases of Campylobacter jejuni to stress tolerance and virulence. Appl Environ Microbiol. 2007;73(24):7803–13. doi: 10.1128/AEM.00698-07

[78] Burnside K, Lembo A, de Los Reyes M, et al. Regulation of hemolysin expression and virulence of staphylococcus aureus by a serine/threonine kinase and phosphatase. PloS One. 2010;5(6):e11071. doi: 10.1371/journal.pone.0011071

[79] Rocco CJ, Bakaletz LO, Goodman SD, et al. Targeting the HUβ protein prevents porphyromonas gingivalis from entering into preexisting biofilms. J Bacteriol. 2018;200(11). doi: 10.1128/JB.00790-17

[80] Li SX, Wu HT, Liu YT, et al. The F1Fo-ATP synthase beta subunit is required for Candida albicans pathogenicity due to its role in carbon flexibility. Front Microbiol. 2018;9:1025. doi: 10.3389/fmicb.2018.01025

[81] Geddes JM, Croll D, Caza M, et al. Secretome profiling of Cryptococcus neoformans reveals regulation of a subset of virulence-associated proteins and potential biomarkers by protein kinase a. BMC Microbiol. 2015;15:206. doi: 10.1186/s12866-015-0532-3

[82] Frees D, Brondsted L, Ingmer H. Bacterial proteases and virulence. Subcell Biochem. 2013;66:161–192.

[83] Lynskey NN, Reglinski M, Calay D, et al. Multifunctional mechanisms of immune evasion by the streptococcal complement inhibitor C5a peptidase. PLOS Pathog. 2017;13(8):e1006493. doi: 10.1371/journal.ppat.1006493

[84] Clarke SC, Dumesic PA, Homer CM, et al. Integrated activity and genetic profiling of secreted peptidases in Cryptococcus neoformans reveals an aspartyl peptidase required for low pH survival and virulence. PLOS Pathog. 2016;12(12):e1006051. doi: 10.1371/journal.ppat.1006051

[85] Tai SS, Yu C, Lee JK. A solute binding protein of Streptococcus pneumoniae iron transport. FEMS Microbiol Lett. 2003;220(2):303–8. doi: 10.1016/S0378-1097(03)00135-6

[86] Wang Z, Bie P, Cheng J, et al. The ABC transporter YejABEF is required for resistance to antimicrobial peptides and the virulence of Brucella melitensis. Sci Rep. 2016;6(1):31876. doi: 10.1038/srep31876

[87] Moreno-Altamirano MM, Paredes-Gonzalez IS, Espitia C, et al. Bioinformatic identification of mycobacterium tuberculosis proteins likely to target host cell mitochondria: virulence factors? Microb Inform Exp. 2012;2(1):9. doi: 10.1186/2042-5783-2-9

[88] Zijnge V, Kieselbach T, Oscarsson J, et al. Proteomics of protein secretion by *aggregatibacter actinomycetemcomitans*. PloS One. 2012;7(7):e41662. doi: 10.1371/journal.pone.0041662

[89] Stobernack T, Glasner C, Junker S, et al. Extracellular proteome and citrullinome of the oral *pathogen porphyromonas gingivalis*. J Proteome Res. 2016;15(12):4532–43. doi: 10.1021/acs.jproteome.6b00634

[90] Terrasse R, Amoroso A, Vernet T, et al. *Streptococcus pneumoniae* GAPDH is released by cell lysis and interacts with Peptidoglycan. PloS One. 2015;10(4):e0125377. doi: 10.1371/journal.pone.0125377

[91] Jeffery CJ. Moonlighting proteins. Trends Biochem Sci. 1999;24(1):8–11. doi: 10.1016/S0968-0004(98)01335-8

[92] Henderson B, Martin AC. Protein moonlighting: a new factor in biology and medicine. Biochem Soc Trans. 2014;42(6):1671–1678. doi: 10.1042/BST20140273

[93] Henderson B, Martin A, Andrews-Polymenis HL. Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. Infect Immun. 2011;79(9):3476–91. doi: 10.1128/IAI.00179-11

[94] Wang G, Xia Y, Cui J, et al. The roles of moonlighting proteins in bacteria. Curr Issues Mol Biol. 2014;16:15–22.

[95] Ribeiro LA, Azevedo V, Le Loir Y, et al. Production and targeting of the *Brucella abortus* antigen L7/L12 in *Lactococcus lactis*: a first step towards food-grade live vaccines against brucellosis. Appl Environ Microbiol. 2002;68(2):910–6. doi: 10.1128/AEM.68.2.910-916.2002

[96] Oliveira SC, Splitter GA. Immunization of mice with recombinant L7/L12 ribosomal protein confers protection against *Brucella abortus* infection. Vaccine. 1996;14(10):959–962. doi: 10.1016/0264-410X(96)00018-7

[97] Tunio SA, Oldfield NJ, Berry A, et al. The moonlighting protein fructose-1, 6-bisphosphate aldolase of *Neisseria meningitidis*: surface localization and role in host cell adhesion. Mol Microbiol. 2010;76(3):605–615. doi: 10.1111/j.1365-2958.2010.07098.x

[98] Modun B, Morrissey J, Williams P. The staphylococcal transferrin receptor: a glycolytic enzyme with novel functions. Trends Microbiol. 2000;8(5):231–7. doi: 10.1016/S0966-842X(00)01728-5

[99] Terao Y, Yamaguchi M, Hamada S, et al. Multifunctional glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pyogenes* is essential for evasion from neutrophils. J Biol Chem. 2006;281(20):14215–23. doi: 10.1074/jbc.M513408200

[100] Madureira P, Baptista M, Vieira M, et al. *Streptococcus agalactiae* GAPDH is a virulence-associated immunomodulatory protein. J Immunol. 2007;178(3):1379–1387. doi: 10.4049/jimmunol.178.3.1379

[101] Lu GT, Xie JR, Chen L, et al. Glyceraldehyde-3-phosphate dehydrogenase of *xanthomonas campestris* pv. *campestris* is required for extracellular polysaccharide production and full virulence. Microbiology. 2009;155(Pt 5):1602–1612. doi: 10.1099/mic.0.023762-0

[102] Liu Y, Chen S, Zhang J, et al. Growth, microcystin-production and proteomic responses of *Microcystis aeruginosa* under long-term exposure to amoxicillin. Water Res. 2016;93:141–52. doi: 10.1016/j.watres.2016.01.060

[103] Barnard-Britson S, Chi X, Nonaka K, et al. Amalgamation of nucleosides and amino acids in antibiotic biosynthesis: discovery of an L-threonine: uridine-5'-aldehyde transaldolase. J Am Chem Soc. 2012;134(45):18514–18517. doi: 10.1021/ja308185q

[104] Rayah A, Kanellopoulos JM, Di Virgilio F. P2 receptors and immunity. Microbes Infect. 2012;14(14):1254–62. doi: 10.1016/j.micinf.2012.07.006

[105] Lee EJ, Groisman EA. Control of a Salmonella virulence locus by an ATP-sensing leader messenger RNA. Nature. 2012;486(7402):271–5. doi: 10.1038/nature11090

[106] Mempin R, Tran H, Chen C, et al. Release of extracellular ATP by bacteria during growth. BMC Microbiol. 2013;13(1):301. doi: 10.1186/1471-2180-13-301

[107] Coutinho-Silva R, Ojcius DM. Role of extracellular nucleotides in the immune response against intracellular bacteria and protozoan parasites. Microbes Infect. 2012;14(14):1271–7. doi: 10.1016/j.micinf.2012.05.009

[108] Dapunt U, Giese T, Stegmaier S, et al. The osteoblast as an inflammatory cell: production of cytokines in response to bacteria and components of bacterial biofilms. BMC Musculoskelet Disord. 2016;17(1):243. doi: 10.1186/s12891-016-1091-y

[109] Oscarsson J, Karched M, Thay B, et al. Proinflammatory effect in whole blood by free soluble bacterial components released from planktonic and biofilm cells. BMC Microbiol. 2008;8(1):206. doi: 10.1186/1471-2180-8-206

[110] Lee VT, Schneewind O. Protein secretion and the pathogenesis of bacterial infections. Genes Dev. 2001;15(14):1725–52. doi: 10.1101/gad.896801

[111] Finlay BB, Falkow S. Common themes in microbial pathogenicity revisited. Microbiol Mol Biol Rev. 1997;61(2):136–69. doi: 10.1128/mmbr.61.2.136-169.1997

[112] Bhardwaj RG, Al-Khabbaz A, Karched M. Cytokine induction of peripheral blood mononuclear cells by biofilms and biofilm supernatants of *Granulicatella and abiotrophia* spp. Microb Pathog. 2018;114:90–4. doi: 10.1016/j.micpath.2017.11.037

[113] Fletcher JM, Nair SP, Ward JM, et al. Analysis of the effect of changing environmental conditions on the expression patterns of exported surface-associated proteins of the oral pathogen Actinobacillus actinomycetemcomitans. Microb Pathog. 2001;30(6):359–68. doi: 10.1006/mpat.2000.0439

[114] Graf AC, Leonard A, Schauble M, et al. Virulence factors produced by staphylococcus aureus biofilms have a moonlighting function contributing to biofilm integrity. Mol & Cell Proteomics. 2019;18(6):1036–1053. doi: 10.1074/mcp.RA118.001120

[115] Pavkova I, Kopeckova M, Klimentova J, et al. The multiple localized glyceraldehyde-3-phosphate dehydrogenase contributes to the attenuation of the Francisella tularensis dsbA deletion mutant. Front Cell Infect Microbiol. 2017;7:503. doi: 10.3389/fcimb.2017.00503