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The effects of dietary *Bacillus cereus* QSI-1 on skin mucus proteins profile and immune response in Crucian Carp (*Carassius auratus gibelio*)

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ABSTRACT

The objective of this study was to investigate the effect of dietary quorum quenching bacterium *Bacillus cereus* QSI-1 on skin mucus protein pattern and innate immune response in Crucian Carp (*Carassius auratus gibelio*). The differential proteomes of skin mucus of Crucian Carp were analyzed after administration of *Bacillus cereus* QSI-1 by isobaric tags for relative and absolute quantitation (iTRAQ) labeling, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). A total of 1974 proteins were quantified. Using a 1.5-fold change in expression as a physiological significant benchmark, 264 differentially expressed proteins were reliably quantified by iTRAQ analysis, including 130 up- and 134 down-regulated proteins after dietary *Bacillus cereus* QSI-1. Some Proteins that were involved in immunity included protein S100, annexin, histone H3, lymphocyte cytosolic protein 1, heat shock protein, L-plastin, keratin 91, etc. Furthermore, fish fed 5×10^8 CFU/g *Bacillus cereus* QSI-1 supplemented diet showed an increase in alternative complement activity and lysozyme activity but expressed a decrease in superoxide dismutase activity in skin mucus ($P < 0.05$). However, administration of *Bacillus cereus* QSI-1 had no significant effects on total immunoglobulin level ($P > 0.05$). These results demonstrated that dietary administration of *Bacillus cereus* QSI-1 affects skin mucus protein profile and innate immune response in Crucian Carp, and also can enhance the disease resistance of Crucian Carp against *A. hydrophila*. This is the first report on proteomics analysis of skin mucus proteins in Crucian Carp after administration of quorum quenching bacterium *Bacillus cereus*, and the results will help to understand the mucosal immune responses to probiotics at the protein level in fish.

1. Introduction

Antibiotics play an important role in bacterial diseases control and growth promotion in animal husbandry. However, the abusive use of antibiotics and other antimicrobial agents lead to the development of drug-resistant bacteria [1]. Therefore, it is essential to find effective alternative approaches to the antibiotic. Probiotics are viable cell preparations that have beneficial effects on the health of the host by improving its intestinal balance via production of nutrients, enhancing immune responses and improving the water quality in aquaculture [2,3]. In recent years, probiotics have been increasingly used as a biological control in preventing diseases in aquaculture [3], which has made aquaculture products more acceptable to consumers.

Previous studies have shown that *Bacillus cereus* strain QSI-1 can decrease the pathogenicity of *Aeromonas hydrophila* YJ-1 in zebrafish (*Danio rerio*) and Goldfish (*Carassius auratus*) models [4,5]. We hypothesized that *Bacillus cereus* QSI-1 plays important roles in enhancing innate immune responses in fish. This hypothesis was examined by measuring skin mucus protein profiles and humoral immune response in Crucian Carp (*Carassius auratus gibelio*) in response to dietary supplementation with *Bacillus cereus* strain QSI-1.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strain used in this study was *Bacillus cereus* QSI-1 (the isolate was formally named as *Bacillus* sp. QSI-1 and was identified as *Bacillus cereus* and deposited at the China General Microbiological Culture Collection Center as CGMCC 15979) which can produce quorum quenching enzyme [4]. *Bacillus cereus* strain QSI-1 was cultured in Luria-Bertani (LB) broth medium at 37 °C, *A. hydrophila* YJ-1 was cultured in LB medium at 28 °C. For long-term storage, bacterial strains were preserved at −80 °C in LB containing 15% (v/v) glycerol.

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2.2. Experimental fish

Mixed sexes of healthy Crucian Carp (*Carassius auratus gibelio*) with no signs of disease, and having a mean body length 10.4 ± 0.45 cm and weight 15.2 ± 0.53 g were purchased from a local aquaculture farm at Nanjing, Jiangsu province, China. All the fishes were acclimatized for 14 days in 60 L tanks containing aerated fresh water at a temperature of 25 ± 2.0 °C, about 30% of the water in all aquariums was exchanged every day. The fishes were fed with 1.5% basal diet per body weight twice a day at 06:30 a.m. and 18:30 p.m. This study was approved by the China Pharmaceutical University Animal Care and Use Committee, and all animal experimental procedures including treatment, housing, husbandry, and slaughtering conditions were in accordance with the guidelines of the Instituted Animal Care and Use Committee of China Pharmaceutical University.

2.3. Diet preparation and experiment design

A basal pelleted diet comprising 25% soybean meal, 15% fish meal, 30% mustard oil cake, 20% rice bran, 5% corn starch, 3.5% sunflower oil and 1.5% vitamin and mineral mixture (every gram of vitamin-mineral mixture provided Vitamin A, 8000 IU; Vitamin B_2, 2.8 mg; Vitamin B_{12}, 5 μg; Vitamin D_3, 1500 IU; Vitamin E, 5 mg; Vitamin K_3, 5 mg; Vitamin PP, 12.5 mg; D Calcium antothenate, 5 mg; Copper Sulphate, 0.7 mg; Zinc Sulphate, 2.5 mg; Ferrous Sulphate, 6.2 mg; Potassium Iodide, 0.4 mg; Manganese Sulphate, 3.8 mg and Sorbitol, 20 mg) was prepared. Proximate analysis of the basal feed performed according to the AOAC (Association of Official Analytical Chemists) method [6] revealed 29.8% crude protein, 5.29% crude lipid and 13.1% ash. The basal diet was used as the control diet. For the preparation of *Bacillus cereus* QSI-1 containing diets, *Bacillus cereus* QSI-1 were grown in nutrient broth in a rotary shaker at 37 °C with 120 rpm. After incubation for up to 80% spore formation, the cells were harvested through centrifugation (5000 g) to obtain a pellet. The pellet was then re-suspended in saline solution and mixed with basal diet @ 5×10^8 CFU/g. To achieve accurate final concentrations in the diet, the bacterial suspension was slowly added to the dough with gradual mixing in the laminar airflow chamber under sterilized conditions. The resultant feed was oven-dried at 45 °C for 2–4 h and stored at −20 °C in sealed plastic Ziploc bags until used. To ensure high *Bacillus cereus* QSI-1 level in the supplemented feed, fresh diets were prepared on weekly basis. Two experimental treatments were carried out with randomly taken Crucian Carp in six plastic aquaria. Fish were fed with basal diet or *Bacillus cereus* QSI-1 diet and equal amount were provided for 15 days. The experiments were run in triplicates.

2.4. Sample collection

Six fish each from each group were anesthetized in MS-222 and killed by a blow to the head for sampling. Mucus collection was done from normal skin surface avoiding collection of blood along with mucus. Mucus was scraped off with sterile micro slides, transferred into an Eppendorf tube and were immediately frozen at −80 °C until used.

2.5. Skin mucus protein extraction, digestion, iTRAQ labeling and LC-MS/MS and bioinformatics analysis

Modified TCA/acetone method was used for the preparation of mucus proteins [7]. Briefly, skin mucus sample was added to a 4-fold volume of ice-cold acetone containing 0.1% dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF), and precipitated at −20 °C overnight, the supernatant was discarded following centrifugation at 15,000 rpm/min for 20 min at 4 °C. After repeating this step one more time, the pellets were then lyophilized and stored at −80 °C. 100 μg skin mucus proteins were re-suspended in 800 μL lysis buffer (7MUrea, 2MThiourea, 4% CHAPS, 65 mM DTT, 0.5% Bio-Rad Ampholyte and 1 mM PMSF). After mixing, the samples were sonicated with 200 W for 5 min and then centrifuged, the resulting supernatant protein samples were stored at −80 °C. The protein concentration was quantified by the Bradford method using a commercial protein assay kit (PTM, Hangzhou, China).

The iTRAQ assays were performed as described by Martin et al. [8]. Protocol-iTRAQ chemistry labeling reagents were obtained from Applied Biosystems. iTRAQ labeling was performed with 100 μg of protein samples according to the manufacturer's protocol, using an 8 × plex iTRAQ Multiplex Kit (Applied Biosystems). The labeled samples were purified using a strong cation exchange chromatography (SCX) column (Michrom Bioresources, Inc., CA, USA), and separated by liquid chromatography (LC) using an Agilent 1200 HPLC system (Agilent, Shanghai, China). Each peptide fraction was vacuum-dried and re-suspended in Nano-RPLC Buffer A and then was loaded onto a C18 trap column equipped with an Eksigent nanoLC-UltraTM 2D system (Dublin, CA). LC-ESI-MS/MS analysis was performed using a Triple TOF 5600 system (AB SCIEX) fitted with a Nanospray III source (AB SCIEX) using a pulled quartz tip as the emitter (New Objectives). Data were acquired using an ion spray voltage of 2.5 kV, a curtain gas at 30 psi, and a nebulizer gas at 15 psi. A series of survey scans were acquired in 250 ms to enable an information-dependent acquisition model. A total of 30 product ion scans were collected, which exceeded a threshold of 120 counts per second incorporating a charge-state of +2 to +5 for each cycle.

iTRAQ data from three biological replicates were analyzed by Protein Pilot Software v. 5.0 (SCIEX, USA), then protein identification was performed using the most recently updated fish UniProt protein database. To calculate the relative protein levels, proteins with a statistically significant label ratio of ≥1.5 ($p < 0.05$) were considered to be differentially expressed proteins.

Functional annotations of differentially accumulated protein species were performed using Gene Ontology (http://www.geneontology.org). The clusters of orthologous groups of proteins (COG) (http://www.ncbi.nlm.nih.gov/COG/) database was used to functionally classify differentially abundant protein species (DAPS), and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/or http://www.kegg.jp/) was used to predict the main metabolic pathways and biochemical signal transduction pathways that DAPS were involved in. A p-value≤0.05 was used as the threshold to determine the significant enrichments identified by the GO and KEGG pathways [9,10].

2.6. Immunological assays for immune parameters in skin mucus

Lysozyme activity, total immunoglobulin M levels, alternative complement pathway activity (C3) and superoxide dismutase (SOD) activity in Crucian Carp skin mucus were detected using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Youxuan BioEngineering, Shanghai, China) according to the manufacturer's instructions.

2.7. Assessment of the protective effect of Bacillus cereus QSI-1 on A. hydrophila infection

After the 10 day feeding experiment, Crucian Carp were tested intraperitoneally with 0.1 mL of *A. hydrophila* YJ-1 suspension containing 2.0×10^5 cells at the dose causing 50% mortality (LD50). The mortality in each group was recorded daily and dead fish were removed from the aquaria daily. The Protective effects were evaluated by recording the accumulated mortalities over 10 days.

2.8. Statistical analysis

Statistical analysis was performed using a One way ANOVA followed by Duncan's multiple-range test. The significant difference levels

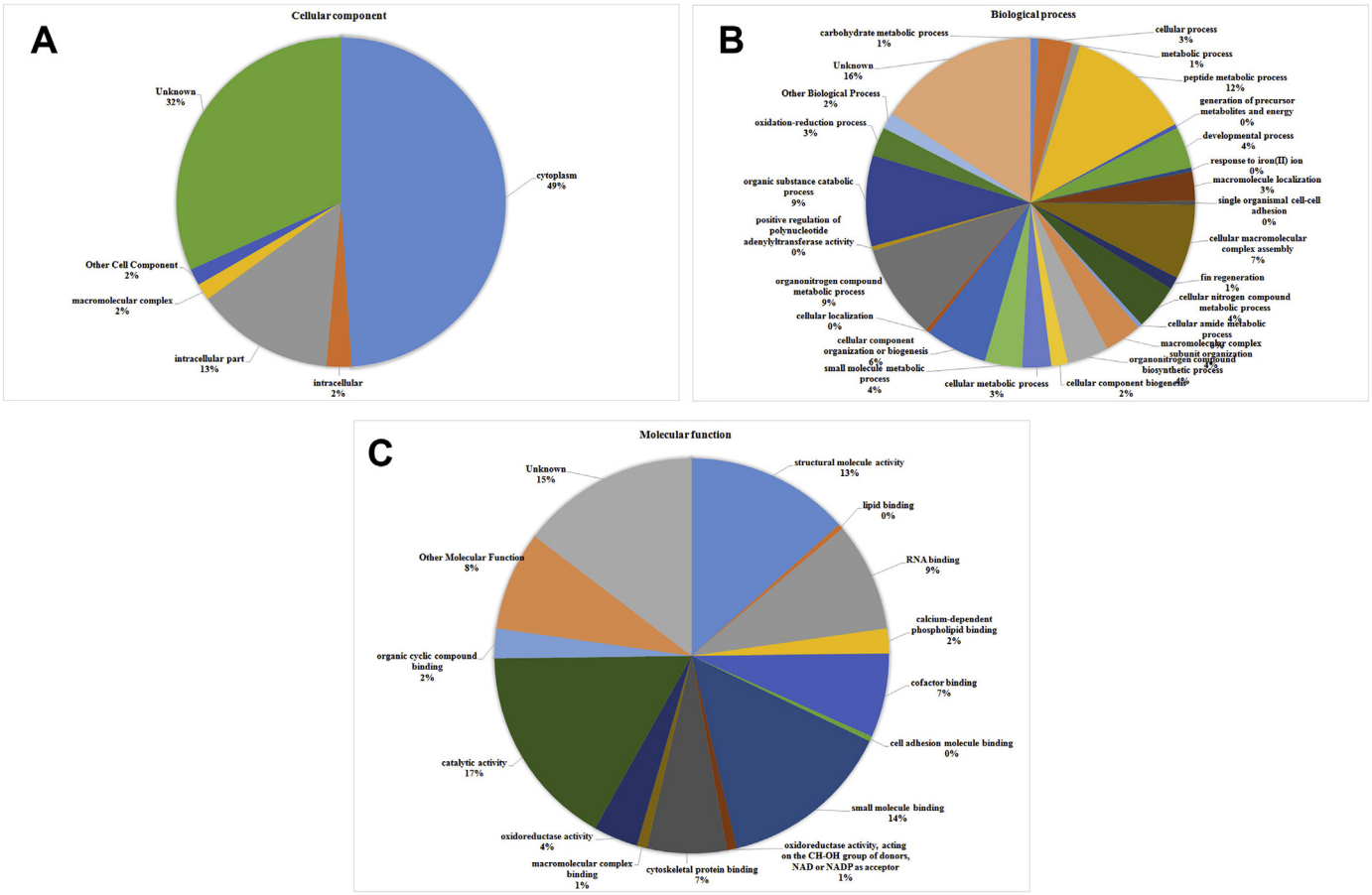

Fig. 1. Gene ontology (GO) analysis of differentially expressed proteins in mucus based on cellular component (A), biological process (B) and molecular function (C).

was considered when $P < 0.05$. The statistical analysis was performed by SPSS software 19 (SPSS, USA).

3. Results

3.1. Protein profiling and iTRAQ quantification

Compared with the control group, a total of 1974 distinct proteins were identified. The cellular component by GO analysis of the peptides is presented in Fig. 1A. Using GO analyses, the proteins/peptides can be categorized into several biological processes example cellular process, metabolic process, peptide metabolic process, oxidation-reduction process, etc with respect to the two regulatory stages, biological regulation and regulation of biological process (Fig. 1B). The major molecular functions of peptides obtained by GO analysis were binding (42%) and catalytic activity (17%), structural molecule activity (13%), oxidoreductase activity (4%) and others (Fig. 3C). Using a 1.5-fold increase or decrease in protein expression as a benchmark for physiologically significant change, 264 proteins were further quantified as showing differential expression by iTRAQ analysis, including 130 up-regulated proteins and 134 down-regulated proteins in skin mucus of Crucian Carp in response to *Bacillus cereus* QSI-1 administration.

The KEGG pathway analysis for the differential expressed proteins is shown in Table 1. The 264 identified proteins were sub-divided into 47 categories based on comparisons with the KEGG database http://www.genome.ad.jp/kegg/.These pathways included metabolic pathways, glycolysis/gluconeogenesis, ribosome, carbon metabolism, synthesis and degradation of ketone bodies, Biosynthesis of amino acids, Fructose and mannose metabolism, butanoate metabolism, pentose phosphate pathway, protein processing in endoplasmic reticulum, etc. The KEGG analysis revealed that most metabolic pathways, including those involved in protein processing in the endoplasmic reticulum, ribosome, synthesis and degradation of ketone bodies, NOD-like receptor signaling pathway, proteasome, peroxisome, adherens junction, and progesterone-mediated oocyte maturation, were significantly improved. The results suggesting these physiological processes are major priorities for common carp in response to *Bacillus cereus* QSI-1.

Among them, the proteins that were involved in skin immunity is shown in Table 2, which included protein S100, annexin, histone H3, lymphocyte cytosolic protein 1, heat shock protein 90, autoantigen La, heat shock protein 8, proteasome 26S subunit and vinculin, etc. All these immune-related proteins expression were up-regulated after *Bacillus cereus* QSI-1 administration.

3.2. Immunological assays

The effects of feeding Crucian Carp with *Bacillus cereus* QSI-1 on the immune parameters in carp skin mucus were observed (Fig. 2). The alternative complement pathway activity (C3), and lysozyme activities in the *Bacillus cereus* QSI-1 fed group increased significantly compared to normal diets group ($P < 0.05$) whereas the superoxide dismutase (SOD) activities in the *Bacillus cereus* QSI-1 fed group decreased significantly compared to normal diets group ($P < 0.05$). There was no differential changes in skin mucus between normal diets and *Bacillus cereus* QSI-1 groups.

3.3. Immune protection against A. hydrophila infection

The protective effect of the diet supplemented with *Bacillus cereus* QSI-1 on Crucian Carp against *A. hydrophila* infection was assessed. Cumulative mortality was calculated and was shown in Fig. 3. The results clearly indicate that *Bacillus cereus* QSI-1 plays a vital role in

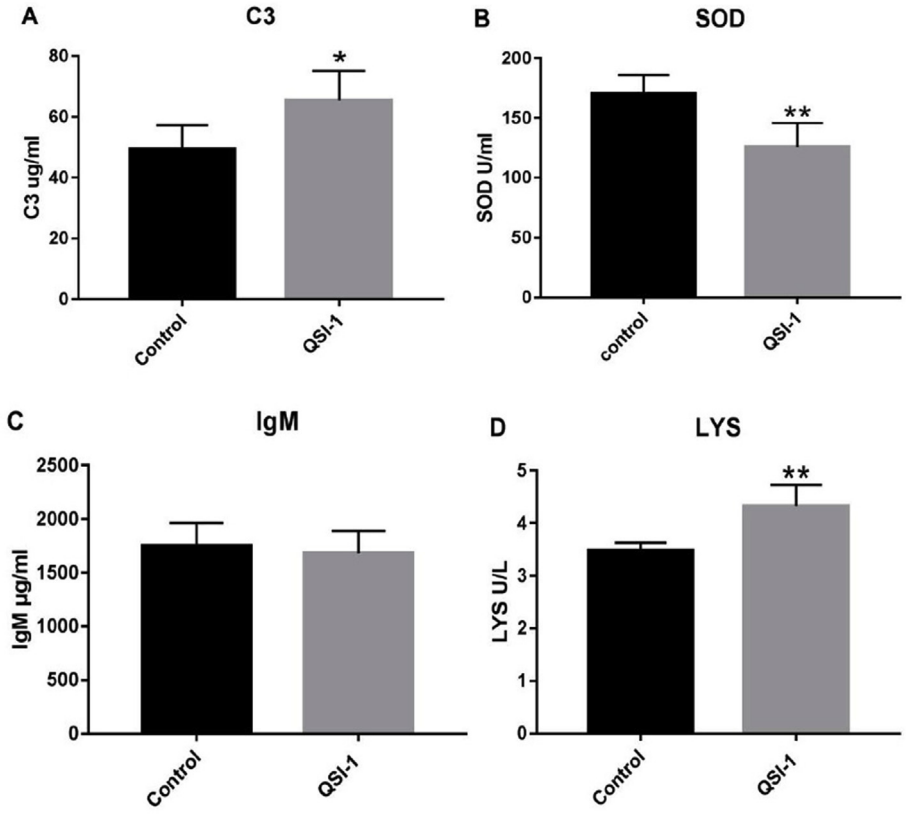

Fig. 2. Comparison of alternative complement activity (C3) (μg/ml) (A), superoxide dismutase (SOD) (U/ml) (B), total immunoglobulin (μg/ml) (C), and lysozyme activities (U/ml) (D) in skin mucus of Crucian Carp after 15 days with *Bacillus cereus* QSI-1 administration.

prevention of mortality of Crucian Carp by *A. hydrophila.* The cumulative mortality of *Bacillus cereus* QSI-1 group was only 16.7%, as compared to the control group i.e 46.7% 10 days after the *A. hydrophila* challenge.

4. Discussion

Fish skin mucus plays an important role in the host defense mechanism against infection through non-specified immune system and it is the first line of defense that prevents fish against infection [11,12]. The skin mucus contains many innate immune components such as complement, lysozyme, superoxide dismutase, secretory immunoglobulins, protease and lectins [13,14]. Probiotic bacteria, including *Bacillus cereus*, *Lactobacilli* sp., *Enterococcus* sp. and some yeast, are considered to have beneficial effects to the host. Probiotic bacteria are known to be immune stimulators with immuno-modulatory activities, and can stimulate both specific and innate immune systems in fish, and showed promising beneficial effects on serum and mucosal immunity [15]. The use of probiotic bacteria not only stimulate fish immune responses but also improves water quality, which acts as a means

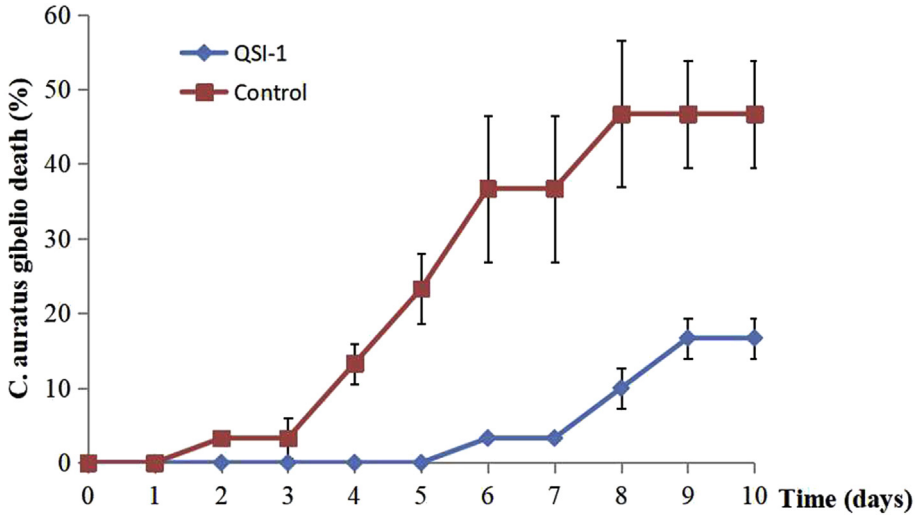

Fig. 3. Protective effect of *Bacillus cereus* QSI-1 on Crucian Carp after *A. hydrophila* YJ-1 challenged. Error bars represent standard SD of the mean values of results from three replicate experiments.

Table 1
Differentially expressed proteins involved in metabolic pathways in the skin mucus of *Crucian Carp* by using iTRAQ after *Bacillus cereus* QSI-1 administration.

Pathway Name	Accession	Protein Name	Peptides(95%)	%Cov	Fold Change
Glycolysis/Gluconeogenesis	W5LED4	L-lactate dehydrogenase	9	36.52	0.644
	W5LP80	Fructose-bisphosphate aldolase	20	51.10	0.643
	H2LIY2	Fructose-bisphosphate aldolase	12	43.68	0.634
	W5LJD0	Triosephosphate isomerase	13	57.89	0.629
	W5LB62	Glyceraldehyde-3-phosphate dehydrogenase	24	42.99	0.609
	W5KT62	Aldo-keto reductase family 1, member A1b (aldehyde reductase)	3	20.99	0.602
	W5LI76	Glyceraldehyde-3-phosphate dehydrogenase	11	39.64	0.284
Ribosome	H2L7U1	60S ribosomal protein L27	2	41.18	0.638
	W5KKN5	Ribosomal protein S18	6	49.40	0.595
	H2N0P8	60S ribosomal protein L18a	4	23.50	0.535
	W5L1E1	40S ribosomal protein S4	4	36.88	0.478
	H2MCW3	Ribosomal protein S25	2	31.97	0.325
	W5KFP8	40S ribosomal protein S8	4	22.12	2.520
	W5KRP9	40S ribosomal protein SA	23	42.95	1.575
Carbon metabolism	W5LP80	Fructose-bisphosphate aldolase	20	51.10	0.643
	H2LIY2	Fructose-bisphosphate aldolase	12	43.68	0.634
	W5LJD0	Triosephosphate isomerase	13	57.89	0.629
	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
	W5LI76	Glyceraldehyde-3-phosphate dehydrogenase	11	39.64	0.284
Synthesis and degradation of ketone bodies	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
	W5L150	6-phosphogluconate dehydrogenase, decarboxylating	8	29.61	1.845
Biosynthesis of amino acids	W5LP80	Fructose-bisphosphate aldolase	20	51.10	0.643
	H2LIY2	Fructose-bisphosphate aldolase	12	43.68	0.634
	W5LJD0	Triosephosphate isomerase	13	57.89	0.629
	W5LI76	Glyceraldehyde-3-phosphate dehydrogenase	11	39.64	0.284
Fructose and mannose metabolism	W5LP80	Fructose-bisphosphate aldolase	20	51.10	0.643
	H2LIY2	Fructose-bisphosphate aldolase	12	43.68	0.634
	W5LJD0	Triosephosphate isomerase	13	57.89	0.629
Butanoate metabolism	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
	W5LH87	3-hydroxymethyl-3-methylglutaryl-CoA lyase	2	21.36	1.845
Metabolic pathways	W5LED4	L-lactate dehydrogenase	9	36.52	0.644
	W5LP80	Fructose-bisphosphate aldolase	20	51.10	0.643
	H2LIY2	Fructose-bisphosphate aldolase	12	43.68	0.634
	W5LJD0	Triosephosphate isomerase	13	57.89	0.629
	W5LB62	Glyceraldehyde-3-phosphate dehydrogenase	24	42.99	0.609
	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
	W5KT62	Aldo-keto reductase family 1, member A1b (aldehyde reductase)	3	20.99	0.602
	H2MB41	GDP-mannose 4,6-dehydratase	5	47.35	0.529
	W5KW55	UDP-*N*-acetylglucosamine pyrophosphorylase 1	5	18.53	0.524
	W5LI76	Glyceraldehyde-3-phosphate dehydrogenase	11	39.64	0.284
	W5K4J9	4-aminobutyrate aminotransferase	1	13.97	0.224
	W5LH87	3-hydroxymethyl-3-methylglutaryl-CoA lyase	2	21.36	1.845
	H2LBW3	Lissencephaly-1 homolog	7	35.85	1.771
	W5K632	Adenylosuccinate synthetase isozyme 2	3	15.68	1.758
	W5LNV1	Glycine *C*-acetyltransferase	4	29.66	1.550
Pentose phosphate pathway	W5LP80	Fructose-bisphosphate aldolase	20	51.10	0.643
	H2LIY2	Fructose-bisphosphate aldolase	12	43.68	0.634
Propanoate metabolism	W5LED4	L-lactate dehydrogenase	9	36.52	0.644
	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
Pyruvate metabolism	W5LED4	L-lactate dehydrogenase	9	36.52	0.644
	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
Valine, leucine and isoleucine degradation	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
	W5LH87	3-hydroxymethyl-3-methylglutaryl-CoA lyase	2	21.36	1.845
Amino sugar and nucleotide sugar metabolism	H2MB41	GDP-mannose 4,6-dehydratase	5	47.35	0.529
	W5KW55	UDP-*N*-acetylglucosamine pyrophosphorylase 1	5	18.53	0.524
Protein processing in endoplasmic reticulum	W5K5X9	Sec23 homolog A, COPII coat complex component	4	19.35	0.635
	H2MXE1	Uncharacterized protein	2	16.31	3.105
	W5LMY2	Heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	21	31.82	2.317
Terpenoid backbone biosynthesis	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
Glycerophospholipid metabolism	H2L974	Glycerol-3-phosphate dehydrogenase [NAD(+)]	8	48.90	0.624
	W5K4J9	4-aminobutyrate aminotransferase	1	13.97	0.224
Pentose and glucuronate interconversions	W5KT62	Aldo-keto reductase family 1, member A1b (aldehyde reductase)	3	20.99	0.602
Porphyrin and chlorophyll metabolism	W5LNV1	Glycine *C*-acetyltransferase	4	29.66	1.550
Glyoxylate and dicarboxylate metabolism	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
Retinol metabolism	W5KH83	V-type proton ATPase subunit G	4	30.51	0.369
Alanine, aspartate and glutamate metabolism	W5K632	Adenylosuccinate synthetase isozyme 2	3	15.68	1.758
Starch and sucrose metabolism	H2MB41	GDP-mannose 4,6-dehydratase	5	47.35	0.529
Ether lipid metabolism	H2LBW3	Lissencephaly-1 homolog	7	35.85	1.771
Fatty acid degradation	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
Glycine, serine and threonine metabolism	W5LNV1	Glycine *C*-acetyltransferase	4	29.66	1.550
Tryptophan metabolism	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
NOD-like receptor signaling pathway	W5LMY2	Heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	21	31.82	2.317

(*continued on next page*)

Table 1 (*continued*)

Pathway Name	Accession	Protein Name	Peptides(95%)	%Cov	Fold Change
Cysteine and methionine metabolism	W5LED4	L-lactate dehydrogenase	9	36.52	0.644
Proteasome	W5KSX0	Proteasome 26S subunit, non-ATPase 11b	5	38.15	1.921
Fatty acid metabolism	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
Tight junction	W5KH07	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B	4	28.96	0.652
	H2LX46	Vinculin a	14	28.55	1.960
Glycerolipid metabolism	W5KT62	Aldo-keto reductase family 1, member A1b (aldehyde reductase)	3	20.99	0.602
Lysine degradation	W5KG73	Acetyl-CoA acetyltransferase 1	11	35.89	0.606
PPAR signaling pathway	W5K2L3	Apolipoprotein A-Ia	1	17.25	0.558
Ribosome biogenesis in eukaryotes	W5L7X6	SNU13 homolog, small nuclear ribonucleoprotein a (U4/U6.U5)	6	36.72	0.530
Inositol phosphate metabolism	W5LJD0	Triosephosphate isomerase	13	57.89	0.629
Peroxisome	W5LH87	3-hydroxymethyl-3-methylglutaryl-CoA lyase	2	21.36	1.845
mRNA surveillance pathway	W5KH07	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B	4	28.96	0.652
Adherens junction	H2LX46	Vinculin a OS	14	28.55	1.960
Progesterone-mediated oocyte maturation	W5LMY2	Heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	21	31.82	2.317
Oocyte meiosis	W5L258	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide b	16	73.36	1.929
Cell cycle	W5L258	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide b	16	73.36	1.929
RNA transport	W5K484	Eukaryotic translation initiation factor 3 subunit A	9	44.01	0.582
Insulin signaling pathway	H2M751	Flotillin 2a	8	33.59	0.642
Purine metabolism	W5K632	Adenylosuccinate synthetase isozyme 2	3	15.68	1.758
Adrenergic signaling in cardiomyocytes	W5KH07	Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B	4	28.96	0.652
Endocytosis	W5LM81	RAB4B, member RAS oncogene family	6	30.99	0.654

of increasing their survival rate [16].

Dietary administration of probiotics in fish culture offers an eco-friendly pathway instead of antibiotics in growth promotion and disease prevention. Dietary supplementation of probiotics can enhances both serum and mucosal immunity [17]. In this study, we used iTRAQ-based proteomics to investigate the skin mucus proteins profile and immune response of Crucian Carp after oral administration of quorum quenching bacterium *Bacillus cereus* QSI-1. Our results showed that multiple proteins in the skin mucus of Crucian Carp exhibited different expression levels between normal diets and *Bacillus cereus* QSI-1 group. Similar results were obtained from other studies in the case of administration of probiotics. Dietary administration of *L. casei*, *L. acidophilus* and *Bacillus* sp. significantly increased the content of protein in the skin mucus of *Poecilopsis gracilis*, black swordtail (*Xiphophorus helleri*), Atlantic Salmon (*Salmo salar*) and gold fish (*Carassius auratus gibelio*) [18–21].

Oral administration of *Bacillus cereus* QSI-1 also showed positive effects on innate immune response of Crucian Carp including serum and skin mucus immune. Among the differentially expressed proteins, some proteins were related to immunity of fish, such as protein S100, annexin, histone H3, lymphocyte cytosolic protein 1, heat shock protein 90, autoantigen La, heat shock protein 8, proteasome 26S subunit and vinculin, etc., and the alternative complement pathway activity (C3) and lysozyme activities were also increased after administration of *Bacillus cereus* QSI-1. Differentially expressed proteins especially immune response associated proteins, such as lysozyme, complement C3, natural killer cell enhancing factor and nonspecific cytotoxic cell receptor protein 1 were in the skin mucus of stressed and non-stressed gilthead seabream after probiotic *Shewanella putrefaciens* Pdp11 intake were detected by 2-DE of skin mucus followed by LC–MS/MS analysis [22]. Protein S 100 is a multigenic family of EF-hand calcium (Ca(2+))-binding proteins that can bind Zn^{2+}, Mn^{2+}, and other transition metals, and play key roles in the innate immune response to pathogens [23]. Here we found that after *Bacillus cereus* QSI-1 administration, the level of protein S100 increased 37.9808 fold compared to control diet. Kuo et al. [24] showed the mRNA expression of S100a9 (S100 calcium binding protein A9) was up-regulated in response to synbiotic *Bifidobacterium animalis* Subspecies *lactis* and inulin supplemented diets in

Table 2
Immune-related proteins identified by peptide sequence analysis in the skin mucus of *Crucian Carp* by using iTRAQ after *Bacillus cereus* QSI-1 administration.

Accession	Name	Peptides(95%)	%Cov	Fold Change
W5LAN5	Protein S100	3	26.09	37.9808
O93446	Annexin	3	11.57	5.9080
W5KFV0	Heterogeneous nuclear ribonucleoprotein A1b	2	22.22	5.2011
W5KKI5	DDRGK domain containing 1	1	14.57	4.0174
W5KAB9	Annexin	5	17.60	3.3807
H2M8F5	Histone H3	8	48.94	2.7564
W5K5G3	Family with sequence similarity 49, member Bb	6	32.31	2.7432
H2LQN3	Annexin	15	25.92	2.7258
W5L056	Lymphocyte cytosolic protein 1 (L-plastin)	27	58.20	2.7145
W5LMY2	Heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	21	31.82	2.3169
W5K0S8	Keratin 91	58	57.55	2.1751
W5KZE6	Sjogren syndrome antigen B (autoantigen La)	1	19.75	2.0672
W5LMZ9	Annexin	6	47.66	1.6893
O93445	Annexin	4	31.86	1.6813
H2LY52	Annexin	7	38.65	1.6295
W5KA74	Heat shock protein 8	35	54.62	1.6258
W5KSX0	Proteasome 26S subunit, non-ATPase 11b	5	38.15	1.921
H2LX46	Vinculin a OS	14	28.55	1.960
W5LH87	3-hydroxymethyl-3-methylglutaryl-CoA lyase	2	21.36	1.845

mice. Annexins are glucocorticoid-inducible anti-inflammatory proteins, they have multiple functions, such as modulation of reactive oxygen species (ROS) and regulation of inflammatory response [25,26]. The results of our study revealed that an increase annexins in carp skin mucus after *Bacillus cereus* QSI-1 administration. The complement system and lysozyme are crucial components in targeting and lysing pathogens, our study showed oral administration of *Bacillus cereus* QSI-1 can increased C3 and lysozyme level in skin mucus. Similarly, enhanced activities of complement and lysozyme level have been demonstrated in different fish with different probiotics. Pourgholam et al. [27] reported that dietary supplementation of *L. plantarum* can improve alternative complement activity (ACH50) and lysozyme activity of Siberian sturgeon juvenile. Lysozyme activity increased in tilapia *Oreochromis mossambicus* that fed a probiotic *B. licheniformis* Dahb1 [28]. A commercial probiotic BS containing *B. subtilis* and *B. licheniformis* can increase *C*-lysozyme, heat shock protein 70, β-defensin, transforming growth factor beta level and can enhance the immune response and provoke significant up-regulation of immune-related genes of tilapia (*Oreochromis niloticus*) [29]. Cerezuela et al. [30] reported that dietary supplements with probiotic *Bacillus cereus* in gilthead seabream (*Sparus aurata* L.), the enzymatic activities, IgM level in skin mucus were significantly higher and immune-related genes were also up-regulated.

In conclusion, the results of this present work indicated that dietary administration of quorum quenching bacterium *Bacillus cereus* QSI-1 can alter skin mucus protein profile and up-regulate immune-associated proteins. Furthermore, the administration of *Bacillus cereus* QSI-1 also has positive effects on innate immune response in Crucian Carp. The administration of quorum quenching bacterium could be considered as an alternative pathway for control of bacterial infection instead of antibiotics in aquaculture. Moreover, there is not enough information about the mechanisms of probiotics, so further research is still needed to clarify its relationship with fish health and how they fight against a bacterial pathogen.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.04.014.

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