**UNIVERSIDADE FEDERAL DE MINAS GERAIS**
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# INSIGHTS INTO THE DIVERSITY, ADAPTATION AND VIRULENCE OF FISH-PATHOGENIC *Streptococcus agalactiae* STRAINS THROUGH TRANSCRIPTOMIC AND PROTEOMIC ANALYSIS

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**Belo Horizonte**
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**Thesis presented to Veterinary School of the Federal University of Minas Gerais as partial fulfillment of the requirements for the degree of Doctor in Animal Science.**

**Area of concentration: Preventive Veterinary Medicine**

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Louis Pasteur

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## SUMMARY

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| µm | Micrometer |
| 2D | Two-dimensional Gel Electrophoresis |
| AQUACEN | National Reference Laboratory for Aquatic Animal Diseases |
| AQUAVET | Laboratory of Aquatic Animal Diseases |
| ATP | Adenosine triphosphate |
| BHI | Brain Heart Infusion agar |
| BHIT | Brain Heart Infusion agar supplemented with 0.05 % Tween 80 |
| BIGSdb | Bacterial Isolate Genome Sequence Database |
| BURST | Based Upon Related Sequences |
| CC | Clonal complex |
| cDNA | Complementary Deoxyribonucleic Acid |
| CDS | Coding Sequences |
| CFU | Colony-forming Unit |
| CHAPS | 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate |
| COG | Cluster of Orthologous Genes |
| Cy3 | 1,1′-bis(3-hydroxypropyl)-3,3,3′,3′-tetramethylindocarbocyanine |
| DEG | Differentially Expressed Genes |
| DEP | Differentially Expressed Proteins |
| DIA | Data-independent Acquisition |
| DNA | Deoxyribonucleic Acid |
| DTT | Dithiothreitol |
| eV | Eletron-volt |
| FAO | Food and Agriculture Organization of the United Nations |
| FDR | False Discovery Rate |
| fg | Femtogram |
| FWHM | Full Width at Half Maximum |
| g | Gram |
| GBS | Group B *Streptococcus* |
| h | Hour |
| IBGE | Brazilian Institute of Geography and Statistics |
| kDa | Kilodalton |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| kg | Kilogram |
| LAMP | Loop-mediated Isothermal Amplification |
| LC-HDMS$^E$ | Liquid Chromatography- High Definition Mass Spectrometry |
| LC-MS | Liquid Chromatopgraphy-tandem Mass Spectrometry |
| LC-UDMS$^E$ | Liquid Chromatography-Ultra Definition Mass Spectrometry |
| LD | Lethal Dose |
| MALDI | Matrix-assisted Laser Desorption Ionization |
| mg | Milligram |
| MIC | Minimum inhibitory concentration |
| mL | Milliliter |
| MLST | Multilocus Sequence Typing |
| mM | Millimolar |
| mRNA | Messenger Ribonucleic Acid |
| ng | Nanogram |
| NGS | Next-generation Sequencing |
| NT | Non-typeable |

| | |
|---|---|
| º C | Celsius degrees |
| OD | Optical Density |
| PCA | Principal Component Analysis |
| PCR | Polymerase Chain Reaction |
| PFGE | Pulsed-field Gel Electrophoresis |
| pg | Picogram |
| pH | Potential of Hydrogen |
| PLGS | ProteinLynx Global Server |
| pmol | Picomolar |
| PPC | Predicted Protein Cluster |
| PPI | Protein-protein Interaction |
| ppm | Parts per Million |
| PSE | Potentially Surface-exposed |
| PTS | Phosphoenolpyruvate-dependent Phosphotransferase System |
| QIP | Progenesis QI for Proteomics |
| qPCR | Real-time Quantitative Polymerase Chain Reaction |
| qRT-PCR | Real-time Quantitative Reverse Transcription Polymerase Chain Reaction |
| RIN | Ribonucleic acid Integrity Number |
| RNA | Ribonucleic Acid |
| RNA-seq | Ribonucleic acid sequencing |
| rRNA | Ribosomal Ribonucleic Acid |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| SCOTS | Selective Capture of Transcribed Sequences |
| SDC | Sodium Deoxycholate |
| ST | Sequence Type |
| TCS | Two-component Regulatory System |
| TFA | Trifluoroacetic Acid |
| THA | Todd-Hewitt agar |
| TIC | Total Ion Account |
| TOF | Time of Flight |
| tRNA | Transfer Ribonucleic Acid |
| TSA | Tryptic Soy agar |
| UFMG | Federal University of Minas Gerais |
| UPLC | Ultra-Performance Liquid Chromatography |
| wgMLST | Whole Genome Multilocus Sequence Typing |
| WGS | Whole Genome Sequencing |
| xg | Number of Times the Gravitational Force |

## RESUMO

A infecção por *Streptococcus agalactiae* (GBS) é uma das principais doenças detectadas na piscicultura mundial. Essa bactéria é o principal patógeno de tilápias, uma commodity global do setor aquícola, causando surtos de septicemia e meningoencefalite. Os isolados brasileiros de GBS oriundos de peixes possuem diferentes genótipos quando avaliados pela técnica de MLST, predominando os ST-260, ST-927 e as linhagens não tipáveis. Considerando o relacionamento evolutivo entre esses genótipos e por eles representarem as principais linhagens que infectam peixes no país, se torna necessário entender as características de metabolismo, adaptação e patogenicidade destes grupos genéticos e o seu relacionamento com o hospedeiro aquático. Para avaliar a expressão de transcritos e proteínas dos isolados de GBS, dois experimentos foram conduzidos. O primeiro experimento avaliou o pan-proteoma destes mesmos genótipos, comparou a expressão diferencial de proteínas entre os isolados obtidos de peixes e ser humano usando LC-UDMS$^{E}$ e identificou *in silico* proteínas antigênicas conservadas que poderiam ser usadas como possíveis alvos para a produção de vacinas. Enquanto que o segundo experimento objetivou avaliar o transcriptoma e o proteoma de uma linhagem de GBS obtido de peixe no Brasil, sendo o experimento conduzido em diferentes temperaturas de cultivo e analisados usando as técnicas de microarranjos e cromatografia líquida associada com espectrometria de massa (LC-HDMS$^{E}$). No primeiro experimento, um total de 1065 proteínas corresponderam ao pan-proteoma dos isolados de GBS oriundos de peixes, sendo 989 identificadas em todos os isolados simultaneamente (core proteoma), 62 compartilhadas em pelo menos 2 isolados (proteoma acessório) e 14 exclusivamente expressas entre cada amostra. O alto grau de conservação de proteínas entre os isolados avaliados com diferentes STs sugerem que utilização de vacinas monovalentes podem ser efetivas contra as diferentes variantes genéticas circulantes no país. Nós observamos que as proteínas identificadas no pan-proteoma refletem na habilidade adaptativa dos isolados de peixes em responder aos fatores estressantes impostos pelo ambiente aquático e que permitem a sobrevivência e multiplicação bacteriana em peixes. Um total de 215 e 269 proteínas foram up- e down-reguladas, respectivamente, em isolados obtidos de peixes em comparação ao isolado de ser humano. Por fim, independente da similaridade do conteúdo de proteínas, a expressão global de proteínas entre os isolados de GBS obtidos de peixes e ser humano foram diferentes, sugerindo uma distinta adaptação em mamíferos e hospedeiros aquáticos na regulação do proteoma. No segundo experimento, a análise de transcriptoma detectou um total de 107 genes diferencialmente expressos no isolado SA53 a 32 ºC quando comparado com 22 ºC. Enquanto que na análise de proteoma foram detectadas 81 proteínas diferencialmente expressas. Os resultados demonstraram que a temperatura é capaz de regular a expressão diferencial de genes e proteínas, principalmente daquelas envolvidas com a expressão de fatores de virulência, metabolismo, adaptação e resistência ao estresse térmico. Em conjunto os dois experimentos providenciaram novos conhecimentos sobre diversidade, adaptação e virulência de isolados patogênicos de GBS obtidos de peixes através das análises de transcriptoma e proteoma.



Palavras-chave: estreptococose, transcritos, proteínas, peixe, ser humano, expressão diferencial

## ABSTRACT

*Streptococcus agalactiae* (GBS) infection is one the main diseases diagnosed in worldwide fish farming. This bacterium is a major pathogen for the Nile tilapia, a global commodity of the aquaculture sector, causing outbreaks of septicemia and meningoencephalitis. The Brazilian GBS fish strains have distinct known genotypes, when evaluated by the MLST technique, predominantly the ST-260, ST-927 and the non-typeable lineage. Considering the evolutionary relationship between these genotypes and for representing the major lineages that infects fish in Brazil, it becomes necessary to understand the specific characteristics of metabolism, adaptation and pathogenicity of these genetic groups as well as their relationships with the aquatic host. In order to evaluate the transcript and protein expression on GBS strains, two experiment trials were performed. The first trial evaluated the pan-proteome of these same genotypes, compared the differential expression of proteins identified between isolates from fish and human using a LC-UDMS$^{E}$, and identified *in silico* conserved antigenic proteins that can be used as target in vaccine design. The second trial aimed to evaluate the transcriptome and proteome of a Brazilian fish-adapted GBS strain, being cultured *in vitro* under different temperatures and analyzed using microarray and liquid chromatography-mass spectrometry label-free shotgun (LC-HDMS$^{E}$) approaches. In the first trial, a total of 1,065 protein clusters corresponded to pan-proteome of GBS fish strains, being 989 identified in all GBS fish strains (core proteome), 62 shared by at least two strains (accessory proteome) and 14 were exclusively expressed to each strain. The high degree of conservation among strains with different STs suggests that monovalent vaccines may be effective against different genetic variants. We also observed that the identified proteins in pan-proteome reflect the adaptive ability of the GBS fish strains in the response to stress factors imposed by the aquatic environment allowing the bacterial survival and multiplication in the fish host. A total of 215 and 269 proteins from GBS fish strains were up- and down-regulated, respectively, in comparison to human isolate. Regardless of the similarities in protein content, the global protein expression of the GBS human strain was different from the GBS fish strains suggesting distinct adaptations to mammal and fish host at the proteome level. In the second trial, the transcriptomic analysis detected 107 genes as being differentially expressed in SA53 at 32 ºC when compared with 22 ºC. While in proteomic analysis were detected 81 differentially expressed proteins. The results demonstrated that the temperature regulates the differential expression of genes and proteins, mainly those involved with the expression of virulence factors, metabolism, adaptation and bacterial resistance to thermal stress. Together, the two experiments provided news insights into the diversity, adaptation and virulence of fish-pathogenic GBS strains through transcriptomic and proteomic analysis.



Keywords: streptococcosis, transcripts, proteins, fish, human, differential expression

# 1. GENERAL INTRODUCTION

In the last decades, the aquaculture industry has become consolidated as the world's fastest growing sector for animal production. According to the Food and Agriculture Organization of the United Nations (FAO), this sector demonstrated an average annual growth rate of 3.2% between 1961 and 2013. The global fish production in 2014 was 167.2 million tons, including fisheries production and aquaculture (FAO, 2016).

Brazil is one of the major producer countries of fish mainly due to its territorial extension, with more than two thirds of its land being located in tropical regions possesses several hydrographic basins, a vast maritime territory as well as large quantities and diversity of fish native species. Currently, Brazil is the 14th largest producer of fish in the world (FAO, 2016). In 2015, the national production reached a volume of more than one million tones, taking into account fisheries production (~500 thousand tons) and aquaculture (592,23 thousand tons) (KUBITZA, 2015; IBGE, 2016). The revenue generated from aquaculture in this same year reached R$4.61 billion (IBGE, 2016). In 2016, the Brazilian fish production increased approximately 4.4% in relation to 2015, with Nile tilapia (*Oreochromis niloticus*), tambaqui (*Colossoma macropomum*) and pacific white shrimp (*Litopenaeus vannamei*) being the main aquatic organisms produced (IBGE, 2016).

The higher potential for aquatic organism production in Brazil, however, is challenged by several obstacles that are hindering the development of the sector. Among them, the occurrence of outbreaks of infectious diseases is a limiting factor for the different aquaculture branches. The bacterial microorganisms have been commonly associated with outbreaks in fish (FIGUEIREDO e LEAL, 2012). The infections caused by *Streptococcus agalactiae*, *Streptococcus iniae*, *Streptococcus dysgalactiae*, *Aeromonas hydrophila*, *Flavobacterium columnare* and *Francisella noatunensis* subsp. *orientalis* (FNO) are the main health problems detected in farm-raised Nile tilapia (FIGUEIREDO *et al.*, 2005; SALVADOR *et al.*, 2005; MIAN *et al.*, 2009; CARVALHO-CASTRO *et al.*, 2010; NETTO *et al.*, 2011; FIGUEIREDO *et al.*, 2012b; LEAL *et al.*, 2014; CHIDEROLI *et al.*, 2017), whereas *Weissella ceti* and *Photobacterium damselae* susbp. *piscicida* have caused mortalities in farm producers of rainbow trout (*Oncorhynchus mykiss*) and cobia (*Rachycentron canadum*), respectively (FIGUEIREDO *et al.*, 2011; FIGUEIREDO *et al.*, 2012a).

Among these pathogens, *Streptococcus agalactiae* (GBS) have been responsible for significant economic losses in fish farms annually, due to high mortality rates, reduction of productive performance and increase in production costs (MIAN *et al.*, 2009). In Brazil, GBS infection in fish were reported in 12 states by AQUAVET – Laboratory of Aquatic Animal Diseases and AQUACEN – National Reference Laboratory for Aquatic Animal Diseases, both located at the Federal University of Minas Gerais (UFMG). GBS have been isolated from several fish species in Brazil since 2001, such as Nile tilapia, Amazon catfish (*Leiarius marmoratus* x *Pseudoplatystoma corruscans*), curimba (*Prochilodus lineatus*) and cará (*Geophagus brasiliensis*) (SALVADOR *et al.*, 2005; MIAN *et al.*, 2009; GODOY *et al.*, 2013; LEIRA, 2016).

Recently, a work evaluating the genomic diversity of 39 Brazilian fish-adapted GBS strains resulted in the identification of three genetic groups based to multilocus sequence typing (MLST) technique: ST-260, ST-927 and non-typeable (NT) lineage, being the isolates considered closely related and with a high genomic similarity between them (BARONY *et al.*, 2017). Considering the evolutionary relationship between these genotypes and for representing the major lineages that infects fish in Brazil, it becomes necessary to understand the specific characteristics of

metabolism, adaptation and pathogenicity of these genetic groups and their relationships with the aquatic host.

In this context, the evaluation of the genes involved in infectivity, virulence or adaptation of GBS to a particular niche in the host environment can be accomplished by studying the global expression of the functional genome of the microorganism through transcriptomic and proteomic assays.

## 1.1. REFERENCES


BARONY, G. M.; TAVARES, G. C.; PEREIRA, F. L. *et al.* Large-scale genomic analyses reveal the population structure and evolutionary trends of *Streptococcus agalactiae* strains in Brazilian fish farms. *Sci. Rep.*, v. 7, n. 1, p. 13538, 2017.

CARVALHO-CASTRO, G. A.; LOPES, C. O.; LEAL, C. A. G. *et al.* Detection of type III secretion system genes in *Aeromonas hydrophila* and their relationship with virulence in Nile tilapia. *Vet. Microbiol.*, v. 144, n. 3, p. 371-376, 2010.

CHIDEROLI, R. T.; AMOROSO, N.; MAINARDI, R. M. *et al.* Emergence of a new multidrug-resistant and highly virulent serotype of *Streptococcus agalactiae* in fish farms from Brazil. *Aquaculture*, v. 479, p. 45-51, 2017.

FAO. The State of World Fisheries and Aquaculture 2016: contributing to food security and nutrition for all. 2016. Disponível em: < http://www.fao.org/3/a-i5555e.pdf >. Acesso em: 15 dez. 2017.

FIGUEIREDO, H. C. P.; COSTA, F. A. A.; LEAL, C. A. G. *et al. Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. *Vet. Microbiol.*, v. 156, n. 3, p. 359-366, 2012a.

FIGUEIREDO, H. C. P; LEAL, C. A. G. Infecções por *Streptococcus* spp. em peixes. In: SILVA-SOUZA, A. T.; LIZAMA, M. A. P., *et al*. *Patologia e Sanidade de Organismos Aquáticos*. Maringá: Editora ABRAPOA, 2012b. cap. 12, p.275-292.

FIGUEIREDO, H. C. P.; KLESIUS, P. H.; ARIAS, C. R. *et al.* Isolation and characterization of strains of *Flavobacterium columnare* from Brazil. *J. Fish Dis.*, v. 28, n. 4, p. 199-204, 2005.

FIGUEIREDO, H. C. P.; LEAL, C. A. G.; COSTA, F. A. A. Fotobacteriose em bijupirá cultivado: uma "velha" doença em uma produção "jovem". *Panorama da Aquicultura*, v. 21, n. 125, p. 30-35, 2011.

FIGUEIREDO, H. C. P.; NETTO, L. N.; LEAL, C. A. G. *et al. Streptococcus iniae* outbreaks in Brazilian Nile tilapia (*Oreochromis niloticus* L.) farms. *Braz. J. Microbiol.*, v. 43, n. 2, p. 576-580, 2012b.

GODOY, D. T.; CARVALHO-CASTRO, G. A.; LEAL, C. A. G. *et al.* Genetic diversity and new genotyping scheme for fish pathogenic *Streptococcus agalactiae*. *Lett. Appl. Microbiol.*, v. 57, n. 6, p. 476-483, 2013.

IBGE. Instituto Brasileiro de Geografia e Estatística. Produção da Pecuária Municipal. 2016. Disponível em: < http://www.ibge.gov.br/home/estatistica/economia/ppm/2016/ >. Acesso em: 15 dez. 2017.

KUBITZA, F. Aquicultura no Brasil: principais espécies, áreas de cultivo, rações, fatores limitantes e desafios. *Panorama da Aquicultura*, v. 25, n. 150, p. 10-23, 2015.

LEAL, C. A. G.; TAVARES, G. C.; FIGUEIREDO, H. C. Outbreaks and genetic diversity of *Francisella noatunensis* subsp *orientalis* isolated from farm-raised Nile tilapia (*Oreochromis niloticus*) in Brazil. *Genet. Mol. Res.*, v. 13, n. 3, p. 5704-5712, 2014.

LEIRA, M. H. *Susceptibilidade de serrasalminídeos e seus híbridos a parasitos em sistema de recirculação indoor e de peixes em policultivo a bactérias.* 2016. 98f. Tese (Doutorado) - Universidade Federal de Lavras, Lavras, MG.

MIAN, G. F.; GODOY, D. T.; LEAL, C. A. G. *et al.* Aspects of the natural history and virulence of *S. agalactiae* infection in Nile tilapia. *Vet. Microbiol.*, v. 136, n. 1–2, p. 180-183, 2009.

NETTO, L. N.; LEAL, C. A. G.; FIGUEIREDO, H. C. P. *Streptococcus dysgalactiae* as an agent of septicaemia in Nile tilapia, *Oreochromis niloticus* (L.). *J. Fish Dis.*, v. 34, n. 3, p. 251-254, 2011.

SALVADOR, R.; MULLER, E. E.; FREITAS, J. C. D. *et al.* Isolation and characterization of *Streptococcus* spp. Group B in Nile tilapias (*Oreochromis niloticus*) reared in hapas nets and earth nurseries in the northern region of Parana State, Brazil. *Cienc. Rural*, v. 35, n. 6, p. 1374-1378, 2005.

# 2. OBJECTIVES

The aims of this study were: to identify and quantitate the pan-proteome of the main genotypes of Brazilian fish-adapted GBS strains, to identify conserved antigenic proteins that can be used as target in vaccine design, and to compare the differential expression of proteins identified between isolates from fish and human sources looking for host-related adaptations. In addition, the global expression of the functional genome of a Brazilian fish-adapted GBS strain was evaluated at transcript and protein level under *in vitro* growth in different temperatures using microarray and liquid chromatography-mass spectrometry label-free shotgun approaches, respectively.

# 3. CHAPTER 1. LITERATURE REVIEW

## 3.1. *Streptococcus agalactiae*

Belonging to the Streptococcaceae family, the *Streptococcus* genus covers a broad range of bacterial species associated with infectious process in mammals, including humans, aquatic animals, as well as microorganisms saprophytic or non-pathogenic (JOHRI *et al.*, 2006; EVANS *et al.*, 2008; HAENEN *et al.*, 2013). Currently, 122 species and 23 bacterial subspecies have been described as belonging to this genus (LPSN, 2017).

*Streptococcus agalactiae* (Lancefield's group B Streptococcus, GBS) has been characterized as etiological agent of diseases since 19$^{th}$ century (BISHARAT *et al.*, 2004). This bacterium exhibits as spherical or ovoid cells, Gram-positive, catalase-negative, non-motile, non-spore forming, facultatively anaerobic, with a diameter of 0.5-2.0 µm, arranged in pairs or short chains, that require nutritionally rich media for their growth. GBS grow in temperature between 25ºC and 45ºC (VOS *et al.*, 2009). Some strains may produce orange or yellow pigment and have fermentative metabolism, producing mainly lactic acid as the end product of the carbohydrate metabolism (NIZET, 2002). GBS produce hemolysins and the strains can be classified as α-, β- or non-hemolytic (VOS *et al.*, 2009).

GBS is a microorganism widely found colonizing the digestive and genitourinary tract of humans and animals (MAIONE *et al.*, 2005). This bacterium can also be found in the mammary gland of ruminants, causing mastitis (BROCHET *et al.*, 2006). Differences between strains obtained from humans and animals have already been described. Human GBS strains are hemolytic and does not use lactose as energy source, whereas, bovine GBS strains are commonly non-hemolytic and use the lactose as energy source (YILDIRIM *et al.*, 2002). In addition, fish-adapted GBS strains have also been described as non-hemolytic (FIGUEIREDO *et al.*, 2006).

This bacterium causes septicemia and meningitis in human newborns (MAIONE *et al.*, 2005; JOHRI *et al.*, 2006), clinical and subclinical mastitis in bovine (KEEFE, 1997) and septicemia and meningoencephalitis in fish (MIAN *et al.*, 2009). However, GBS has also been associated with some occasional outbreaks in other animal species such as chicken, camel, dog, rabbit, ovine, goat, horse, cat, frog, guinea pig, mice, monkey, dolphin, seal, shrimp, crocodile, emerald monitor, turtle, snake, pig, nutria, elephant and ray (AMBORSKI *et al.*, 1983; ELLIOTT *et al.*, 1990; WIBAWAN *et al.*, 1993; KEEFE, 1997; LÄMMLER *et al.*, 1998; BERRIDGE *et al.*, 2001; FORTIN e HIGGINS, 2001; EVANS *et al.*, 2002; YILDIRIM *et al.*, 2002; HETZEL *et al.*, 2003; MAIONE *et al.*, 2005; ZAPPULLI *et al.*, 2005; BISHOP *et al.*, 2007; HERNÁNDEZ *et al.*, 2009; KESKIN *et al.*, 2011; DELANNOY *et al.*, 2013; REN *et al.*, 2014; ARMAS *et al.*, 2017; EISENBERG *et al.*, 2017; MORACH *et al.*, 2017; BOWATER *et al.*, 2018; ZHOU *et al.*, 2018).

## 3.2. FISH-PATHOGENIC *Streptococcus agalactiae*

Among the streptococci, six species have been reported as the main etiological agents of septicemia and meningoencephalitis in fish: *Streptococcus agalactiae* (EVANS *et al.*, 2002), *Streptococcus iniae* (EL AAMRI *et al.*, 2010), *Streptococcus dysgalactiae* (NOMOTO *et al.*, 2006), *Streptococcus parauberis* (DOMEÉNECH *et al.*, 1996), *Streptococcus phocae* (ROMALDE *et al.*, 2008) and *Streptococcus ictaluri* (SHEWMAKER *et al.*, 2007).

Among these, the GBS infection has been verified in many species of marine and freshwater fish worldwide, causing on high morbidity and mortality rates within the farms (EVANS *et al.*, 2002; MIAN *et al.*, 2009). The first streptococcal infection in fish was described in 1957, in Japan, from diseased rainbow trout (HOSHINA *et al.*, 1958), but the specific species was not determined at

that time. Only in 1966, GBS was confirmed as etiological agent of outbreak of infectious disease in fish, in the United States, from golden shiners (*Notemigonus crysoleucas*) with numerous inflamed areas in the tegument (ROBINSON e MEYER, 1966). Since then, this bacterium has been identified in several other fish species and in different countries worldwide, affecting mainly the production of tilapines (MIAN *et al.*, 2009).

### 3.2.1. MORPHOLOGY AND PHENOTYPIC CHARACTERIZATION

GBS is a Gram-positive cocci, which occurs in short chains in different culture media, forming non-pigmented colonies of 0.6-1.2 µm diameter (Figure 1). The bacterium is non-motile, non-spore forming, facultatively anaerobic, catalase-negative, oxidase-negative and is capable of lactic acid fermentation (PEREIRA *et al.*, 2013). Fish GBS strains do not grow at 10ºC or 45ºC, or in medium supplemented with 40% bile or 6.5% (weigth/vol) of sodium chloride, however it grows at pH 9.5 (AUSTIN e AUSTIN, 2007).

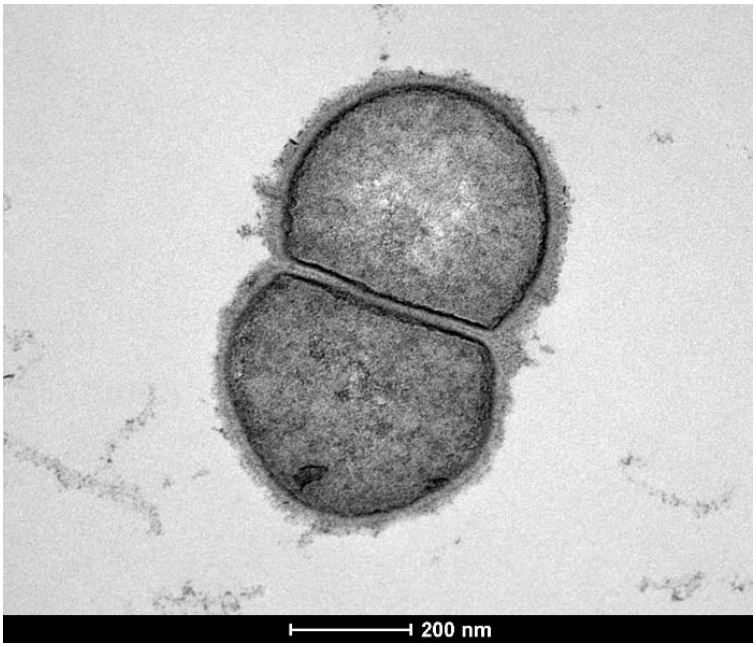


Figure 1. Transmission electron microscopy of fish GBS strain SA95

The fish GBS strains are not capable to hydrolyze urea and starch or to ferment sorbitol, mannose and xylulose. On the other hand, the hippurate hydrolysis and ribose fermentation have been demonstrated in biochemical assays. The reaction of Voges-Proskauer and pyrrolidonyl arylamidase are described as positive and negative, respectively for all strains. Other biochemical parameters such as the hydrolysis of arginine and aesculin, or the fermentation of trehalose, lactose and inulin vary according to the strains evaluated (EVANS *et al.*, 2008; EVANS *et al.*, 2015). As to the production of hemolysis in blood agar, fish GBS strains can be hemolytic or non-hemolytic, being the CAMP reaction positive only in hemolytic isolates (EVANS *et al.*, 2002; MIAN *et al.*, 2009; AREECHON *et al.*, 2016).

### 3.2.2. GEOGRAPHICAL DISTRIBUTION AND GENETIC DIVERSITY

The GBS infection in fish occurs in all continents (Figure 2). Outbreaks of the disease were reported in Australia, Belgium, Brazil, Canada, China, Colombia, Costa Rica, Ecuador, Egypt, Ghana, Honduras, Indonesia, Iran, Ireland, Israel, Japan, Kuwait, Malaysia, Mexico, Peru, Philippines, Singapore, Taiwan, Thailand, United Kingdom, USA and Vietnam (ELDAR *et al.*,

1994; EVANS *et al.*, 2002; EVANS *et al.*, 2008; CONROY, 2009; HERNÁNDEZ *et al.*, 2009; AMAL *et al.*, 2012; BOWATER *et al.*, 2012; ROSINSKI-CHUPIN *et al.*, 2013; RUANE *et al.*, 2013; KAYANSAMRUAJ *et al.*, 2014b; BARATO *et al.*, 2015; SOTO *et al.*, 2015; ASENCIOS *et al.*, 2016; CHU *et al.*, 2016; DENG *et al.*, 2017; KALIMUDDIN *et al.*, 2017; SHOEMAKER *et al.*, 2017a; VERNER-JEFFREYS *et al.*, 2018).

The evaluation of genetic diversity of the isolates using different genotyping tools associated with molecular epidemiology data of the disease have contributed to a more in-depth knowledge about the pathogen and its distribution, populational structure and transmission among animals (LIMANSKY *et al.*, 1998). Several genotyping methods have been used to study the population structure of fish GBS strains including capsular serotyping (SHOEMAKER *et al.*, 2017a), pulsed-field gel electrophoresis (PFGE) (PEREIRA *et al.*, 2010; DELANNOY *et al.*, 2013) and multilocus sequence typing (MLST) (EVANS *et al.*, 2008; GODOY *et al.*, 2013).

The serotyping of the capsular polysaccharide has been used to discriminate GBS strains in ten different serotypes (Ia, Ib, II-IX) (LANCEFIELD, 1934; SLOTVED *et al.*, 2007; KONG *et al.*, 2008). The capsule is one of the main virulence factors of this bacterium and is involved in the evasion of the host immune system due to the presence of sialic acid in its constitution, which is also found in the host cell surfaces, preventing the recognition of the pathogen (CHAFFIN *et al.*, 2005; CIESLEWICZ *et al.*, 2005). The evolution of the different capsular types observed in GBS strains probably occurs by the selection pressure imposed by the host (CIESLEWICZ *et al.*, 2005). The fish GBS strains belong to serotypes Ia, Ib, II and III, being the serotype Ib is commonly known to be a non-hemolytic strain (SHOEMAKER *et al.*, 2017a). The serotype Ia has been more detected in diseased fish from Kuwait, Thailand and China (EVANS *et al.*, 2008; LIU *et al.*, 2013b; KAYANSAMRUAJ *et al.*, 2015), while the serotype Ib is more prevalent in Latin America, Ghana and Australia (BARATO *et al.*, 2015; BARONY *et al.*, 2017; BOWATER *et al.*, 2017; VERNER-JEFFREYS *et al.*, 2018). The serotype II was identified in Australia (BOWATER *et al.*, 2012). Finally, the serotype III has been identified in Brazil and Southeast Asia (CHIDEROLI *et al.*, 2017; KALIMUDDIN *et al.*, 2017). The serotypes Ia, II and III belong to β-hemolytic strains (BOWATER *et al.*, 2012; AREECHON *et al.*, 2016). Among all these serotypes, the III stands out due to its characterized zoonotic potential and the possibility of transmission between fish and humans through the ingestion of raw fish (TAN *et al.*, 2016).

The PFGE technique is commonly used as a molecular typing tool for several strains that cause outbreaks of infectious disease in humans and other animals (SHIMA *et al.*, 2006). This methodology allows the separation of large DNA fragments from the reorientation of DNA in gels by the action of alternating electric fields (MAGALHÃES *et al.*, 2005). PEREIRA *et al.* (2010) compared GBS strains obtained from humans ($n$ =11), bovine ($n$ = 9) and fish ($n$ = 27) by PFGE and found different genetic patterns among the hosts. In summary, they showed that all bovine GBS strains presented distinct genetic profiles, however, none of them showed a genetic relationship with GBS fish strains. Similarly, human GBS strains were more genetically related to each other, being observed a genetic relationship between them and fish GBS strains. Among fish GBS strains, the authors identified five genetic patterns in 27 evaluated strains, however, one specific PFGE profile was predominant in seven out of the nine farms studied, being considered at that time the main genetic type involved in the meningoencephalitis in Nile tilapia in Brazil. In another study, DELANNOY *et al.* (2013) evaluated 34 GBS isolates obtained from aquatic animals in different countries by PGFE and found 13 pulsotypes that were indistinguishable from multiple isolates from a single outbreak and from isolates from different host species or countries, being each outbreak considered, therefore, epidemiologically independent. However, the PFGE technique is time-consuming and an intensively laborious methodology, restricting its use only in reference laboratories (LIMANSKY et al., 1998).

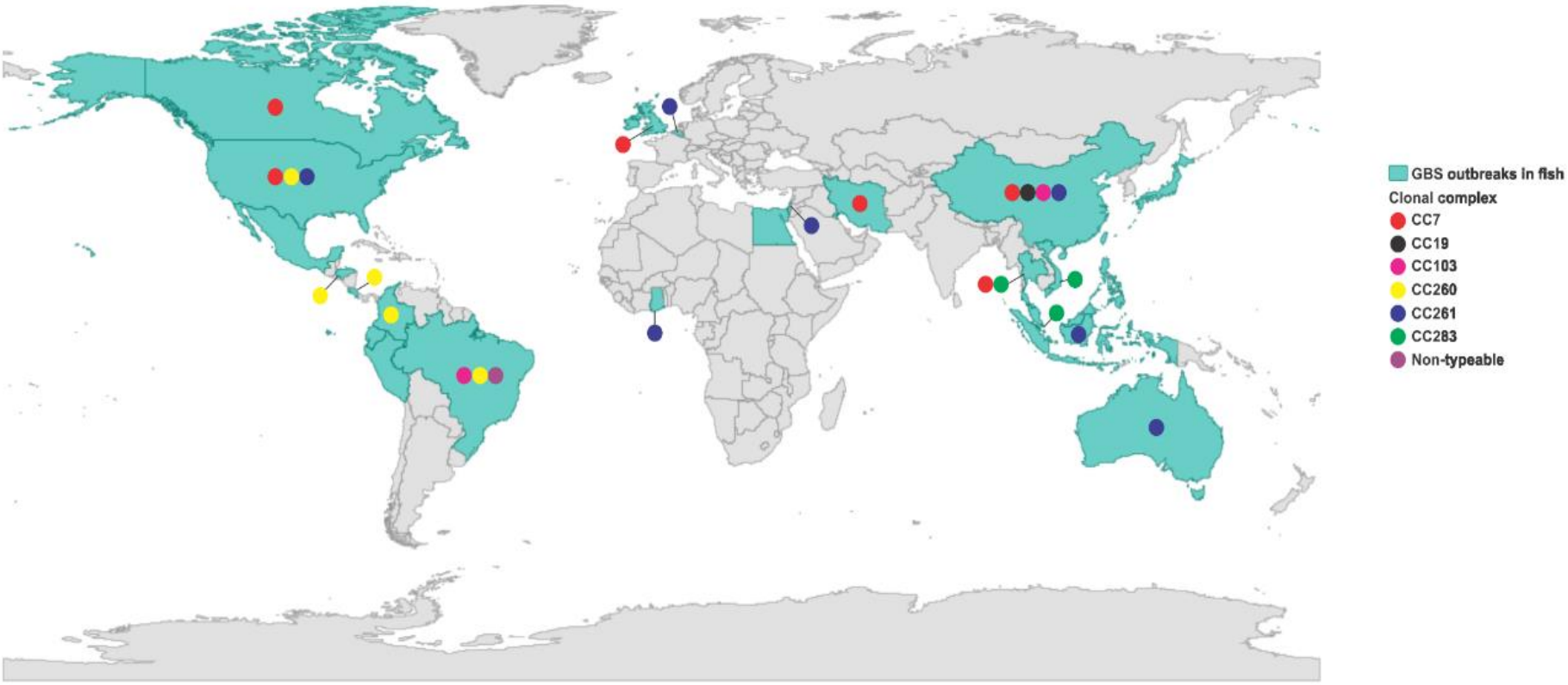


Figure 2. Map of global distribution of GBS outbreaks in fish farms and the main genetic types detected in these countries

The MLST is considered the gold standard method for the evaluation of the genetic diversity of GBS strains since it is sufficiently discriminatory for epidemiological studies and able to provides a precise and unambiguous way of characterizing the isolates of these pathogen (JONES *et al.*, 2003). This technique is a robust tool that allows a better epidemiological and evolution analyses, as well as pathogen monitoring, when compared to PFGE, due the interlaboratory reproducibility, ease of data analysis thought comparison of sequences queried against a central and curated web-based MLST database (JOHNSON *et al.*, 2007; THOMAS e PETTIGREW, 2009; AL NAKIB *et al.*, 2011). The MLST grouped different strains according to their nucleotide sequences or allelic profiles from a set of housekeeping genes of a specific pathogen. Therefore, the proximity between different isolates of a microorganism can be verified by allelic profile comparisons (JONES *et al.*, 2003). The combination of different alleles (*adhP, pheS, atr, glnA, sdhA, glcK* e *tkt*) generates a profile which represent a ST (*sequence type*) for GBS.

The database with all alleles and STs already described is public available, which makes the MLST technique of easy interpretation, and, until the present date, there are 1193 STs described on the *Streptococcus agalactiae* MLST database (https://pubmlst.org/sagalactiae/) (JOLLEY e MAIDEN, 2010). The STs are still grouped in clonal complexes (CC), evaluating the isolates ancestry thought *eBURST* analysis by BURST (*Based Upon Related Sequences*) algorithm. STs that not grouped in CC are designed as *singletons* (FEIL *et al.*, 2004). The identification of CC is an important tool for epidemiological studies. For example, the CC61 and CC67 are exclusively composed of strains isolated from bovine with mastitis, and the CC1, CC17 e CC19 possesses strains obtained from human with clinical manifestation of GBS infection (SPRINGMAN *et al.*, 2014; BERGAL *et al.*, 2015).

Moreover, there are two clonal complexes (CC260 and CC261) and one non-typeable lineage that is composed only of strains isolated from diseased fish and encompasses the STs 246, 257, 259, 260, 261, 891, 927 (BARONY *et al.*, 2017) (Figure 2). Other fish GBS strains were also grouped in the following STs: ST-1, ST-6, ST-7, ST-19, ST-23, ST-24, ST-28, ST-103, ST-167, ST-258, ST-283, ST-335, ST-485, ST-491, ST-500, ST-651, ST-652, ST-735 and ST-861 (EVANS *et al.*, 2008; DELANNOY *et al.*, 2013; GODOY *et al.*, 2013; CHEN *et al.*, 2015; CHAU *et al.*, 2017; ZHANG *et al.*, 2017). All STs associated with GBS infection and colonization in fish and its geographical distribution are showed in Table 1 and Figure 2.

ST-7, ST-103 and ST-283 possesses isolates described in different hosts. The ST-7 belongs to CC7 and encompasses strains obtained from dolphins, fish and humans (EVANS *et al.*, 2008; LIU *et al.*, 2013b). Recent studies from China and Thailand have demonstrated that new GBS strains, associated with outbreaks of encephalitis in tilapia have emerged and they belong to this typical clonal complex of infection in humans (CC7) (LIU *et al.*, 2013b; KAYANSAMRUAJ *et al.*, 2015) (Figure 2). On the other hand, the ST-103 belongs to CC103 and possesses isolates from cattle, guinea pig, cat and human, being identified in cultured fish in Brazil and China (GODOY *et al.*, 2013; ZHANG *et al.*, 2017). This ST is commonly reported among bovine GBS strains from Brazil, China and Denmark, and is well adapted to the oral-fecal transmission route in cows and to environmental survival (JØRGENSEN *et al.*, 2016; CARVALHO-CASTRO *et al.*, 2017; PANG *et al.*, 2017). This generates speculations about a possible transmission of GBS ST-7 between human and fish and GBS ST-103 between cattle and fish (LIU *et al.*, 2013b; CHEN *et al.*, 2015), but this epidemiological linkage still unconfirmed. However, in 2015, episodes of bacteremia and meningitis in human associated with raw fish consumption were investigated in Singapore, being GBS serotype III ST-283 diagnosed in these cases (TAN *et al.*, 2017). Therefore, this specific genotype has been considered a potential zoonotic agent to humans (RAJENDRAM *et al.*, 2016; KALIMUDDIN *et al.*, 2017). Besides Singapore, this ST was detected in fish in Thailand (DELANNOY *et al.*, 2013; AREECHON *et al.*, 2016).

Table 1. Main GBS sequence type associated with infection in fish and its global distribution.

| | ST | CC | Country | Reference |
|---|---|---|---|---|
| Fish host | 257 | - | Brazil | EVANS *et al*., 2008 |
| | 258 | - | Israel | EVANS *et al*., 2008 |
| | 259 | 260 | USA | EVANS *et al*., 2008 |
| | 260 | 260 | Brazil, Honduras, Colombia, Costa Rica, USA | EVANS *et al*., 2008; DELANNOY *et al*., 2013; GODOY *et al*., 2013; ROSINSKI-CHUPIN *et al*., 2013 |
| | 246/261 | 261 | Israel, Australia, Belgium, USA, Ghana, China, Indonesia | DELANNOY *et al*., 2013; ROSINSKI-CHUPIN *et al*., 2013; LUSIASTUTI *et al.,* 2014; PRIDGEON e ZHANG, 2014; DEFENG *et al*., 2017; VERNER-JEFFREYS *et al*., 2018 |
| | 552/553/NT | - | Brazil | GODOY *et al*., 2013; BARONY *et al*., 2017 |
| | 891 | 261 | China | DENG *et al*., 2017 |
| | 927 | 260 | Brazil | BARONY *et al*., 2017 |
| Multiple host | 1 | 1 | Singapore | CHAU *et al*., 2017 |
| | 6 | 7 | USA | ROSINSKI-CHUPIN *et al*., 2013 |
| | 7 | 7 | Kuwait, GB, Thailand, Canada, China, Taiwan | DELANNOY *et al*., 2013; LIU et al., 2013b; KAYANSAMRUAJ *et al*., 2015; CHU *et al*., 2016 |
| | 19 | 19 | China | ZHANG *et al*., 2017 |
| | 23 | 23 | Singapore | CHAU *et al.*, 2017 |
| | 24 | 24 | Singapore | CHAU *et al.*, 2017 |
| | 28 | 28 | Singapore | CHAU *et al.*, 2017 |
| | 103 | 103 | Brazil, China | GODOY *et al*., 2013; ZHANG *et al*., 2017 |
| | 167 | 1 | Singapore | CHAU *et al.*, 2017 |
| | 283 | 283 | Thailand, Singapore | DELANNOY *et al*., 2013; KALIMUDDIN *et al.,* 2017 |
| | 335 | 19 | Singapore | CHAU *et al.*, 2017 |
| | 485 | 485 | Singapore | CHAU *et al.*, 2017 |
| | 491 | 283 | Vietnam | DELANNOY *et al*., 2013 |
| | 500 | 7 | Thailand | DELANNOY *et al*., 2013 |
| | 651 | 103 | Singapore | CHAU *et al.*, 2017 |
| | 652 | 12 | Singapore | CHAU *et al.*, 2017 |
| | 735 | 7 | China | CHEN *et al*., 2015 |
| | 861 | 19 | Singapore | CHAU *et al.*, 2017 |

Although the MLST is still the most commonly used technique for GBS genotyping, its resolution is insufficient for some genetic analysis. This methodology cannot distinguish some isolates from human and cattle (SØRENSEN *et al.*, 2010), or clones involved in the same outbreaks in fish farms (GODOY *et al.*, 2013), due to the low variation that can occur in housekeeping genes. The low discriminatory power and resolution of phylogenetic inferences using the MLST approach enables the utilization of alternative technologies with higher resolution capacities such as the whole genome sequencing (WGS). In this context, the BIGSdb (*Bacterial Isolate Genome Sequence Database*) (JOLLEY e MAIDEN, 2010) is an alternative to manipulate WGS data from microbial genomes and to perform focused analysis using extended MLST genotyping methods, which may comprise thousands of genes through a gene-by-gene approach. This approach known as *whole genome* MLST (wgMLST) performed by the BIGSdb software has been used to discriminate isolated strains from different regions, as well as to differentiate clonal lineages from a given bacterial species obtained from the same host, or from the same outbreak (MAIDEN *et al.*, 2013; CODY *et al.*, 2014). We performed a wgMLST with the GBS genomes of fish strains available on GenBank (https://www.ncbi.nlm.nih.gov/genome/genomes/186?) plus other *in house* database (n= 55) to depict the population structure of fish GBS strains in the world which were grouped by ST or CC (Figure 3).

The wgMLST analysis takes into account the changes in alleles that may occur due to a simple genetic event, from horizontal gene transfer that is considered to be of extreme importance for the strain differentiation (HASSAN *et al.*, 2000) and the main mechanism for the dissemination and selection of virulence and antimicrobial resistance genes in bacterial pathogens (BECEIRO *et al.*, 2013). Using this methodology in 42 genomes of fish GBS strains, BARONY *et al.* (2017) identified four groups based on the "All loci" comparison, being these groups mainly related to

sequence types. These authors established two eBurst groups in all Brazilian fish-adapted GBS strains: the CC260 and one group composed by ST-257 and non-typeable strains, resulting in the deconstruction of previously described CC552. However, BARONY *et al.* (2017) also observed that there was a relative genetic distance between the strains tested, suggesting that each Brazilian isolate belongs to a single clone, characterizing, therefore, a diverse populational structure for GBS strains

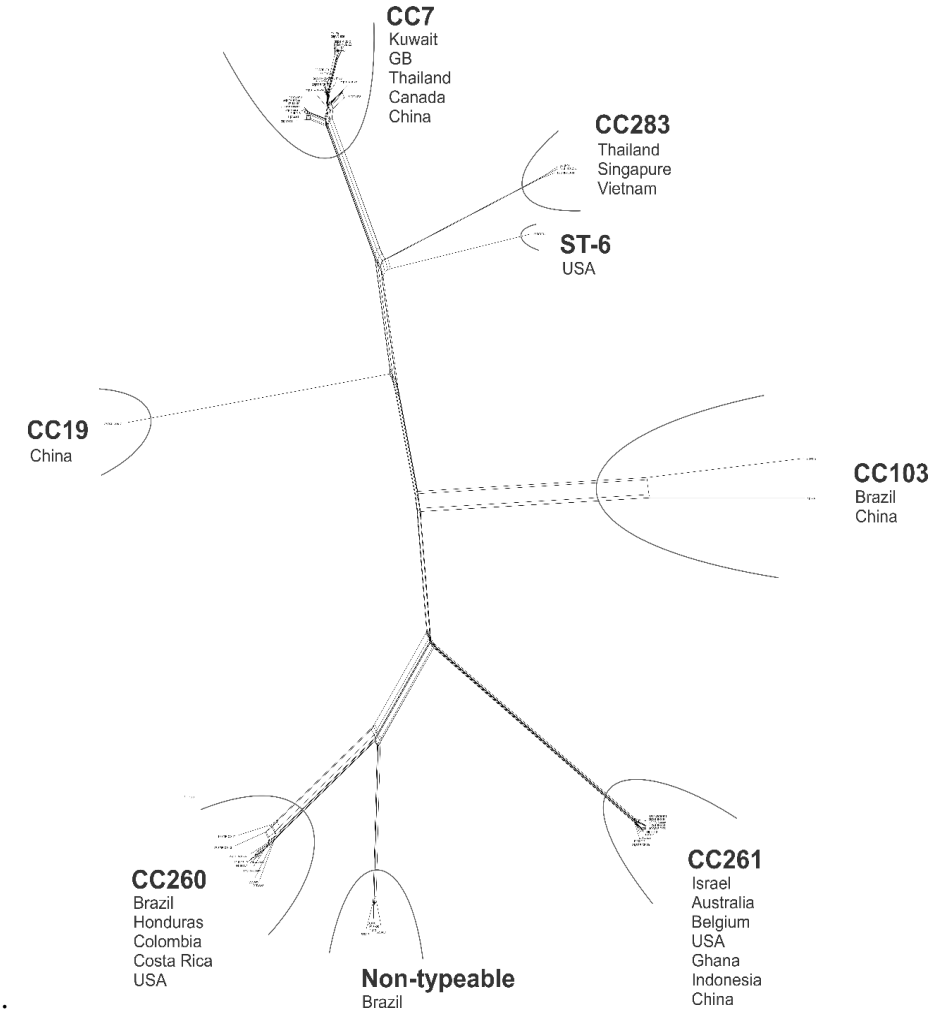


Figure 3. Phylogenomic NeighborNet network of wgMLST data of 55 fish GBS strains. The isolates grouped in clusters based on assignment to clonal complex (CC) or sequence type (ST).

### 3.2.3. SUSCEPTIBLE SPECIES, TRANSMISSION AND RISK FACTORS

The GBS cause disease in a wide range of fish species. At the moment, there are 41 fish species among captive and wild fish being susceptible to GBS infection, with tilapia and its hybrids the most affected ones (Table 2).

In fish polyculture systems or in aquatic environments shared between farmed fish and wild fish, where infected tilapia cohabit with other fish species, can provide pathways for GBS transmission. However, in this system few fish species demonstrated to be susceptible to GBS infection, including curimba and cará, while other species such as piracanjuba (*Brycon orbignyanus*), dourado (Salminus brasiliensis), cascudo (*Hypostomus plecostomus*), nicuros (*Pimelodus*

*blochii*), bocachico (*Prochilodus magdalenae*) and cachama blanca (*Piaractus brachypomus*) are known to be resistant to the infection when cultured together with susceptible and infected fish in Brazilian and Colombian farms (IREGUI *et al.*, 2014; LEIRA, 2016).

Table 2. Current fish species naturally and experimentally susceptible to GBS infection.

| Host | Country | Reference |
|---|---|---|
| *Acanthopagrus schlegelii* | Taiwan | CHU *et al*., 2016 |
| *Arius felis* | USA | PLUMB *et al*., 1974 |
| *Bidyanus bidyanus* | Taiwan | CHU *et al*., 2016 |
| *Brevoortia patronus* | USA | PLUMB *et al*., 1974 |
| *Cirrhinus molitorella* | China | LI-HUI *et al*., 2015 |
| *Cynoscion nothus* | USA | PLUMB *et al*., 1974 |
| *Danio rerio** | Finland | PATTERSON *et al*., 2012 |
| *Dasyatis sp.* | USA | PLUMB *et al*., 1974 |
| *Epinephelus lanceolatus* | Australia | BOWATER *et al*., 2012 |
| *Fundulus grandis* | USA | SOTO *et al*., 2015 |
| *Garra rufa* | Ireland | RUANE *et al*., 2013 |
| *Geophagus brasiliensis* | Brazil | LEIRA, 2016 |
| *Hypophthalmichthys nobilis* | China | Genbank |
| *Lagodon rhomboides* | USA | PLUMB *et al*., 1974 |
| *Lateolabrax japonicus* | Taiwan | CHU *et al*., 2016 |
| *Lates calcarifer* | Taiwan | CHU *et al*., 2016 |
| *Leiostomus xanthurus* | USA | PLUMB *et al*., 1974 |
| *Lepomis cyanellus** | USA | ROBINSON e MEYER, 1966 |
| *Lepomis macrochirus** | USA | ROBINSON e MEYER, 1966 |
| *Liza klunzingeri* | Kuwait | EVANS *et al*., 2002 |
| *Liza macrolepis* | Taiwan | CHU *et al*., 2016 |
| *Micropogon undulates* | USA | PLUMB *et al*., 1974 |
| *Mikrogeophagus ramirezi* | Australia | DELANNOY *et al*., 2013 |
| *Morone chrysops x Morone saxatilis* | USA | SOTO *et al*., 2015 |
| *Mugil cephalus* | USA | PLUMB *et al*., 1974 |
| *Notemigonus crysoleucas* | USA | ROBINSON e MEYER, 1966 |
| *Oncorhyncus mykiss* | Israel | ELDAR *et al.*, 1994 |
| *Oreochromis sp.* | Brazil, Thailand, Vietnam, Colombia, Costa Rica, Honduras, Belgium | MIAN *et al*., 2009; DELANNOY *et al*., 2013 |
| *Oryzias javanicus** | Malaysia | AMAL *et al*., 2017 |
| *Pampus argenteus* | Kuwait | DUREMDEZ *et al.*, 2004 |
| *Pomatonus saltatrix* | USA | BAYA *et al*., 1990 |
| *Prochilodus lineatus* | Brazil | LEIRA, 2016 |
| *Pseudoplatystoma facium x Leiarius marmoratus* | Brazil | GODOY *et al*., 2013 |
| *Puntius conchonius* | Australia | DELANNOY *et al*., 2013 |
| *Rhamdia quelen** | Brazil | BALDISSERA *et al*., 2017 |
| *Schizopygopsis pylzovi* | China | ZHANG *et al*., 2017 |
| *Schizothorax prenanti* | China | GENG *et al*., 2012 |
| *Scortum barcoo* | China | LIU *et al*., 2014 |
| *Sparus auratus* | Kuwait | EVANS *et al*., 2002 |
| *Thunnus albacares* | Taiwan | CHU *et al*., 2016 |
| *Trachinotus blochii* | Singapore | CHONG *et al*., 2017 |

* induced susceptibility

The GBS transmission occurs through direct contact between diseased and health fish, cannibalism of the dead or moribund fish and by indirect contact through the bacterium present in the water, allowing the disease to gradually manifest in different production systems (SHOEMAKER *et al.*, 2017b).

The introduction of new batches of fish in the farms has been considered the most important way of a bacterial pathogen entry into the system (VENDRELL *et al.*, 2006). Besides, fish can be carriers of GBS without showing signs of disease, being, therefore, a significant source of infection for the epidemiological dynamics of streptococcosis (TAVARES *et al.*, 2016). A diseased or carrier fish usually shed GBS via gills, mucus and fecal routes (PASNIK *et al.*, 2009; TAVARES *et al.*, 2016), infecting the remaining healthy fish in the farm. However, the time period that the agent remains in the water after being eliminated from a infected fish is indeterminate. KAYANSAMRUAJ *et al.* (2014b) isolated GBS from fish, water samples and

sediments collected during a period of 9 months from commercial fish farms in Thailand. These authors demonstrated that there is a shedding of the pathogen from fish to the environment, however, the study did not correlate the GBS identification with the viability of the bacterium in the environment, since the month that did not have isolation of the pathogen from a diseased fish, there was also no detection from the environmental samples.

Some studies have indicated that the main route of entry for GBS in fish is the gastrointestinal tract by ingestion of contaminated water (IREGUI *et al.*, 2016) or infected fish (BOWATER *et al.*, 2012). Other GBS exposure routes in fish were already demonstrated, using cohabitation assays, injections, immersion baths and gill inoculations (MIAN *et al.*, 2009; DELAMARE-DEBOUTTEVILLE *et al.*, 2015). The intraperitoneal injection has been extensively used in experimental infection, however this exposure route does not simulate the conditions encountered in the field, since the bacterium needs to overcome all natural barriers found in the aquatic host to cause the disease, and in this type of exposure such barriers are supplanted. Through GBS injection into the fish, the first clinical signs appeared 20 h post-infection and the first mortalities were observed at 72 h post-infection (MIAN *et al.*, 2009; TAVARES *et al.*, 2016). In the cohabitation assay, the healthy fish began to present clinical signs 24-72h after introduction of the diseased fish, and 100% of mortality rate could be verified after 10 days of fish contact (MIAN *et al.*, 2009). The transmission route via water-born exposure might occurred, however the mortality rate is very low as compared to the injection challenge (DELAMARE-DEBOUTTEVILLE *et al.*, 2015). Finally, the gill inoculation demonstrated that the gill tissue is an important site for GBS infection in fish since it cause a mortality rate of 33% (MIAN *et al.*, 2009).

The vertical transmission of GBS was already demonstrated. PRADEEP *et al.* (2016) detected GBS in testis and gonads of tilapia breeding and 10 and 30-day-old larval from positive breeders for the disease, demonstrating, thus, a high potential of tilapia broodstock for transmission of the bacterium via gametes.

High mortalities rates have been verified in GBS outbreaks in Nile tilapia, with the increase of water temperatures (above 27ºC), intensive husbandry, high stocking density and poorly environmental conditions (high ammonia and low dissolved oxygen levels) representing the main risk factors associated with the occurrence of disease in fish farms (MIAN *et al.*, 2009; ZAMRI-SAAD *et al.*, 2014; AMAL *et al.*, 2015). The infection by this pathogen have been commonly observed in fish weighting under 50 g but it affects mainly adult fish in growth stage, with an average weight of 500 g (MIAN *et al.*, 2009).

### 3.2.4. CLINICAL SIGNS AND PATHOGENICITY

GBS infection cause septicemic disease, being able to invade and multiply in different organs of the infected fish. However, it is believed that the brain tissue is the site of predilection since the bacterium causes meningoencephalitis, which, consequently, culminates in clinical signs of loss of orientation and erratic swimming. Other clinical signs that may be observed in diseased fish are: anorexia, lethargy, melanosis, dorsal rigidity, C-shape body curvature, exophthalmia, corneal opacity, peri-orbital or intraocular hemorrhage, diffuse hemorrhage in the integument and musculoskeletal tissue and ascites (Figure 4) (EVANS *et al.*, 2002; HERNÁNDEZ *et al.*, 2009; MIAN *et al.*, 2009; AMAL *et al.*, 2012; BOWATER *et al.*, 2012; DELAMARE-DEBOUTTEVILLE *et al.*, 2015).

At necropsy, it can be observed: accumulation of serosanguinous fluid in the fish celomatic cavity, pale liver, hepatomegaly, splenomegaly, cloudy meninges or cerebro-spinal fluid, and yellow or dark red nodules in the muscular tissue (BOWATER *et al.*, 2012; LI *et al.*, 2014b).

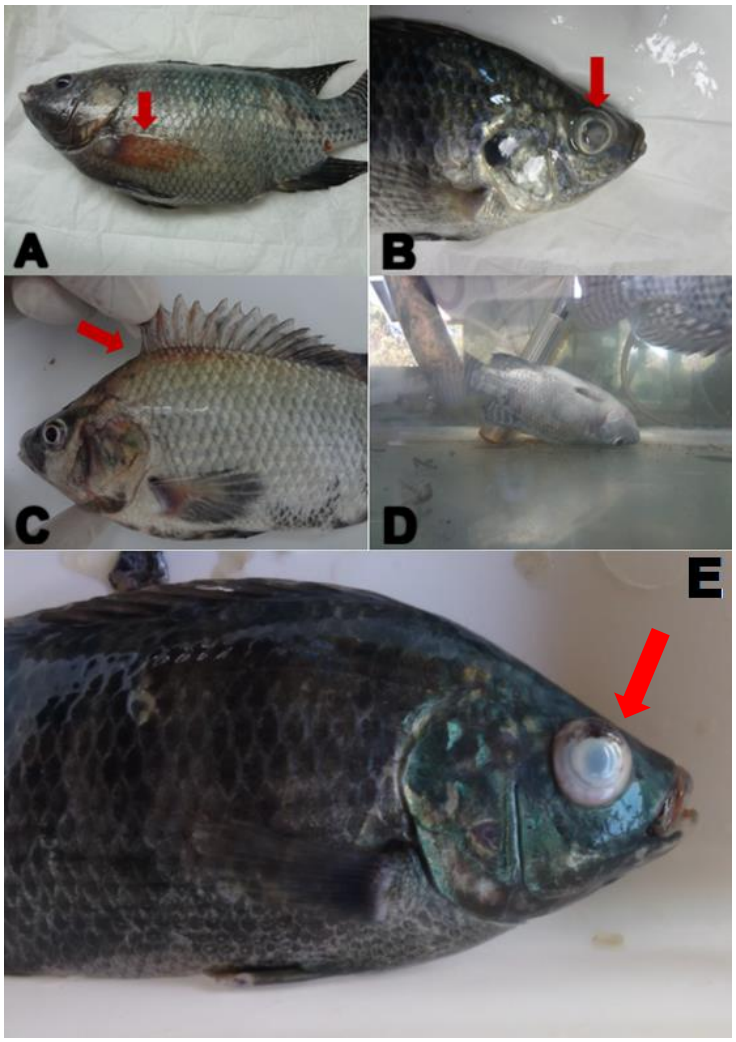


Figure 4. Main clinical signs observed in experimentally infected Nile tilapia with GBS. A. ascites and hemorrhagic fins (arrow), B. corneal opacity (arrow), C. hemorrhage in the integument (arrow), D. loss of orientation and erratic swimming, E. exophthalmia (arrow).

Histopathological alterations in systemic streptococcosis have been observed in many organs and tissues, through a mixed mononuclear inflammatory response especially in the brain, heart and eyes (ZAMRI-SAAD *et al.*, 2010; SOTO *et al.*, 2016). The diseased fish may have diffuse granulomatous meningitis; multifocal branchiitis, with proliferation and hyperplasia of gill lamellar epithelium and fusion of adjacent gill lamellae; blood vessels dilated and congested with bacteria circulating within macrophages; endophthalmos and choroiditis with different degrees of severity of granulomatous inflammation in the tissues; keratitis with cornea ulcerated and edema between adjacent stromal layers; granulomatous epicarditis, pericarditis, myocarditis and endocarditis; interstitial nephritis; hepatitis; gastritis; enteritis with the presence of bacteria in the intestinal lumen and lamina propria; pancreatitis; peritonitis; nodular granulomatous splenitis with congestion and hemorrhage of spleen tissue, following by degeneration and necrosis of ellipsoids, and depletion of the white pulp; skeletal muscle myositis with presence granulomatous nodule of central necrosis and accumulation of bacteria within the granulomas; and finally, ulcerative and hyperemic dermatitis were also reported (LI *et al.*, 2014b; DELAMARE-DEBOUTTEVILLE *et al.*, 2015; SOTO *et al.*, 2016).

The pathogenesis of GBS infection in fish is poorly understood. It is known that an important step in the GBS pathogenesis in humans is the ability to cross the blood-brain barrier, however, few

studies specifically tested the function of genes involved in this process (DORAN e NIZET, 2004; JOHRI *et al.*, 2006). In fish, this pathogen has also the ability to cross the blood-brain barrier, however, according to the literature reviewed, there is no mention on how it happens. Isolates from different hosts most probably share common virulence factors allowing the microorganism to colonize, spread and cross the blood-brain barrier of fish, causing meningoencephalitis (PEREIRA *et al.*, 2010).

The main virulence factors described for GBS, regardless the host species, are adhesins, invasins and evasins. Among the adhesin, fibrinogen-binding proteins (*fbsA* and *fbsB*), fibronectin-binding protein (*pavA*), immunogenic bacterial adhesin (*bibA*), laminin-binding proteins (*lmb*) and pilus proteins (PI1, PI2b or PI2a) standouts. The main representatives of invasins are β-hemolysin (*cylE*), CAMP factor (*cfb*), C-α protein (*bca*) and rib surface protein. While that C-β protein (*bac*), capsular polysaccharide proteins (*cps*), C5a peptidase (*scpB*) and serine protease (*cspA*) are described as immune-evasion proteins (SANTI *et al.*, 2007; RAJAGOPAL, 2009; LIN *et al.*, 2011). These virulence factors have already been associated with infection in humans and murine models, but the participation of them in the pathogenesis of the disease in fish are unknown. GODOY *et al.* (2013) demonstrated that the Brazilian GBS fish strains have virulence genes involved with cell adhesion and invasion, such as *iagA*, *cfb*, *fbsA*, *fbsB* and *gbs2018* (*bibA*), as well as genes related with cytotoxicity and cell lysis (*cylE* and *hylB*). The absence of *bac*, *bca* and *scpB* genes involved in adhesion, invasion and immune evasion were also found to be absent in the whole genome of GBS SA20 strain (PEREIRA *et al.*, 2013). The identification of these genes in the strains evaluated may contribute to a significant increase in virulence in the fish host, reducing then the median lethal dose (LD50), however, the involvement of these genes in the bacterial virulence has not yet been proven.

*In vivo* assays have demonstrated the ability of human and bovine GBS strains to cause clinical signs and mortalities in experimentally infected tilapia (PEREIRA *et al.*, 2010; CHEN *et al.*, 2015). However, despite being the same bacterial species, fish GBS strains were more pathogenic when compared to human and cattle isolates, due to the high mortality rates observed using fish isolates (PEREIRA *et al.*, 2010). Different results in pathogenicity were also observed between fish GBS strains with diverse genotypes. EVANS *et al.* (2015) used five strains with different sequence types (ST-257, ST-258, ST-259, ST-260 and ST-7) obtained from three fish species (tilapia, hybrid striped bass and mullet) in a standardizing condition for experimental infection in Nile tilapia. It was observed that the adapted host isolates (ST-257, ST-258, ST-259 and ST-260) were more virulent (LD50 below $10^4$ cfu $mL^{-1}$) than the strains shared between fish and humans (ST-7 – LD50 = 7.2 x $10^5$ cfu $mL^{-1}$). It is noteworthy that Brazilian fish-adapted GBS strains were extremely virulent to Nile tilapia (LD50 = 6.1 x $10^1$ cfu $mL^{-1}$) as previously described (MIAN *et al.*, 2009).

## 3.2.5. DIAGNOSTIC METHODS

The diagnoses and identification of GBS infection in fish can be performed through the association of clinical signs with laboratory findings. Due to the wide range of susceptible hosts and general clinical signs verified in cases of infections in fish caused by different species of *Streptococcus* genus, the laboratory diagnosis is indispensable for the determination of the etiological agent involved in the outbreaks (MATA *et al.*, 2004). Therefore, the GBS diagnosis is based on isolation and identification of the microorganism.

Moribund fish can be collected and immediately sent to diagnostic laboratories on ice (ASSIS *et al.*, 2017b). The predilection tissues of sampling to perform bacteriology vary according to the disease and bacterium associated with infection. In short, GBS infection have been associated with septicemia and meningoencephalitis, being best indicated the sampling of nervous tissue, organs highly vascularized or that play immunological functions such as kidney, liver and spleen

(NOGA, 2000). Eye, heart and intestine are also used for disease diagnosis (EVANS *et al.*, 2002; IREGUI *et al.*, 2016; FACIMOTO *et al.*, 2017). In addition, kidney aspiration and venipuncture have been considered nonlethal sampling methods feasible and secure for obtaining kidney and blood samples of Nile tilapia for GBS infection diagnosis (TAVARES *et al.*, 2016).

In the laboratory, the diagnosis can be performed by bacteriology and molecular methods. For bacteriology, swabs of tissues aseptically sampled are streaked on generic culture media as blood agar, BHI (Brain Heart Infusion) agar, Tryptic Soy agar (TSA) and Todd-Hewitt agar (THA) (EVANS *et al.*, 2002; MIAN *et al.*, 2009), selective media as Columbia blood agar and chromID Strepto B agar (PRETTO-GIORDANO *et al.*, 2010; KOH *et al.*, 2017) or enriched on Lim broth or Granada biphasic broth with posterior subculture in culture media to select GBS in samples considered to be contaminated (EVANS *et al.*, 2010; TAVARES *et al.*, 2016).

After the incubation period of 48-72h at 28 ºC, the bacterial identification is based on the evaluation of characteristics of bacterial colonies, visualization of cell morphology under a light microscope (Gram stain), type of hemolysis, structural pattern of capsular antigen (Lancefiled group antigen) and biochemical assays (catalase and oxidase, for example) (SHOEMAKER *et al.*, 2017b). The utilization of commercial kits for phenotypical characterization of GBS as RAPID32 and API20 Strep have showed good applicability, accuracy and time saving (MIAN *et al.*, 2009). However, the isolation and bacterial characterization using biochemical/phenotypical tests can lead to misidentifications or a lack of species-level resolution (ASSIS *et al.*, 2017a). Thus, complementary molecular methods for diagnosis are necessary.

The identification of GBS by molecular biology methods can be performed using different techniques, such as PCR amplification and sequencing of the 16S rRNA gene (MIAN *et al.*, 2009), species-specific PCR (BERRIDGE *et al.*, 2001), species-specific qPCR (SU *et al.*, 2016), multiplex PCR (MATA *et al.*, 2004), nested-PCR (JIMÉNEZ *et al.*, 2011), loop-mediated isothermal amplification (LAMP) assay (SUEBSING *et al.*, 2013), and matrix-assisted laser desorption ionization (MALDI)- time of flight (TOF) mass spectrometry (ASSIS *et al.*, 2017a).

Among these techniques, 16S rRNA gene sequencing and GBS-specific PCR have been widely used for definitive diagnosis of the disease. From PCR amplification of universal primers for housekeeping genes (16S rRNA gene) in bacterial pathogens, following by sequencing of obtained amplicons, it became possible to compare the DNA sequence of a determined isolate with others deposited in public databases, such as the NCBI (www.ncbi.nlm.nih.gov/BLAST). Sequences identified with similarity ≥ 97% are considered to be the same bacterial species (NGUYEN *et al.*, 2016).

The GBS-specific PCR uses primers of the 16S-23S intergenic spacer regions (IGS). This technique allows the confirmation of GBS strains in suspect bacterial isolates on culture media since no amplicons are produced during amplification from related *Streptococcus* spp. This methodology is also used to detect the pathogen from tissue samples from diseased fish (BERRIDGE *et al.*, 2001). The same set of primers used by BERRIDGE *et al.* (2001) were used in an multiplex PCR approach to allow the simultaneous detection of Gram-positive fish pathogens such as GBS, *Streptococcus iniae*, *Streptococcus parauberis* and *Lactococcus garvieae*. The results demonstrated that this assay is an effective tool for the rapid and specific detection of GBS infection using both pure culture (detection limit = 250 to 125 cells) and tissue from fish (detection limit = 12000 cells/g) (MATA *et al.*, 2004). Similarly, a nested PCR using 16S-23S rRNA gene are also performed for GBS identification in naturally infected fish and in frozen and paraffin-wax-embedded tissues (JIMÉNEZ *et al.*, 2011). The nested PCR showed a high level of sensitivity for GBS detection in these samples, with detection limits varying of 6.95 pg to 1.58 fg from DNA extracted of each sample (JIMÉNEZ *et al.*, 2011).

Interestingly, a qPCR assay was also developed using the 16S-23S rRNA gene. This assay provides a rapid, specific and sensitive method for the identification and precise quantitation of GBS in fish tissues, demonstrating that brain ($10^5$ copies per mg tissue), eye ($10^4$ copies), spleen ($10^4$ copies) and kidney ($10^4$ copies) were the tissues with the higher bacterial load after experimental infections (SU *et al.*, 2016). A previous study compared the frequency of detection of GBS in different tissues of experimentally infected tilapia using the species-specific PCR developed by MATA *et al.* (2004) with the species-specific qPCR described by SU *et al.* (2016) (TAVARES *et al.*, 2016). In this study, qPCR was more sensitive than conventional PCR, detecting GBS in 95.3% of infected fish against 51.1% of detection of the other technique.

The other two techniques (LAMP and MALDI-TOF) can only be performed using pure culture. The LAMP assay is a diagnostic tool that when combined with a dye indicator allows the visual identification of the pathogen. For GBS diagnosis, this technique was used to the detection of the bacterium in different tissues of broodstock fish, including ovary and testis. In addition, milt, egg and larvae from broodstock were also investigated and confirmed as positive for this infection (SUEBSING *et al.*, 2013; PRADEEP *et al.*, 2016). On the other hand, the MALDI-TOF approach allows the identification of bacterial species through of comparison of peptide mass fingerprints from the device database with the GBS fish strains being efficiently detected by this technology (ASSIS *et al.*, 2017a).

### 3.2.6. TREATMENT, PREVENTION AND CONTROL

The treatment of GBS infection is performed through the oral administration of antibiotics such as amoxicillin, enrofloxacin, oxytetracycline, florfenicol and others (AISYHAH *et al.*, 2015). Some of these drugs have demonstrated activity *in vitro* against fish GBS strains as showed in Table 3. The main methods used for the *in vitro* determination of GBS sensitivity to antibiotics are disc diffusion assays and the determination of minimum inhibitory concentration (MIC) (FARIA *et al.*, 2014). However, besides the antimicrobial susceptibility, other factors can influence the therapeutic effectiveness such as the pharmacokinetic and pharmacodynamic of the antibiotic, maximum plasma concentration, tissue distribution and drug dosage (MILLER e REIMSCHUESSEL, 2006).

As GBS crosses the blood-brain barrier causing meningoencephalitis in fish, the antibiotic must be capable to reach the brain tissue. However, information on the capacity of antibiotics, such as florfenicol and oxytetracycline to pass the blood-brain barrier is scarce. Other problems associated with the utilization of antibiotics are the reduction of feeding by the fish, with the anorexia being one of the first physiological changes induced by GBS infection. The antibiotic therapy is limited, since it avoids the occurrence of the disease in healthy fish, treat the initial stages of disease, but do not cure fish with clinical signs (FARIA *et al.*, 2014).

Table 3. Antimicrobial susceptibility of GBS strains to main antibiotics used in fish farms

| | **Antibiotic** | **Reference** |
|---|---|---|
| Sensitive | Chloramphenicol, Erythromycin, Rifampicin, Ampicillin, Sulfamethoxazole/trimethoprim, Tetracycline, Oxytetracycline, Gentamicin, Ciprofloxacin, Amoxicillin/clavulanic acid, Linomycin, Cephalexin, Nitrofurantoin, Ceftiofur, Florfenicol, Penicillin, Imipenem, Ceftriaxone and Streptomycin | EVANS *et al*., 2002; ABUSELIANA *et al.*, 2010; FARIA *et al*., 2014; AISYHAH *et al*., 2015; SOTO *et al*., 2015; CHIDEROLI *et al.*, 2017 |
| Resistant | Rifampin, Ampicillin, Amoxicillin/clavulanic acid, Streptomycin, Kanamycin, Neomycin, Amikacin, Enrofloxacin, Ciprofloxacin, Norfloxacin, Marbofloxacin, Gentamicin, Tobramycin, Sulfamethoxazole/trimethoprim, Tetracycline, Oxytetracycline, Oxolinic acid, Florfenicol, Nitrofurantion, Penicilin and Erythromycin | EVANS *et al*., 2002; ABUSELIANA *et al.*, 2010; FARIA *et al*., 2014; AISYHAH *et al*., 2015; SOTO *et al*., 2015; CHIDEROLI *et al.*, 2017 |

*In vivo* studies were conducted using the oral administration of florfenicol and oxytetracycline in Nile tilapia to evaluate the therapeutic efficacy of both antibiotics against GBS. In the florfenicol

trial, the standard dose of antibiotic (10 mg $kg^{-1}$) was not able to control the GBS infection in fish experimentally infected, showing a mortality rate of 90% during the treatment period (10 days) (OLIVEIRA, 2016). On the other hand, during the same period, the doses of 20 and 40 mg $kg^{-1}$ were efficient to control the infectious process. However, during the 20 days post-treatment, cumulative mortalities were verified in all groups treated, demonstrating that the treatment was not able to thwart the infection in fish regardless of the dose administrated, allowing, the pathogen transmission to healthy fish in cohabitation assays (OLIVEIRA, 2016). Regarding the oxytetracycline trial, it was observed that this antibiotic reduced significantly the number of dead Nile tilapia after the drug administration (24 hours before experimental infection, 1 and 24 hours post-infection) when compared to the control group (without antibiotic administration). However, after the experimental period, it was possible to reisolate the bacterium from the brain and kidney tissues from apparently healthy fish of all treated groups, characterizing thus the carrier state of the infection (FARIA *et al.*, 2014). Similar results were observed in trials with florfenicol, showing that both antibiotics may not control the infection and that the pathogen can remain viable in the different fish tissues, resulting, therefore, in the maintenance of the bacterium in fish farms.

The control of GBS infection can be performed using integrated approaches of fish health management including: water treatment with commercial biological filters and solid removal; monitoring of water quality (water temperature, dissolved oxygen, ammonia, nitrate, iron and alkalinity), good management practices, minimizing unnecessary handling or transportation of fish, consequently reducing the stress of fish; reduction of fish stocking densities according to the size of cages, fish sizes or types of culture systems; partial reduction or stop feeding to control or reduce mortality rates during outbreaks; frequent removal of moribund and dead fish; periodic cleaning and disinfection of all production units and equipments; acquisition of pathogen-free fish and periodic laboratory testings to certify that fish are GBS free; maintenance and monitoring of fish production and maintenance of health records (mortality rate, growth parameters, introduction or returning of fish and others) (ZAMRI-SAAD *et al.*, 2014).

Vaccination is an alternative method to prevention and control of streptococcosis in fish farms. Different experimental GBS vaccines have been developed and were extensively reviewed by LIU *et al.* (2016) and MIYABE *et al.* (2017). The commercial vaccine, AQUAVAC® Strep Sa, developed by Merck Animal Health, is available in different countries since 2011. In experimental trials, this vaccine was shown to be safe and able to induce effective protection in Nile tilapia challenged with GBS biotype 2 (non-hemolytic strains) (MERCK ANIMAL HEALTH, 2012). However, further studies are necessary to evaluate the relationships between genetic variability, antigenic profile and protection capacity against heterologous challenge induced by this vaccine. Therefore, this commercial vaccine should be tested against the various other fish GBS strains, especially with different genotypes and serotypes for a better verification of its effectiveness.

## 3.3. OMICS-BASED APPROACHES FOR THE STUDY OF FISH-PATHOGENIC STRAINS OF *Streptococcus agalactiae*

To provide new knowledge about diversity, pathogenesis and environmental adaptation of fish-pathogenic *Streptococcus agalactiae* strains, studies using omics-based approaches may be conducted.

Omics is a collective concept of high-throughput approaches for better understanding of fundamental biological processes using integrative strategies including genomics, transcriptomics and proteomics (MORRISON *et al.*, 2006). These technologies allow the elucidation of specific features of a given microorganism, such as physiological and pathogenic characteristics. However, it generates large datasets that will require an integrative approach using bioinformatic strategies to enable biological data analyses (YANG *et al.*, 2012).

### 3.3.1. GENOMICS

The knowledge about the nucleotide sequences of a bacterial genome provides important information about the existing genes , as well as their functions and localization, assisting, in this way, the study of gene expression, such as the transcription and translation of genetic information from genome, as well as enabling evolutionary trend studies of the pathogen (MADIGAN *et al.*, 2010).

With the advent of new sequencing technologies, known as next-generation sequencing (NGS), the identification of complete set of genes of a microorganism can be performed (MAIDEN *et al.*, 2013). By means of sequencing platforms like 454 GS FLX system (Roche), Illumina GA IIx (Illumina), HeliScope (Helicos), SOLiD system (ABI), PacBio RS system (Pacific Biosciences), Ion Torrent (Life Technologies) and MinION (Oxford Nanopore), the number of genomes available and the information generated by them increased rapidly (ZHANG *et al.*, 2011; HEATHER e CHAIN, 2016).

These platforms have been used to study the whole genome sequencing of several GBS strains, which are currently available in the NCBI database (http://www.ncbi.nlm.nih.gov/genome/genomes/186?). A total of 995 GBS genomes can be accessible, with 92 GBS strains having complete genomes, 146 draft genomes e 757 *contigs* (GLASER *et al.*, 2002; TETTELIN *et al.*, 2005; RICHARDS *et al.*, 2011; CALIOT *et al.*, 2012; WANG *et al.*, 2012; PEREIRA *et al.*, 2013; ROSINSKI-CHUPIN *et al.*, 2013; ZUBAIR *et al.*, 2013; PRIDGEON e ZHANG, 2014; HARDEN *et al.*, 2016). Of these, 93 genomes, among complete, draft or *contigs* sequences, represent isolates obtained from fish.

The GBS genome can be divided into core genome (genes present in all strains of this species), dispensable genome (genes present in some but not all strains) and unique genes (genes found exclusively in a given strain). The association of these three categories of genes represents the pan-genome, that is, the repertoire of all existing genes of the bacterial species studied (TETTELIN *et al.*, 2005; WANG *et al.*, 2015).

Comparative genome analysis of GBS strains obtained from different hosts and/or different geographic regions have allowed further understanding of the potential mechanism of evolution, virulence, immunogenicity and adaptation to a specific host (WANG *et al.*, 2015; DELANNOY *et al.*, 2016). Furthermore, this analysis also enables the search for dispensable genes, deletions or gene mutations, and the identification of genomic islands involved in pathogenicity as well as to predict possible proteins translated from bacterial genomes (SOARES *et al.*, 2016; WANG *et al.*, 2015).

In this context, LIU *et al.* (2013b) performed a comparison of 15 GBS genomes from different origins and observed that the pan-genome of this bacterial species consisted of 4,730 genes, with 1,202 belonging to the core genome, 1,388 being dispensable genomes and 2,040 being unique genes. The phylogenetic analysis of these 15 genomes showed a close relationship between strains from the same host as the clade was formed only by fish GBS strains (SA20-06 and STIR-CD-17), bovine GBS strain (FSL S3-026) and human GBS strains (18RS21 and 2603V/R, 515 and NEM316, COH1 and GBS00112). The authors observed that the Chinese fish GBS strains (GD201008-001 and ZQ0910) were very similar at the gene content level with the A909 strain (GBS human strain) since they grouped at the same evolutionary branch based on phylogenetic analysis. Similarly, the Thai fish GBS strains (FNA07 and FPrA02) also showed significant similarity to these Chinese GBS strains through comparative genomics, suggesting a possible common ancestor among these isolates with the sequence type ST-7 (KAYANSAMRUAJ *et al.*, 2015).

In another study, ROSINSKI-CHUPIN *et al.* (2013) sequenced seven GBS strains obtained from fish and frogs, and then performed phylogenetic analysis of these genomic sequences with other

sequences obtained from human and bovine strains. These authors observed that the GBS strains from aquatic animals belonged to three distinct genetic groups (CC7, ST-261 and ST-260) that differed by their adaptive survival strategies inside the host. It is known that fish GBS strains belonging to CC7 have the potential to colonize and infect multiple hosts, and these authors demonstrated that, at the genomic level, these fish GBS strains were indistinguishable from the human GBS strain with the same sequence type (A909 strain). These results corroborate with the study performed by LIU *et al.* (2013b). On the other hand, analyses conducted with the genomes of ST-260 and ST-261 strains indicated that they had no ancestry with any human or bovine GBS strains evaluated, demonstrating a specific adaptation to the aquatic animals. ROSINSKI-CHUPIN *et al.* (2013) have suggested that a reductive evolution might have contributed to the constitution of this distinct lineage. They identified a high number of pseudogenes among GBS fish strains, as well as deletions, insertions and nucleotide substitutions that were responsible for gene disruptions. Similarly, they found that approximately 60% of the genes involved in bacterial pathogenicity that were described as important virulence genes in human GBS strains, were affected by the reduction of the genome in the fish GBS strains. And these isolates (ST260-261) had also reduced ability to use various carbon sources as compared to the ST-7 strains (ROSINSKI-CHUPIN *et al.*, 2013).

Comparative genomics analysis was also used to evaluate the overall gene differences of a pathogenic fish GBS strain (HN016) as compared to its non-virulent variant (YM001) obtained after successive passages in culture medium (WANG *et al.*, 2015). In this study, WANG *et al.* (2015) observed that 10 functional genes were deleted in YM001 strain and that this could be the main reason for the loss of virulence in tilapia experimentally infected with $10^9$ CFU $fish^{-1}$ (there was no clinical signs or mortality associated with this bacterial dosage). These 10 genes encode proteins involved in bacterial growth and metabolism, and were not previously characterized as virulence genes, however, from this study, the authors suggested that they are important for the pathogenesis of GBS strains. WANG *et al.* (2015) pointed out that the deletion of these 10 functional genes had little effect on other virulence-related genes, which have contributed to the preservation of bacterial antigenicity during the virulence attenuation process by culture passages.

Alternatively, the allelic variation of GBS genes, previously identified by the MLST technique, can also be evaluated through the complete set of genes of the microorganism (whole genome), using a gene-to-gene approach (wgMLST). To date, wgMLST was used in the analysis of fish GBS strains by evaluating the number of identical genes of an isolate of the bacterium (SA20) that was sequenced using three different commercial kits and compared to the previously assembled reference genome (PEREIRA *et al.*, 2016). Furthermore, wgMLST was used to evaluate the population structure of fish-adapted GBS strains obtained from different fish farms in Brazil (BARONY *et al.*, 2017). In this work, advances in epidemiogenomics were observed, seeing that new genetic groups of GBS were identified in the country and that each isolate analyzed belonged to a single clone. Moreover, the study performed by BARONY *et al.* (2017) was the first study to estimate the emergency period of the mains STs involved with outbreaks diseases in fish (~585 years ago).

### 3.3.2. TRANSCRIPTOMICS

The transcriptome profile is a method that is able to analyze the gene expression in cells, tissues or organisms (ZHANG *et al.*, 2013a). The gene expression pattern may vary depending on the physiological or pathological conditions. In the case of infections by microorganisms, different patterns of expression can occur, resulting from different combinations of genes, activation and deactivation of events involved in transcription and translation, such as processing differential of mRNA, post-translational modifications and others (KOIZUMI, 2004).

The search for virulence and adaptation genes in GBS strains have also been performed by studies involving transcriptomic analysis. The GBS pathogenesis is a complex process that involves the

global remodeling of the transcriptome and not only the positive regulation of some virulence-related genes (MEREGHETTI *et al.*, 2008b). In this way, virulence factors that are responsible for bacterial persistence and dissemination in a given host can be studied using transcriptome assays, either by qRT-PCR or by selective capture of transcribed sequences (SCOTS). The latter can be conducted through the analysis of few transcripts, or by evaluating the global transcript analysis by microarray or NGS sequencing (RNA-seq) (SKVORTSOV e AZHIKINA, 2010; GUO *et al.*, 2014).

Studies have demonstrated that the GBS transcriptome is influenced by environmental variations, being observed the remodeling of transcript profile at different stages of bacterial growth (SITKIEWICZ e MUSSER, 2009), at different incubation temperatures (MEREGHETTI *et al.*, 2008b), after incubation in blood (MEREGHETTI *et al.*, 2008a; MEREGHETTI *et al.*, 2009), after incubation in amniotic fluid (SITKIEWICZ *et al.*, 2009), and after incubation in bovine milk (RICHARDS *et al.*, 2013). In addition, differences in the transcript expression under the same experimental condition with isolates obtained from different clinical cases (PAILHORIES *et al.*, 2013) or different hosts (ROSINSKI-CHUPIN *et al.*, 2013) were already performed.

Using the qRT-PCR technique, KAYANSAMRUAJ *et al.* (2014a) evaluated the gene expression of thirteen genes of fish GBS strains related to virulence. These authors cultured a fish GBS strain using two different temperatures (28ºC and 35ºC) and sampled the bacterium at two distinct growth stages (exponential growth phase and stationary phase). These authors observed that during the exponential phase, the expression of *cfb* (cell invasion) and *PI-2b* (adhesion) genes were slightly higher in the strain cultured at 35ºC that the one cultured at 28ºC. On the other hand, some genes were moderately up-regulated at 35ºC during the stationary phase, including *bibA* (adhesion), *sodA* (immune evasion), *dltA* (immune evasion), *cpsX* (immune evasion) and *cpsA* (invasion), while *cylE* (invasion), *cfb* and *PI-2b* were highly up-regulated.

SCOTS is a methodology of RNA analysis based on RT-PCR that also identifies transcribed genes in both *in vivo* and *in vitro* conditions. Bacterial mRNA is extracted, reverse transcribed for cDNA and labeled. Simultaneously, a control mRNA was extracted, labeled with biotin and hybridized with the bacterial cDNA. Then, streptavidin beads removed common genes in all conditions, remaining only the exclusively expressed genes under the desired condition. These genes are then cloned into plasmids, sequenced and identified (WILSON *et al.*, 2010). Using this strategy, GUO *et al.* (2014) identified GBS genes preferentially expressed from the interaction of the bacterium with murine macrophages (RAW264.7). In this case, the control mRNA was obtained from bacteria cultured *in vitro*. These authors found 60 differentially expressed transcripts under the interaction condition, being some of them considered to be virulence factors. Among these transcripts, it can be cited: *ciaH* (involved in bacterial adaptation to environmental changes), *hylB* (allows the dissemination of GBS in host tissues), *gap* (adhesion), *ccpA* (involved with resistance to phagocytosis) and *cov* (involved in cell lysis and survival in phagosomes).

On the other hand, the microarray technique has been widely used in gene expression studies due to its ability to investigate simultaneously thousands of transcripts and to identify differentially expressed genes between diseased and healthy tissues, pharmacogenomic responses and evolution of gene regulation in different species (ZHAO *et al.*, 2014). The microarray technology allows the evaluation of global gene expression as well as comparative analysis between genomes of different strains of the same species whose sequence of the genome is previously known. The latter is conducted through the hybridization of oligonucleotides derived from sequences of known or predicted genes (deposited on glass slides) with a cDNA prepared from total RNA or mRNA isolated from different cells or tissues whose expression pattern is to be compared (MARQUES e SILVA, 2004; MAIER *et al.*, 2009; TIAN *et al.*, 2013). This way, it has been a very useful technique for the transcriptome identification in GBS strains from human and fish hosts.

ROSINSKI-CHUPIN *et al.* (2013) performed a comparative transcriptome analyses using two fish GBS strains (2-22 and CF01173), one GBS frog strain (SS1219) and one human GBS strain (A909). These strains were cultured at 30ºC or 37ºC and subsequently collected after reaching the exponential phase of bacterial growth. Then, total RNA from all strains were extracted, reverse transcribed, labeled with Cy3 or Cy5 and hybridized on microarray slides containing oligonucleotides predicted for three GBS human strains (A909, NEM316 and COH1). The authors identified 1,389 transcripts presents in the four strains evaluated, being observed that CF01173 and A909 strains were closely related. However, the expression of the *srr-1* gene (adhesion and invasion) in the fish GBS strains was 20-fold higher than in the A909 strain. The authors suggested that the expression of this gene in fish GBS strains belonging to ST-7 could facilitate the GBS penetration into the blood-brain barrier and increase the probability of occurrence of neurological disorders in the host.

Regarding the fish GBS strains that are host-adapted (ST260-261), ROSINSKI-CHUPIN *et al.* (2013) detected that 29 genes varied in the same way, at the gene expression level among the isolates, including capsule biosynthesis operon, zoocin, hyaluronidase, two-component regulatory system (TCS) and genes related to energy metabolism. The authors suggested that the observed regulation of gene expression reflects a common mechanism of bacterial adaptation to aquatic environment. In this case, high expression of capsule biosynthesis genes could favor resistance to environmental conditions and to the immune system of the fish host, while the high expression of hyaluronidase could enable the invasion and dissemination of GBS in fish tissues.

Currently, the RNA-seq methodology has replaced the microarray technique to evaluate the transcriptome profile from bacterial pathogens, due to the direct sequencing of the transcripts by NGS (ZHAO *et al.*, 2014). This methodology allows the identification of all transcripts expressed by a pathogen at a given condition, besides allowing the detection of new transcripts, splice junctions, the expression of specific alleles, and corrections of genome annotations, independent on previous genomic annotations, avoiding then problems that may arise from the hybridization as it is observed in the microarray technology (MARTIN e WANG, 2011; ZHAO *et al.*, 2014).

Previous studies were able to analyze the differential gene expression in Nile tilapia tissues after experimental infections using fish GBS strains (ZHANG *et al.*, 2013a; WANG *et al.*, 2016; ZHU *et al.*, 2017), nonetheless no work evaluated the gene expression of the bacterium itself. Therefore, the transcriptome of fish GBS strains until the present date was not carried out using RNA-seq.

### 3.3.3. PROTEOMICS

The proteomic approaches allow the identification and quantification of a set of proteins synthetized by a determined microorganism. The pattern of protein synthesis can be evaluated by protein separation using two-dimensional polyacrylamide (2D) gel electrophoresis, or through the identification and sequencing of polypeptides through mass spectrometry technologies, such as matrix-assisted laser desorption ionization (MALDI)- time of flight (TOF) (BISCH, 2004). Mass spectrometry can be defined as the study of matter through the formation of ions in the gas phase and their characterization by mass, charge, structure or physicochemical properties, using mass spectrometer that measure m/z values and abundance of ions (VESSECCHI *et al.*, 2011).

The association between 2D gel electrophoresis and mass spectrometry was already considered the most appropriate method to recognize and identify proteins from pathogenic microorganisms (CORDWELL *et al.*, 2001) for being a methodology used for the construction of proteomic databases, due to its greater efficiency and high resolution to investigate the complex mixtures of proteins present in cell or tissues (BISCH, 2004). In this context, previous studies performed the comparative proteome analysis among fish GBS strains with different virulence (LI *et al.*, 2014a) and with different genotypes and serotypes (LI *et al.*, 2015), using the association between 2D

electrophoresis and mass spectrometry (MALDI-TOF), found different patterns of proteins expression among the strains evaluated.

Nevertheless, with the technical advances achieved in recent years, such as solubilization of complex samples, pH gradient and detection of proteins present in small quantities, the technique of liquid chromatography associated with mass spectrometry (LC-MS) started to be used and allowed the analysis of complex mixtures of proteins by tryptic digestion without prior gel separation (SILVA *et al.*, 2014; JEONG *et al.*, 2015). This technique had as advantage to have a low detection limit for peptides and proteins (some fentomoles), capability to identify hundreds to thousands of proteins in a simple experiment as well as allowing the study of membrane proteins, poorly accessible by others methods, since the concentration of these proteins after the extraction is too low to be detected in gels (SOLIS e CORDWELL, 2011).

LC-MS is divided into two techniques: stable isotopic labelling (HÖLPER *et al.*, 2014; KOSONO *et al.*, 2015) and label-free quantification (CHENG *et al.*, 2014; SILVA *et al.*, 2014). In the first technique, two solutions containing the proteins to be analyzed are labeled with different molecular mass isotopes, and are mixed, trypsin-digested to obtain peptides and submitted to the LC-MS system (KOSONO *et al.*, 2015). The molecular weight difference allows the identification and quantification of peptides of both samples tested (GYGI *et al.*, 2002), but the labeling occurs after the extraction step, which can lead to a reduction in the precision of the quantification method (PATEL *et al.*, 2009). Alternatively, label-free quantification allows the evaluation of numerous samples at the same time within the LC-MS system, with data-independent acquisition, and the concentration of a given peptide is proportional to its chromatographic area (CHELIUS e BONDARENKO, 2002).

The use of proteomic approaches has enabled the investigation, in prokaryotes, of physiological behaviors, mutations, adaptability to different environmental conditions, presence of proteins involved in virulence, and the identification of putative immunogenic proteins (HUSSAIN e HUYGENS, 2012). Furthermore, this approach has provided new knowledge on GBS pathogenesis for the different hosts studied and characterization of potential vaccine targets (DORO *et al.*, 2009; LI *et al.*, 2016).

Among the strategies used in proteomic studies using fish GBS strains, what stands out is the surfome (LIU *et al.*, 2013a; LI *et al.*, 2016), secretome (LI *et al.*, 2016) and comparative proteome analysis (LI *et al.*, 2014a; LI *et al.*, 2015).

The bacterial surface has been considered of great importance for the understanding of the pathogenesis of an infectious disease. On the surface, it can be found proteins associated with mechanisms of defense and virulence factors, which can promote adhesion and cellular invasion, culminating consequently in the appearance of clinical signs in an infected host (HUGHES *et al.*, 2002). Therefore, surfome is a proteome based method, in which allows the identification of bacterial surface proteins (DORO *et al.*, 2009).

Using 2D gel electrophoresis, LIU *et al.* (2013a) identified 22 immunoreactive spots from surface proteins extracted from a fish GBS strain (GD201008-001 strain) associated with hyperimmune serum, or guinea pig and tilapia antisera after experimental infections with the bacterium. The spots were excised from the 2D gel, digested, and the peptide sequences were determined by the mass spectrometry (MALDI-TOF). Sixteen proteins were identified in this study, such as GAPDH (immune evasion), PI-2b, phosphoglycerate kinase (immunoreactive protein), Sip (adhesion), Srr-1 (adhesion) and ArcB (survival and dissemination in acidic environment) being referenced as virulence factors of GBS strains.

LI *et al.* (2016) identified a total of 40 surface proteins using liquid chromatography-tandem mass spectrometry (LC-MS) after peptide analysis of extracted proteins of a fish GBS strain (THN0901 strain) was conducted. These authors identified membrane proteins, lipoproteins and extracellular proteins involved in virulence (Cell wall surface anchor family protein, IgA Fc receptor, BibA,

D-alanyl-D-alanine carboxypeptidase, Sip, PcsB protein and Hyaluronate lyase) and metabolism (ABC transporter substrate-binding protein, Nickel ABC transporter substrate-binding protein and Amino acid ABC transporter substrate-binding protein).

Apart from surface proteins, extracellular and secreted proteins are important in bacterial pathogenesis, since they also mediate the interaction of the bacterium with the host and by stimulating the immune response. Therefore, the secretome have been associated with adhesion, invasion, immune evasion and spread of bacterium in host tissues. In addition, these proteins can also be used for the development of antibiotics and vaccines (SILVA *et al.*, 2013b).

LI *et al.* (2016) also identified proteins (n = 26) that were secreted by fish GBS strains using LC-MS approach. Among the detected proteins, CAMP factor, Immunogenic secreted protein, Bsp protein, Hyaluronate lyase, Sip, Beta-lactamase, Collagen-binding protein, Reticulocyte-binding protein, PI-2b and CpsD were involved in virulence processes.

Comparative proteomic analysis have also been used to identify virulence factors and to obtain information on physiological and environmental adaptations in different pathogens (SILVA *et al.*, 2013a), including from GBS strains obtained from diseased fish (LI *et al.*, 2014a; LI *et al.*, 2015). LI *et al.* (2015) performed comparative proteomic analysis of nine GBS fish strains. These strains had different serotypes and different genotypes based on PFGE typing. From the association between 2D electrophoresis and mass spectrometry, it was found 43 proteins, with 12 differentially expressed proteins present just in strains with different serotypes (Ia x Ib) independently of their genotype. The following proteins were reported by the authors: cysteine synthetase (involved in homeostasis), SodA, GAPDH, Elongation factor tu (adhesion), fibronectin-binding protein (adhesion) and GrpE (heat shock protein). A lower number of differentially expressed proteins were identified after comparisons of the proteome of strains with the same serotype but with different genotypes. This result suggests that the difference among serotypes might be an essential role for differential protein expression in fish GBS strains.

In order to identify proteins present in a virulent GBS strain, LI *et al.* (2014a) conducted analysis by 2D electrophoresis associated with mass spectrometry, of the proteome of two fish GBS strains: virulent (THN0901 strain) and attenuated (TFJ0901 strain) isolates, both obtained from Nile tilapia. The comparative proteome analyses of these strains showed the average detection of 507 spots per isolate, with 38 spots being exclusively expressed in the TFJ0901 or THN0901 strains. Among these spots, 23 proteins were identified by MALDI-TOF, with 13 being identified exclusively in TFJ0901 and 10 proteins in THN0901. According to the authors, the proteins identified were involved in metabolic pathways and it was suggested that these proteins participate in virulence differentiations between the two strains tested. Among proteins, it was detected DnaJ (heat shock), dihydrolipoamide dehydrogenase (evasion), Thioredoxin (oxidative stress), manganese-dependent inorganic pyrophosphatase (regulation of bacterial growth), elongation factor Tu, bleomycin resistance protein (multidrug resistance) and DivIVA cell division protein (dissemination).

The proteomic techniques used to study fish GBS strains, until the present date, have a low detection capacity of proteins when compared with a high throughput methodology as shotgun proteomics, a label-free approach. From this approach the proteins are measured through a peptide mixture obtained from proteolytic digestion of intact proteins subjected to LC-MS (ZHANG *et al.*, 2013b), allowing the detection of several thousand proteins. Therefore, the utilization of shotgun proteomics can be providing new insights about biological process mechanisms related with GBS infection in aquatic host.

## 3.4. REFERENCES


ABUSELIANA, A.; DAUD, H.; AZIZ, S. A. *et al. Streptococcus agalactiae* the etiological agent of mass mortality in farmed red tilapia (*Oreochromis* sp.). *J. Anim. Vet. Adv.*, v. 9, p. 2640-2646, 2010.

AISYHAH, M. A. S.; AMAL, M. N. A.; ZAMRI-SAAD, M. *et al. Streptococcus agalactiae* isolates from cultured fishes in Malaysia manifesting low resistance pattern towards selected antibiotics. *J. Fish Dis.*, v. 38, n. 12, p. 1093-1098, 2015.

AL NAKIB, M.; LONGO, M.; TAZI, A. *et al.* Comparison of the Diversilab® system with multi-locus sequence typing and pulsed-field gel electrophoresis for the characterization of *Streptococcus agalactiae* invasive strains. *J. Microbiol. Methods*, v. 85, n. 2, p. 137-142, 2011.

AMAL, M. N. A.; SAAD, M. Z.; ZAHRAH, A. S. *et al.* Water quality influences the presence of *Streptococcus agalactiae* in cage cultured red hybrid tilapia, *Oreochromis niloticus* × *Oreochromis mossambicus*. *Aquacult. Res.*, v. 46, n. 2, p. 313-323, 2015.

AMAL, M. N. A.; ZAMRI-SAAD, M.; IFTIKHAR, A. R. *et al.* An outbreak of *Streptococcus agalactiae* infection in cage-cultured golden pompano, *Trachinotus blochii* (Lacépède), in Malaysia. *J. Fish Dis.*, v. 35, n. 11, p. 849-852, 2012.

AMAL, M. N. A.; ZARIF, S. T.; SUHAIBA, M. *et al.* The effect of fish gender on susceptibility to acute *Streptococcus agalactiae* infection in Javanese medaka *Oryzias javanicus*. *Microb. Pathog.*, v. 114, n. 1, p. 251-254, 2018.

AMBORSKI, R. L.; SNIDER, T. G.; THUNE, R. L. *et al.* A non-hemolytic, Group B *Streptococcus* infection of cultured bullfrogs, *Rana catesbeiana*, in Brazil. *J. Wildlife Dis.*, v. 19, n. 3, p. 180-184, 1983.

AREECHON, N.; KANNIKA, K.; HIRONO, I. *et al.* Draft genome sequences of *Streptococcus agalactiae* serotype Ia and III isolates from tilapia farms in Thailand. *Genome Announc.*, v. 4, n. 2, p. e00122-16, 2016.

ARMAS, F.; CAMPERIO, C.; MARIANELLI, C. *In vitro* assessment of the probiotic potential of *Lactococcus lactis* LMG 7930 against ruminant mastitis-causing pathogens. *Plos One*, v. 12, n. 1, p. e0169543, 2017.

ASENCIOS, Y. O.; SÁNCHEZ, F. B.; MENDIZÁBAL, H. B. *et al.* First report of *Streptococcus agalactiae* isolated from *Oreochromis niloticus* in Piura, Peru: molecular identification and histopathological lesions. *Aquacult. Rep.*, v. 4, n. Supplement C, p. 74-79, 2016.

ASSIS, G. B. N.; PEREIRA, F. L.; ZEGARRA, A. U. *et al.* Use of MALDI-TOF mass spectrometry for the fast identification of Gram-positive fish pathogens. *Front. Microbiol.*, v. 8, n. 1492, 2017a.

ASSIS, G. B. N.; TAVARES, G. C.; PEREIRA, F. L. *et al.* Natural coinfection by *Streptococcus agalactiae* and *Francisella noatunensis* subsp. *orientalis* in farmed Nile tilapia (*Oreochromis niloticus* L.). *J. Fish Dis.*, v. 40, n. 1, p. 51-63, 2017b.

AUSTIN, B.; AUSTIN, D. A. *Bacterial fish pathogens: diseases of farmed and wild fish*. Chichester: Praxis Publishing, 2007.

BALDISSERA, M. D.; SOUZA, C. F.; PARMEGGIANI, B. S. *et al. Streptococcus agalactiae* impairs cerebral bioenergetics in experimentally infected silver catfish. *Microb. Pathog.*, v. 111, n. Supplement C, p. 28-32, 2017.

BARATO, P.; MARTINS, E. R.; MELO-CRISTINO, J. *et al.* Persistence of a single clone of *Streptococcus agalactiae* causing disease in tilapia (*Oreochromis* sp.) cultured in Colombia over 8 years. *J. Fish Dis.*, v. 38, n. 12, p. 1083-1087, 2015.

BARONY, G. M.; TAVARES, G. C.; PEREIRA, F. L. *et al.* Large-scale genomic analyses reveal the population structure and evolutionary trends of *Streptococcus agalactiae* strains in Brazilian fish farms. *Sci. Rep.*, v. 7, n. 1, p. 13538, 2017.

BAYA, A. M.; LUPIANI, B.; HETRICK, F. M. *et al.* Association of *Streptococcus* sp. with fish mortalities in the Chesapeake Bay and its tributaries. *J. Fish Dis.*, v. 13, n. 3, p. 251-253, 1990.

BECEIRO, A.; TOMÁS, M.; BOU, G. Antimicrobial resistance and virulence: a successful or deleterious association in the bacterial world? *Clin. Microbiol. Rev.*, v. 26, n. 2, p. 185-230, 2013.

BERGAL, A.; LOUCIF, L.; BENOUARETH, D. E. *et al.* Molecular epidemiology and distribution of serotypes, genotypes, and antibiotic resistance genes of *Streptococcus agalactiae* clinical isolates from Guelma, Algeria and Marseille, France. *Eur. J. Clin. Microbiol. Infect. Dis.*, v. 34, n. 12, p. 2339-48, 2015.

BERRIDGE, B. R.; BERCOVIER, H.; FRELIER, P. F. *Streptococcus agalactiae* and *Streptococcus difficile* 16S–23S intergenic rDNA: genetic homogeneity and species-specific PCR. *Vet. Microbiol.*, v. 78, n. 2, p. 165-173, 2001.

BISCH, P. M. Genômica funcional: proteômica. In: MIR, L. *Genômica*. São Paulo: Atheneu, 2004. cap. 8, p.139-162.

BISHARAT, N.; CROOK, D. W.; LEIGH, J. *et al.* Hyperinvasive neonatal Group B *Streptococcus* has arisen from a bovine Ancestor. *J. Clin. Microbiol.*, v. 42, n. 5, p. 2161-2167, 2004.

BISHOP, E J.; SHILTON, C.; BENEDICT, S. *et al.* Necrotizing fasciitis in captive juvenile *Crocodylus porosus* caused by *Streptococcus agalactiae*: an outbreak and review of the animal and human literature. *Epidemiol. Infect.*, v. 135, n. 8, p. 1248-1255, 2007.

BOWATER, R. O.; DENNIS, M. M.; BLYDE, D. *et al.* Epizootics of *Streptococcus agalactiae* infection in captive rays from Queensland, Australia. *J. Fish Dis.*, v. 41, n. 2, p. 223-232, 2018.

BOWATER, R. O.; FORBES-FAULKNER, J.; ANDERSON, I. G. *et al.* Natural outbreak of *Streptococcus agalactiae* (GBS) infection in wild giant Queensland grouper, *Epinephelus lanceolatus* (Bloch), and other wild fish in northern Queensland, Australia. *Jo. Fish Dis.*, v. 35, n. 3, p. 173-186, 2012.

BROCHET, M.; COUVÉ, E.; ZOUINE, M. *et al.* Genomic diversity and evolution within the species *Streptococcus agalactiae*. *Microbes Infect.*, v. 8, n. 5, p. 1227-1243, 2006.

CALIOT, É.; DRAMSI, S.; CHAPOT-CHARTIER, M.-P. *et al.* Role of the Group B antigen of *Streptococcus agalactiae*: a peptidoglycan-anchored polysaccharide involved in cell wall biogenesis. *Plos Pathog.*, v. 8, n. 6, p. e1002756, 2012.

CARVALHO-CASTRO, G. A.; SILVA, J. R.; PAIVA, L. V. *et al.* Molecular epidemiology of *Streptococcus agalactiae* isolated from mastitis in Brazilian dairy herds. *Braz. J. Microbiol.*, v. 48, n. 3, p. 551-559, 2017.

CHAFFIN, D. O.; MENTELE, L. M.; RUBENS, C. E. Sialylation of Group B streptococcal capsular polysaccharide is mediated by *cpsK* and is required for optimal capsule polymerization and expression. *J. Bacteriol.*, v. 187, n. 13, p. 4615-4626, 2005.

CHAU, M. L.; CHEN, S. L.; YAP, M. *et al.* Group B *Streptococcus* infections caused by improper sourcing and handling of fish for raw consumption, Singapore, 2015–2016. *Emerg. Infect. Dis.*, v. 23, n. 12, p. 2002, 2017.

CHELIUS, D.; BONDARENKO, P. V. Quantitative profiling of proteins in complex mixtures using liquid chromatography and mass spectrometry. *J. Proteome Res.*, v. 1, n. 4, p. 317-23, 2002.

CHEN, M.; WANG, R.; LUO, F.-G. *et al. Streptococcus agalactiae* isolates of serotypes Ia, III and V from human and cow are able to infect tilapia. *Vet. Microbiol.*, v. 180, n. 1–2, p. 129-135, 2015.

CHENG, K.; SLOAN, A.; MCCORRISTER, S. *et al.* Quality evaluation of LC-MS/MS-based *E. coli* H antigen typing (MS-H) through label-free quantitative data analysis in a clinical sample setup. *Proteomics*, v. 8, n. 11-12, p. 963-970, 2014.

CHIDEROLI, R. T.; AMOROSO, N.; MAINARDI, R. M. *et al.* Emergence of a new multidrug-resistant and highly virulent serotype of *Streptococcus agalactiae* in fish farms from Brazil. *Aquaculture*, v. 479, p. 45-51, 2017.

CHONG, S. M.; WONG, W. K.; LEE, W. Y. *et al. Streptococcus agalactiae* outbreaks in cultured golden pomfret, *Trachinotus blochii* (Lacépède), in Singapore. *J. Fish Dis.*, v. 40, n. 7, p. 971-974, 2017.

CHU, C.; HUANG, P.-Y.; CHEN, H.-M. *et al.* Genetic and pathogenic difference between *Streptococcus agalactiae* serotype Ia fish and human isolates. *BMC Microbiol.*, v. 16, n. 1, p. 175, 2016.

CIESLEWICZ, M. J.; CHAFFIN, D.; GLUSMAN, G. *et al.* Structural and genetic diversity of Group B *Streptococcus* capsular polysaccharides. *Infect. Immun.*, v. 73, n. 5, p. 3096-3103, 2005.

CODY, A. J.; BENNETT, J. S.; MAIDEN, M. C. J. Chapter 10 - Multi-locus sequence typing and the gene-by-gene approach to bacterial classification and analysis of population variation. In: GOODFELLOW, M.;SUTCLIFFE, I., *et al*. *Methods in Microbiology*. Academic Press, v. 41, p. 201-219, 2014.

CONROY, G. Tilapia streptococcosis: prevalence of *Streptococcus* species in Latin America and their pathological manifestations. Managing *Streptococcus* in warmwater fish, 2009, Veracruz, Mexico. p.15-20.

CORDWELL, S. J.; NOUWENS, A. S.; WALSH, B. J. Comparative proteomics of bacterial pathogens. *Proteomics*, v. 1, n. 4, p. 461-472, 2001.

DEFENG, Z.; WEI, Y.; XIAOLI, K. *et al.* Molecular characteristics and transmission of *Streptococcus agalactiae* in a major tilapia culturing area of China. *J. Fishery Sci. China*, v. 24, n. 3, p. 606-614, 2017.

DELAMARE-DEBOUTTEVILLE, J.; BOWATER, R.; CONDON, K. *et al.* Infection and pathology in Queensland grouper, *Epinephelus lanceolatus*, (Bloch), caused by exposure to *Streptococcus agalactiae* via different routes. *J. Fish Dis.*, v. 38, n. 12, p. 1021-1035, 2015.

DELANNOY, C. M.; CRUMLISH, M.; FONTAINE, M. C. *et al.* Human *Streptococcus agalactiae* strains in aquatic mammals and fish. *BMC Microbiol.*, v. 13, n. 1, p. 1-9, 2013.

DELANNOY, C. M. J.; ZADOKS, R. N.; CRUMLISH, M. *et al.* Genomic comparison of virulent and non-virulent *Streptococcus agalactiae* in fish. *J. Fish Dis.*, v. 39, n. 1, p. 13-29, 2016.

DENG, Z. B.; ZHANG, Y. W.; GENG, Y. *et al.* A new sequence type (ZST-1) of infectious *Streptococcus agalactiae* from Chinese cyprinid fish, *Schizopygopsis pylzovi*. *Aquaculture*, v. 468, n. Part 1, p. 496-500, 2017.

DOMEÉNECH, A.; DERENAÁANDEZ-GARAYZÁBAL, J. F.; PASCUAL, C. *et al.* Streptococcosis in cultured turbot, *Scopthalmus maximus* (L.), associated with *Streptococcus parauberis*. *J. Fish Dis.*, v. 19, n. 1, p. 33-38, 1996.

DORAN, K. S.; NIZET, V. Molecular pathogenesis of neonatal Group B streptococcal infection: no longer in its infancy. *Mol. Microbiol.*, v. 54, n. 1, p. 23-31, 2004.

DORO, F.; LIBERATORI, S.; RODRÍGUEZ-ORTEGA, M. J. *et al.* Surfome analysis as a fast track to vaccine discovery: identification of a novel protective antigen for Group B *Streptococcus* hypervirulent strain COH1. *Mol. Cell. Proteomics*, v. 8, n. 7, p. 1728-1737, 2009.

DUREMDEZ, R.; AL-MARZOUK, A.; QASEM, J. A. *et al.* Isolation of *Streptococcus agalactiae* from cultured silver pomfret, *Pampus argenteus* (Euphrasen), in Kuwait. *J. Fish Dis.*, v. 27, n. 5, p. 307-310, 2004.

EISENBERG, T.; RAU, J.; WESTERHÜS, U. *et al. Streptococcus agalactiae* in elephants – a comparative study with isolates from human and zoo animal and livestock origin. *Vet. Microbiol.*, v. 204, n. Supplement C, p. 141-150, 2017.

EL AAMRI, F.; PADILLA, D.; ACOSTA, F. *et al.* First report of *Streptococcus iniae* in red porgy (*Pagrus pagrus*, L.). *J. Fish Dis.*, v. 33, n. 11, p. 901-905, 2010.

ELDAR, A.; BEJERANO, Y.; BERCOVIER, H. *Streptococcus shiloi* and *Streptococcus difficile*: two new streptococcal species causing a meningoencephalitis in fish. *Curr. Microbiol.*, v. 28, n. 3, p. 139-143, 1994.

ELLIOTT, J. A.; FACKLAM, R. R.; RICHTER, C. B. Whole-cell protein patterns of nonhemolytic Group B, type Ib, streptococci isolated from humans, mice, cattle, frogs, and fish. *J. Clin. Microbiol.*, v. 28, n. 3, p. 628-630, 1990.

EVANS, J. J.; BOHNSACK, J. F.; KLESIUS, P. H. *et al.* Phylogenetic relationships among *Streptococcus agalactiae* isolated from piscine, dolphin, bovine and human sources: a dolphin and piscine lineage associated with a fish epidemic in Kuwait is also associated with human neonatal infections in Japan. *J. Med. Microbiol.*, v. 57, n. 11, p. 1369-1376, 2008.

EVANS, J. J.; KLESIUS, P. H.; GILBERT, P. M. *et al.* Characterization of β-haemolytic Group B *Streptococcus agalactiae* in cultured seabream, *Sparus auratus* L., and wild mullet, *Liza klunzingeri* (Day), in Kuwait. *J. Fish Dis.*, v. 25, n. 9, p. 505-513, 2002.

EVANS, J. J.; PASNIK, D. J.; KLESIUS, P. H. A commercial rapid optical immunoassay detects *Streptococcus agalactiae* from aquatic cultures and clinical specimens. *Vet. Microbiol.*, v. 144, n. 3, p. 422-428, 2010.

EVANS, J. J.; PASNIK, D. J.; KLESIUS, P. H. Differential pathogenicity of five *Streptococcus agalactiae* isolates of diverse geographic origin in Nile tilapia (*Oreochromis niloticus* L.). *Aquacult. Res.*, v. 46, n. 10, p. 2374-2381, 2015.

FACIMOTO, C. T.; CHIDEROLI, R. T.; GONÇALVES, D. D. *et al.* Whole-Genome sequence of *Streptococcus agalactiae* strain S13, isolated from a fish eye from a Nile tilapia farm in southern Brazil. *Genome Announc.*, v. 5, n. 35, p. e00917-17, 2017.

FARIA, F. C.; LEAL, C. A. G.; CARVALHO-CASTRO, G. A. *et al.* Carrier state induced by oxytetracycline therapy against streptococcosis in Nile tilapia, *Oreochromis niloticus* (L.). *J. Fish Dis.*, v. 37, n. 9, p. 853-857, 2014.

FEIL, E. J.; LI, B. C.; AANENSEN, D. M. *et al.* eBURST: Inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J. Bacteriol.*, v. 186, n. 5, p. 1518-1530, 2004.

FIGUEIREDO, H. C. P.; CARNEIRO, D. O.; FARIA, F. C. *et al. Streptococcus agalactiae* associado à meningoencefalite e infecção sistêmica em tilápia-do-nilo (*Oreochromis niloticus*) no Brasil. *Arq. Bras. Med. Vet. Zootec.*, v. 58, p. 678-680, 2006.

FORTIN, M.; HIGGINS, R. Mixed infection associated with a Group B *Streptococcus* in a dog. *Can. Vet. J.*, v. 42, n. 9, p. 730-730, 2001.

GENG, Y.; WANG, K. Y.; HUANG, X. L. *et al. Streptococcus agalactiae*, an emerging pathogen for cultured ya-fish, *Schizothorax prenanti*, in China. *Transbound. Emerg. Dis.*, v. 59, n. 4, p. 369-375, 2012.

GLASER, P.; RUSNIOK, C.; BUCHRIESER, C. *et al.* Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Mol. Microbiol.*, v. 45, n. 6, p. 1499-1513, 2002.

GODOY, D. T.; CARVALHO-CASTRO, G. A.; LEAL, C. A. G. *et al.* Genetic diversity and new genotyping scheme for fish pathogenic *Streptococcus agalactiae*. *Lett. Appl. Microbiol.*, v. 57, n. 6, p. 476-483, 2013.

GUO, C. M.; CHEN, R. R.; KALHORO, D. H. *et al.* Identification of genes preferentially expressed by highly virulent piscine *Streptococcus agalactiae* upon interaction with macrophages. *Plos One*, v. 9, n. 2, p. 13, 2014.

GYGI, S. P.; RIST, B.; GRIFFIN, T. J. *et al.* Proteome analysis of low-abundance proteins using multidimensional chromatography and isotope-coded affinity tags. *J. Proteome Res.*, v. 1, n. 1, p. 47-54, 2002.

HAENEN, O. L.; EVANS, J. J.; BERTHE, F. Bacterial infections from aquatic species: potential for and prevention of contact zoonoses. *Rev. Sci. Tech.*, v. 32, n. 2, p. 497-507, 2013.

HARDEN, L. K.; MORALES, K. M.; HUGHEY, J. R. Complete genome sequence of nonhemolytic *Streptococcus agalactiae* serotype V strain 1, isolated from the buccal cavity of a canine. *Genome Announc.*, v. 4, n. 1, p. e01612-15. 2016.

HASSAN, A. A.; ABDULMAWJOOD, A.; YILDIRIM, A. O. *et al.* Identification of streptococci isolated from various sources by determination of *cfb* gene and other CAMP-factor genes. *Can. J. Microbiol.*, v. 46, n. 10, p. 946-951, 2000.

HEATHER, J. M.; CHAIN, B. The sequence of sequencers: the history of sequencing DNA. *Genomics*, v. 107, n. 1, p. 1-8, 2016.

HERNÁNDEZ, E.; FIGUEROA, J.; IREGUI, C. Streptococcosis on a red tilapia, *Oreochromis* sp., farm: a case study. *J. Fish Dis.*, v. 32, n. 3, p. 247-252, 2009.

HETZEL, U.; KÖNIG, A.; YILDIRIM, A. Ö. *et al.* Septicaemia in emerald monitors (*Varanus prasinus* Schlegel 1839) caused by *Streptococcus agalactiae* acquired from mice. *Vet. Microbiol.*, v. 95, n. 4, p. 283-293, 2003.

HÖLPER, S.; RUHS, A.; KRÜGER, M. Stable isotope labeling for proteomic analysis of tissues in mouse. In: WARSCHEID, B. *Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC): Methods and Protocols*. New York, NY: Springer New York, p. 95-106, 2014.

HOSHINA, T.; SANO, T.; MORIMOTO, Y. A *Streptococcus* pathogenic to fish. *J. Tokyo Univ. Fish.*, v. 44, p. 57-58, 1958.

HUGHES, M. J. G.; MOORE, J. C.; LANE, J. D. *et al.* Identification of major outer surface proteins of *Streptococcus agalactiae*. *Infect. Immun.*, v. 70, n. 3, p. 1254-1259, 2002.

HUSSAIN, M. A.; HUYGENS, F. Proteomic and bioinformatics tools to understand virulence mechanisms in *Staphylococcus aureus*. *Curr. Proteomics*, v. 9, n. 1, p. 2-8, 2012.

IREGUI, C.; BARATO, P.; REY, A. *et al.* Epidemiology of *Streptococcus agalactiae* and streptococcosis in tilapia (*Oreochromis* sp). In. *Epidemiology: Theory, Research and Practice*: iConcept Press, cap. 10, 2014.

IREGUI, C. A.; COMAS, J.; VASQUEZ, G. M. *et al.* Experimental early pathogenesis of *Streptococcus agalactiae* infection in red tilapia *Oreochromis* spp. *J. Fish Dis.*, v. 39, n. 2, p. 205-215, 2016.

JEONG, E.-H.; VAIDYA, B.; CHO, S.-Y. *et al.* Identification of regulators of the early stage of viral hemorrhagic septicemia virus infection during curcumin treatment. *Fish Shellfish Immunol.*, v. 45, n. 1, p. 184-193, 2015.

JIMÉNEZ, A.; TIBATÁ, V.; JUNCA, H. *et al.* Evaluating a nested-PCR assay for detecting *Streptococcus agalactiae* in red tilapia (*Oreochromis* sp.) tissue. *Aquaculture*, v. 321, n. 3, p. 203-206, 2011.

JOHNSON, J. K.; ARDUINO, S. M.; STINE, O. C. *et al.* Multilocus sequence typing compared to pulsed-field gel electrophoresis for molecular typing of *Pseudomonas aeruginosa*. *J. Clin. Microbiol.*, v. 45, n. 11, p. 3707-3712, 2007.

JOHRI, A. K.; PAOLETTI, L. C.; GLASER, P. *et al.* Group B *Streptococcus*: global incidence and vaccine development. *Nat. Rev. Microbiol.*, v. 4, n. 12, p. 932-942, 2006.

JOLLEY, K. A.; MAIDEN, M. C. BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics*, v. 11, n. 1, p. 1-11, 2010.

JONES, N.; BOHNSACK, J. F.; TAKAHASHI, S. *et al.* Multilocus sequence typing system for Group B *Streptococcus*. *J. Clin. Microbiol.*, v. 41, n. 6, p. 2530-2536, 2003.

JØRGENSEN, H. J.; NORDSTOGA, A. B.; SVILAND, S. *et al. Streptococcus agalactiae* in the environment of bovine dairy herds – rewriting the textbooks? *Vet. Microbiol.*, v. 184, p. 64-72, 2016.

KALIMUDDIN, S.; CHEN, S. L.; LIM, C. T. K. *et al.* 2015 Epidemic of severe *Streptococcus agalactiae* sequence type 283 infections in Singapore associated with the consumption of raw freshwater fish: a detailed analysis of clinical, epidemiological, and bacterial sequencing data. *Clin. Infect. Dis.***,** v. 64, n. suppl_2, p. S145-S152, 2017.

KAYANSAMRUAJ, P.; PIRARAT, N.; HIRONO, I. *et al.* Increasing of temperature induces pathogenicity of *Streptococcus agalactiae* and the up-regulation of inflammatory related genes in infected Nile tilapia (*Oreochromis niloticus*). *Vet. Microbiol.*, v. 172, n. 1–2, p. 265-271, 2014a.

KAYANSAMRUAJ, P.; PIRARAT, N.; KATAGIRI, T. *et al.* Molecular characterization and virulence gene profiling of pathogenic *Streptococcus agalactiae* populations from tilapia (*Oreochromis* sp.) farms in Thailand. *J. Vet. Diagn. Investig.*, v. 26, n. 4, p. 488-495, 2014b.

KAYANSAMRUAJ, P.; PIRARAT, N.; KONDO, H. *et al.* Genomic comparison between pathogenic *Streptococcus agalactiae* isolated from Nile tilapia in Thailand and fish-derived ST7 strains. *Infect. Genet. Evol.*, v. 36, p. 307-314, 2015.

KEEFE, G. P. *Streptococcus agalactiae* mastitis: a review. *Can. Vet. J.*, v. 38, n. 7, p. 429-437, 1997.

KESKIN, D.; ATAY, O.; KIRKAN, S. *et al.* Detection of *Streptococcus agalactiae* existence within milk samples of hair goats grown in West Anatolia region. *Agric. J.*, v. 6, n. 1, p. 31-34, 2011.

KOH, T. H.; CAO, D. Y.; KHOO, B. C. *et al.* Group B streptococci in sushi and sashimi. *Ann. Acad. Med. Singapore*, v. 46, n. 2, p. 74-75, 2017.

KOIZUMI, S. Application of DNA microarrays in occupational health research. *J. Occup. Health*, v. 46, n. 1, p. 20-5, 2004.

KONG, F.; LAMBERTSEN, L. M.; SLOTVED, H.-C. *et al.* Use of phenotypic and molecular serotype identification methods to characterize previously nonserotypeable Group B streptococci. *J. Clin. Microbiol.*, v. 46, n. 8, p. 2745-2750, 2008.

KOSONO, S.; TAMURA, M.; SUZUKI, S. *et al.* Changes in the acetylome and succinylome of *Bacillus subtilis* in response to carbon source. *Plos One*, v. 10, n. 6, p. e0131169, 2015.

LÄMMLER, C.; ABDULMAWJOOD, A.; WEIß, R. Properties of serological Group B streptococci of dog, cat and monkey origin. *J. Vet. Med., Series B*, v. 45, n. 1-10, p. 561-566, 1998.

LANCEFIELD, R. C. A serological differentiation of specific types of bovine hemolytic streptococci (Group B). *J. Exp. Med.*, v. 59, n. 4, p. 441-458, 1934.

LEIRA, M. H. *Susceptibilidade de serrasalmínideos e seus híbridos a parasitos em sistema de recirculação indoor e de peixes em policultivo a bactérias.* 2016. 98f . Tese (Doutorado) - Universidade Federal de Lavras, Lavras, MG.

LI-HUI, L.; DEFENG, Z.; NING-QIU, L. *et al.* Identification, serotype analysis and drug sensitivity test of *Streptococcus agalactiae* from *Cirrhinus molitorella*. *J. Southern Agric.*, v. 46, n. 11, p. 2053-2058, 2015.

LI, L.; SHI, Y.; WANG, R. *et al.* Proteomic analysis of tilapia *Oreochromis niloticus Streptococcus agalactiae* strains with different genotypes and serotypes. *J. Fish Biol.*, v. 86, n. 2, p.615-636, 2015.

LI, W.; SU, Y. L.; MAI, Y. Z. *et al.* Comparative proteome analysis of two *Streptococcus agalactiae* strains from cultured tilapia with different virulence. *Vet. Microbiol.*, v. 170, n. 1-2, p. 135-143, 2014a.

LI, W.; WANG, H.-Q.; HE, R.-Z. *et al.* Major surfome and secretome profile of *Streptococcus agalactiae* from Nile tilapia (*Oreochromis niloticus*): insight into vaccine development. *Fish Shellfish Immunol.*, v. 55, p. 737-745, 2016.

LI, Y. W.; LIU, L.; HUANG, P. R. *et al.* Chronic streptococcosis in Nile tilapia, *Oreochromis niloticus* (L.), caused by *Streptococcus agalactiae*. *J. Fish Dis.*, v. 37, n. 8, p. 757-763, 2014b.

LIMANSKY, A. S.; SUTICH, E. G.; GUARDATI, M. C. *et al.* Genomic diversity among *Streptococcus agalactiae* isolates detected by a degenerate oligonucleotide-primed amplification assay. *J. Infect. Dis.*, v. 177, n. 5, p. 1308-1313, 1998.

LIN, F. P.-Y.; LAN, R.; SINTCHENKO, V. *et al.* Computational bacterial genome-wide analysis of phylogenetic profiles reveals potential virulence genes of *Streptococcus agalactiae*. *Plos One*, v. 6, n. 4, p. e17964, 2011.

LIU, G.; ZHANG, W.; LU, C. Identification of immunoreactive proteins of *Streptococcus agalactiae* isolated from cultured tilapia in China. *Pathog. Dis.*, v. 69, n. 3, p. 223-231, 2013a.

LIU, G.; ZHU, J.; CHEN, K. *et al.* Development of *Streptococcus agalactiae* vaccines for tilapia. *Dis. Aquat. Org.*, v. 122, n. 2, p. 163-170, 2016.

LIU, G. J.; ZHANG, W.; LU, C. P. Comparative genomics analysis of *Streptococcus agalactiae* reveals that isolates from cultured tilapia in China are closely related to the human strain A909. *BMC Genomics*, v. 14, p. 10, 2013b.

LIU, L.; LI, Y. W.; HE, R. Z. *et al.* Outbreak of *Streptococcus agalactiae* infection in barcoo grunter, *Scortum barcoo* (McCulloch & Waite), in an intensive fish farm in China. *J. Fish Dis.*, v. 37, n. 12, p. 1067-72, 2014.

LPSN. List of Prokaryotic names with Standing in Nomenclature. 2017. Disponível em: < http://www.bacterio.net/streptococcus.html >. Acesso em: 15 dez. 2017.

LUSIASTUTI, A. M.; TEXTOR, M.; SEEGER, H. *et al.* The occurrence of *Streptococcus agalactiae* sequence type 261 from fish disease outbreaks of tilapia *Oreochromis niloticus* in Indonesia. *Aquacult. Res.*, v. 45, n. 7, p. 1260-1263, 2014.

MADIGAN, M. T.; MARTINHO, J. M.; DUNLAP, P. V. *et al. Microbiologia de Brock*. Porto Alegre: Artmed, 2010. 1160p.

MAGALHÃES, V. D.; FERREIRA, J. C.; BARELLI, C. *et al.* Eletroforese em campo pulsante em bacteriologia: uma revisão técnica. *Rev. Instit. Adolfo Lutz*, v. 64, p. 155-161, 2005.

MAIDEN, M. C. J.; VAN RENSBURG, M. J. J.; BRAY, J. E. *et al.* MLST revisited: the gene-by-gene approach to bacterial genomics. *Nat. Rev. Microbiol.*, v. 11, n. 10, p. 728-736, 2013.

MAIER, T.; GÜELL, M.; SERRANO, L. Correlation of mRNA and protein in complex biological samples. *FEBS Lett.*, v. 583, n. 24, p. 3966-3973, 2009.

MAIONE, D.; MARGARIT, I.; RINAUDO, C. D. *et al.* Identification of a universal Group B *Streptococcus* vaccine by multiple genome screen. *Science*, v. 309, n. 5731, p. 148-150, 2005.

MARQUES, M. V.; SILVA, A. M. Genômica funcional: transcriptoma. In: MIR, L. *Genômica*. São Paulo: Atheneu, 2004. cap. 7, p.119-138.

MARTIN, J. A.; WANG, Z. Next-generation transcriptome assembly. *Nat. Rev. Genet.*, v. 12, n. 10, p. 671-682, 2011.

MATA, A. I.; GIBELLO, A.; CASAMAYOR, A. *et al.* Multiplex PCR assay for detection of bacterial pathogens associated with warm-water streptococcosis in fish. *Appl. Environ. Microbiol.*, v. 70, n. 5, p. 3183-7, 2004.

MERCK ANIMAL HEALTH. Development of novel *Streptococcus agalactiae* Biotype 2 vaccine – laboratory challenge trials. 2012. Disponível em: < http://aqua.merck-animal-health.com/binaries/AquaVac_StreSa_tech02_06Nov12_051STREP_tcm56-35565.pdf >. Acesso em: 15 dez. 2017.

MEREGHETTI, L.; SITKIEWICZ, I.; GREEN, N. M. *et al.* Extensive adaptive changes occur in the transcriptome of *Streptococcus agalactiae* (Group B *Streptococcus*) in response to incubation with human blood. *Plos One*, v. 3, n. 9, p. e3143, 2008a.

MEREGHETTI, L.; SITKIEWICZ, I.; GREEN, N. M. *et al.* Remodeling of the *Streptococcus agalactiae* transcriptome in response to growth temperature. *Plos One*, v. 3, n. 7, p. e2785, 2008b.

MEREGHETTI, L.; SITKIEWICZ, I.; GREEN, N. M. *et al.* Identification of an unusual pattern of global gene expression in Group B *Streptococcus* grown in human blood. *Plos One*, v. 4, n. 9, p. e7145, 2009.

MIAN, G. F.; GODOY, D. T.; LEAL, C. A. G. *et al.* Aspects of the natural history and virulence of *S. agalactiae* infection in Nile tilapia. *Vet. Microbiol.*, v. 136, n. 1–2, p. 180-183, 2009.

MILLER, R. A.; REIMSCHUESSEL, R. Epidemiologic cutoff values for antimicrobial agents against *Aeromonas salmonicida* isolates determined by frequency distributions of minimal inhibitory concentration and diameter of zone of inhibition data. *Am. J. Vet. Res.*, v. 67, n. 11, p. 1837-1843, 2006.

MIYABE, F. M.; SUPHORONSKI, S. A.; CHIDEROLI, R. T. *et al.* Systematic review evaluation of vaccine efficacy against *Streptococcus agalactiae* in fish. *Ann. Vaccines Immunizat.*, v. 3, n. 1, p. 1013, 2017.

MORACH, M.; STEPHAN, R.; SCHMITT, S. *et al.* Population structure and virulence gene profiles of *Streptococcus agalactiae* collected from different hosts worldwide. *Eur. J. Clin. Microbiol. Infect. Dis.*, v. 37, n. 3, p. 527-536, 2017.

MORRISON, N.; COCHRANE, G.; FARUQUE, N. *et al.* Concept of sample in omics technology. *OMICS*, v. 10, n. 2, p. 127-137, 2006.

NGUYEN, N.-P.; WARNOW, T.; POP, M. *et al.* A perspective on 16S rRNA operational taxonomic unit clustering using sequence similarity. *NPJ Biofilms Microbiomes*, v. 2, p. 16004, 2016.

NIZET, V. Streptococcal β-hemolysins: genetics and role in disease pathogenesis. *Trends Microbiol.*, v. 10, n. 12, p. 575-580, 2002.

NOGA, E. J. *Fish disease: diagnosis and treatment*. Iowa: Blackwell Publishing, 2000. 367p.

NOMOTO, R.; UNOSE, N.; SHIMAHARA, Y. *et al.* Characterization of Lancefield Group C *Streptococcus dysgalactiae* isolated from farmed fish. *J. Fish Dis.*, v. 29, n. 11, p. 673-682, 2006.

OLIVEIRA, T. F. *Therapeutic efficacy of florfenicol against Streptoccoccus agalactiae infection in Nile tilapia (Oreochromis niloticus)*. 2016. 40f. Dissertação (Mestrado) - Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte.

PAILHORIES, H.; QUENTIN, R.; LARTIGUE, M.-F. The transcription of the *neuD* gene is stronger in serotype III Group B streptococci strains isolated from cerebrospinal fluid than in strains isolated from vagina. *FEMS Microbiol. Lett.*, v. 349, n. 1, p. 71-75, 2013.

PANG, M.; SUN, L.; HE, T. *et al.* Molecular and virulence characterization of highly prevalent *Streptococcus agalactiae* circulated in bovine dairy herds. *Vet. Res.*, v. 48, n. 1, p. 65, 2017.

PASNIK, D. J.; EVANS, J. J.; KLESIUS, P. H. Fecal strings associated with *Streptococcus agalactiae* infection in Nile tilapia, *Oreochromis niloticus*. *Open Vet. Sci. J.*, v. 3, p. 6-8, 2009.

PATEL, V. J.; THALASSINOS, K.; SLADE, S. E. *et al.* A comparison of labeling and label-free mass spectrometry-based proteomics approaches. *J. Proteome Res.*, v. 8, n. 7, p. 3752-3759, 2009.

PATTERSON, H.; SARALAHTI, A.; PARIKKA, M. *et al.* Adult zebrafish model of bacterial meningitis in *Streptococcus agalactiae* infection. *Dev. Comp. Immunol.*, v. 38, n. 3, p. 447-455, 2012.

PEREIRA, F. L.; SOARES, S. C.; DORELLA, F. A. *et al.* Evaluating the efficacy of the new Ion PGM Hi-Q Sequencing Kit applied to bacterial genomes. *Genomics*, v. 107, n. 5, 189-198, 2016.

PEREIRA, U. P.; MIAN, G. F.; OLIVEIRA, I. C. M. *et al.* Genotyping of *Streptococcus agalactiae* strains isolated from fish, human and cattle and their virulence potential in Nile tilapia. *Vet. Microbiol.*, v. 140, n. 1–2, p. 186-192, 2010.

PEREIRA, U. P.; SANTOS, A. R.; HASSAN, S. S. *et al.* Complete genome sequence of *Streptococcus agalactiae* strain SA20-06, a fish pathogen associated to meningoencephalitis outbreaks. *Stand. Genomic Sci.*, v. 8, n. 2, p. 188-97, 2013.

PLUMB, J. A.; SCHACHTE, J. H.; GAINES, J. L. *et al. Streptococcus* sp. from marine fishes along the Alabama and Northwest Florida Coast of the Gulf of Mexico. *Trans. Am. Fish. Soc.*, v. 103, n. 2, p. 358-361, 1974.

PRADEEP, P. J.; SUEBSING, R.; SIRTHAMMAJAK, S. *et al.* Evidence of vertical transmission and tissue tropism of streptococcosis from naturally infected red tilapia (*Oreochromis* spp.). *Aquacult. Rep.*, v. 3, n. Supplement C, p. 58-66, 2016.

PRETTO-GIORDANO, L. G.; MÜLLER, E. E.; FREITAS, J. C. D. *et al.* Evaluation on the pathogenesis of *Streptococcus agalactiae* in Nile tilapia (*Oreochromis niloticus*). *Braz. Arch. Biol. Technol.*, v. 53, p. 87-92, 2010.

PRIDGEON, J. W.; ZHANG, D. Complete genome sequence of a virulent *Streptococcus agalactiae* strain, 138P, isolated from diseased Nile tilapia. *Genome Announc.*, v. 2, n. 2, 2014.

RAJAGOPAL, L. Understanding the regulation of Group B streptococcal virulence factors. *Future Microbiol.*, v. 4, n. 2, p. 201-221, 2009.

RAJENDRAM, P.; KYAW, W. M.; LEO, Y. S. *et al.* Group B *Streptococcus* sequence type 283 disease linked to consumption of raw fish, Singapore. *Emerg. Infect. Dis.*, v. 22, n. 11, p. 1974, 2016.

REN, S. Y.; GENG, Y.; WANG, K. Y. *et al. Streptococcus agalactiae* infection in domestic rabbits, *Oryctolagus cuniculus*. *Transbound. Emerg. Dis.*, v. 61, n. 6, p. e92-e95, 2014.

RICHARDS, V. P.; CHOI, S. C.; BITAR, P. D. P. *et al.* Transcriptomic and genomic evidence for *Streptococcus agalactiae* adaptation to the bovine environment. *BMC Genomics*, v. 14, n. 1, p. 1-15, 2013.

RICHARDS, V. P.; LANG, P.; PAVINSKI BITAR, P. D. *et al.* Comparative genomics and the role of lateral gene transfer in the evolution of bovine adapted *Streptococcus agalactiae*. *Infect. Genet. Evol.*, v. 11, n. 6, p. 1263-1275, 2011.

ROBINSON, J. A.; MEYER, F. P. Streptococcal fish pathogen. *J. Bacteriol.*, v. 92, n. 2, p. 512-512, 1966.

ROMALDE, J. L.; RAVELO, C.; VALDÉS, I. *et al. Streptococcus phocae*, an emerging pathogen for salmonid culture. *Vet. Microbiol.*, v. 130, n. 1–2, p. 198-207, 2008.

ROSINSKI-CHUPIN, I.; SAUVAGE, E.; MAIREY, B. *et al.* Reductive evolution in *Streptococcus agalactiae* and the emergence of a host adapted lineage. *BMC Genomics*, v. 14, n. 1, p. 1-15, 2013.

RUANE, N. M.; COLLINS, E. M.; GEARY, M. *et al.* Isolation of *Streptococcus agalactiae* and an aquatic birnavirus from doctor fish *Garra rufa* L. *Irish Vet. J.*, v. 66, n. 1, p. 16-16, 2013.

SANTI, I.; SCARSELLI, M.; MARIANI, M. *et al.* BibA: a novel immunogenic bacterial adhesin contributing to Group B *Streptococcus* survival in human blood. *Mol. Microbiol.*, v. 63, n. 3, p. 754-767, 2007.

SHEWMAKER, P. L.; CAMUS, A. C.; BAILIFF, T. *et al. Streptococcus ictaluri* sp. nov., isolated from channel catfish *Ictalurus punctatus* broodstock. *Int. J. Syst. Evol. Microbiol.*, v. 57, n. 7, p. 1603-1606, 2007.

SHIMA, K.; YOSHII, N.; AKIBA, M. *et al.* Comparison of PCR-RFLP and PFGE for determining the clonality of enterohemorrhagic *Escherichia coli* strains. *FEMS Microbiol. Lett.*, v. 257, n. 1, p. 124-131, 2006.

SHOEMAKER, C. A.; XU, D. H.; GARCÍA, J. C. *et al.* Capsular typing of *Streptococcus agalactiae* (Lancefield Group B streptococci) from fishbusing multiplex PCR and serotyping. *Bull. Eur. Ass. Fish Pathol.*, v. 37, n. 5, p. 190, 2017a.

SHOEMAKER, C. A.; XU, D. H.; SOTO, E. *Streptococcus iniae* and *S. agalactiae*. In: WOO, P. T. K. e CIPRIANO, R. C. *Fish viruses and bacteria: pathobiology and protection***.** Oxfordshire, UK: CABI, 2017b. cap. 22, p. 298-310.

SILVA, W. M.; CARVALHO, R. D.; SOARES, S. C. *et al.* Label-free proteomic analysis to confirm the predicted proteome of *Corynebacterium pseudotuberculosis* under nitrosative stress mediated by nitric oxide. *BMC Genomics*, v. 15, n. 1, p. 1065, 2014.

SILVA, W. M.; SEYFFERT, N.; CIPRANDI, A. *et al.* Differential exoproteome analysis of two *Corynebacterium pseudotuberculosis* biovar ovis strains isolated from goat (1002) and sheep (C231). *Curr. Microbiol.*, v. 67, n. 4, p. 460-465, 2013a.

SILVA, W. M.; SEYFFERT, N.; SANTOS, A. V. *et al.* Identification of 11 new exoproteins in *Corynebacterium pseudotuberculosis* by comparative analysis of the exoproteome. *Microb. Pathog.*, v. 61–62, p. 37-42, 2013b.

SITKIEWICZ, I.; GREEN, N. M.; GUO, N. *et al.* Transcriptome adaptation of Group B *Streptococcus* to growth in human amniotic fluid. *Plos One*, v. 4, n. 7, p. e6114, 2009.

SITKIEWICZ, I.; MUSSER, J. M. Analysis of growth-phase regulated genes in *Streptococcus agalactiae* by global transcript profiling. *BMC Microbiol.*, v. 9, n. 1, p. 1-11, 2009.

SKVORTSOV, T. A.; AZHIKINA, T. L. A review of the transcriptome analysis of bacterial pathogens in vivo: problems and solutions. *Russ. J. Bioorgan. Chem.*, v. 36, n. 5, p. 550-559, 2010.

SLOTVED, H.-C.; KONG, F.; LAMBERTSEN, L. *et al.* Serotype IX, a proposed new *Streptococcus agalactiae* serotype. *J. Clin. Microbiol.*, v. 45, n. 9, p. 2929-2936, 2007.

SOARES, S. C.; GEYIK, H.; RAMOS, R. T. J. *et al.* GIPSy: Genomic island prediction software. *J. Biotechnol.*, v. 232, p. 2-11, 2016.

SOLIS, N.; CORDWELL, S. J. Current methodologies for proteomics of bacterial surface-exposed and cell envelope proteins. *Proteomics*, v. 11, n. 15, p. 3169-3189, 2011.

SØRENSEN, U. B. S.; POULSEN, K.; GHEZZO, C. *et al.* Emergence and global dissemination of host-specific *Streptococcus agalactiae* clones. *MBio*, v. 1, n. 3, p. e00178-10, 2010.

SOTO, E.; WANG, R.; WILES, J. *et al.* Characterization of isolates of *Streptococcus agalactiae* from diseased farmed and wild marine fish from the U.S. Gulf Coast, Latin America, and Thailand. *J. Aquat. Anim. Health*, v. 27, n. 2, p. 123-134, 2015.

SOTO, E.; ZAYAS, M.; TOBAR, J. *et al.* Laboratory-controlled challenges of Nile tilapia (*Oreochromis niloticus*) with *Streptococcus agalactiae*: comparisons between immersion, oral, intracoelomic and intramuscular routes of infection. *J. Comp. Pathol.*, v. 155, n. 4, p. 339-345, 2016.

SPRINGMAN, A. C.; LACHER, D. W.; WAYMIRE, E. A. *et al.* Pilus distribution among lineages of Group B *Streptococcus*: an evolutionary and clinical perspective. *BMC Microbiol.*, v. 14, n. 1, p. 1-11, 2014.

SU, Y. L.; FENG, J.; LI, Y. W. *et al.* Development of a quantitative PCR assay for monitoring *Streptococcus agalactiae* colonization and tissue tropism in experimentally infected tilapia. *J. Fish Dis.*, v. 39, n. 2, p. 229-238, 2016.

SUEBSING, R.; KAMPEERA, J.; TOOKDEE, B. *et al.* Evaluation of colorimetric loop-mediated isothermal amplification assay for visual detection of *Streptococcus agalactiae* and *Streptococcus iniae* in tilapia. *Lett. Appl. Microbiol.*, v. 57, n. 4, p. 317-324, 2013.

TAN, K.; WIJAYA, L.; CHIEW, H.-J. *et al.* Diffusion-weighted MRI abnormalities in an outbreak of *Streptococcus agalactiae* serotype III, multilocus sequence type 283 meningitis. *J. Magn. Reson. Imaging*, v. 45, n. 2, p. 507-514, 2017.

TAN, S.; LIN, Y.; FOO, K. *et al.* Group B *Streptococcus* serotype III sequence type 283 bacteremia associated with consumption of raw fish, Singapore. *Emerg. Infect. Dis.*, v. 22, n. 11, p. 1970-1973, 2016.

TAVARES, G. C.; COSTA, F. A. D. A.; SANTOS, R. R. D. *et al.* Nonlethal sampling methods for diagnosis of *Streptococcus agalactiae* infection in Nile tilapia, *Oreochromis niloticus* (L.). *Aquaculture*, v. 454, p. 237-242, 2016.

TETTELIN, H.; MASIGNANI, V.; CIESLEWICZ, M. J. *et al.* Genome analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: implications for the microbial "pan-genome". *Proc. Natl. Acad. Sci. USA*, v. 102, n. 39, p. 13950-13955, 2005.

THOMAS, J. C.; PETTIGREW, M. M. Multilocus sequence typing and pulsed field gel electrophoresis of otitis media causing pathogens. *Method. Mol Biol.*, v. 493, p. 179-190, 2009.

TIAN, M.; QU, J.; HAN, X. *et al.* Microarray-based identification of differentially expressed genes in intracellular *Brucella abortus* within RAW264.7 cells. *Plos One*, v. 8, n. 8, p. e67014, 2013.

VENDRELL, D.; BALCÁZAR, J. L.; RUIZ-ZARZUELA, I. *et al. Lactococcus garvieae* in fish: a review. *Comp. Immunol. Microbiol. Infect. Dis.*, v. 29, n. 4, p. 177-198, 2006.

VERNER-JEFFREYS, D. W.; WALLIS, T. J.; CANO CEJAS, I. *et al. Streptococcus agalactiae* multilocus sequence type 261 is associated with mortalities in the emerging Ghanaian tilapia industry. *J. Fish Dis.*, v. 41, n. 1, p. 175-179, 2018.

VESSECCHI, R.; LOPES, N. P.; GOZZO, F. C. *et al.* Nomenclaturas de espectrometria de massas em língua portuguesa. *Quim. Nova*, v. 34, p. 1875-1887, 2011.

VOS, P.; GARRITY, G.; JONES, D. *et al. Bergey's manual of systematic bacteriology - Volume 3: The firmicutes*. New Delhi: Springer-Verlag New York, 2009. 1450p.

WANG, B.; JIAN, J.; LU, Y. *et al.* Complete genome sequence of *Streptococcus agalactiae* ZQ0910, a pathogen causing meningoencephalitis in the GIFT strain of Nile tilapia (*Oreochromis niloticus*). *J. Bacteriol.*, v. 194, n. 18, p. 5132-5133, 2012.

WANG, L.; LIU, P.; WAN, Z. Y. *et al.* RNA-Seq revealed the impairment of immune defence of tilapia against the infection of *Streptococcus agalactiae* with simulated climate warming. *Fish Shellfish Immunol.*, v. 55, p. 679-689, 2016.

WANG, R.; LI, L.; HUANG, Y. *et al.* Comparative genome analysis identifies two large deletions in the genome of highly-passaged attenuated *Streptococcus agalactiae* strain YM001 compared to the parental pathogenic strain HN016. *BMC Genomics*, v. 16, n. 1, p. 1-10, 2015.

WIBAWAN, I. W.; LÄMMLER, C.; SMOLA, J. Properties and type antigen patterns of Group B streptococcal isolates from pigs and nutrias. *J. Clin. Microbiol.*, v. 31, n. 3, p. 762-764, 1993.

WILSON, B. A.; SALYERS, A. A.; WHITT, D. D. *et al. Bacterial pathogenesis: a molecular approach*. Washington: ASM Press, 2010. 540p.

YANG, R.; DU, Z.; HAN, Y. *et al.* Omics strategies for revealing *Yersinia pestis* virulence. *Front. Cell. Infect. Microbiol.*, v. 2, p. 157, 2012.

YILDIRIM, A. Ö.; LÄMMLER, C.; WEIß, R. Identification and characterization of *Streptococcus agalactiae* isolated from horses. *Vet. Microbiol.*, v. 85, n. 1, p. 31-35, 2002.

ZAMRI-SAAD, M.; AMAL, M. N. A.; SITI-ZAHRAH, A. Pathological changes in red tilapias (*Oreochromis* spp.) naturally infected by *Streptococcus agalactiae*. *J. Comp. Pathol.*, v. 143, n. 2–3, p. 227-229, 2010.

ZAMRI-SAAD, M.; AMAL, M. N. A.; SITI-ZAHRAH, A. *et al.* Control and prevention of streptococcosis in cultured tilapia in Malaysia: a review. *Pertanika J. Trop. Agric. Sci.*, v. 37, n. 4, p. 389-410, 2014.

ZAPPULLI, V.; MAZZARIOL, S.; CAVICCHIOLI, L. *et al.* Fatal necrotizing fasciitis and myositis in a captive common bottlenose dolphin (*Tursiops truncatus*) associated with *Streptococcus agalactiae*. *J. Vet. Diagn. Investig.*, v. 17, n. 6, p. 617-622, 2005.

ZHANG, J.; CHIODINI, R.; BADR, A. *et al.* The impact of next-generation sequencing on genomics. *J. Genet. Genomics*, v. 38, n. 3, p. 95-109, 2011.

ZHANG, R.; ZHANG, L. L.; YE, X. *et al.* Transcriptome profiling and digital gene expression analysis of Nile tilapia (*Oreochromis niloticus*) infected by *Streptococcus agalactiae*. *Mol. Biol. Rep.*, v. 40, n. 10, p. 5657-5668, 2013a.

ZHANG, Y.; FONSLOW, B. R.; SHAN, B. *et al.* Protein analysis by shotgun/bottom-up proteomics. *Chem. Rev.*, v. 113, n. 4, p. 2343-2394, 2013b.

ZHANG, Y. W.; GENG, Y.; YU, Z. H. *et al.* Serotypes and molecular typing of *Streptococcus agalactiae* isolated from fish. *Acta Hydrobiol.*, v. 41, n. 4, p. 800-806, 2017.

ZHAO, S.; FUNG-LEUNG, W.-P.; BITTNER, A. *et al.* Comparison of RNA-seq and microarray in transcriptome profiling of activated T cells. *Plos One*, v. 9, n. 1, p. e78644, 2014.

ZHOU, F.; ZHOU, K.; HUANG, J. *et al.* Characterization and expression analysis of a chitinase gene (PmChi-5) from black tiger shrimp (*Penaeus monodon*) under pathogens infection and ambient ammonia-N stress. *Fish Shellfish Immunol.*, v. 72, p. 117-123, 2018.

ZHU, J.; FU, Q.; AO, Q. *et al.* Transcriptomic profiling analysis of tilapia (*Oreochromis niloticus*) following *Streptococcus agalactiae* challenge. *Fish Shellfish Immunol.*, v. 62, n. Supplement C, p. 202-212, 2017.

ZUBAIR, S.; DE VILLIERS, E. P.; YOUNAN, M. *et al.* Genome sequences of two pathogenic *Streptococcus agalactiae* isolates from the one-humped camel *Camelus dromedarius*. *Genome Announc.*, v. 1, n. 4, 2013.

# 4. CHAPTER 2. DELINEATION OF THE PAN-PROTEOME OF FISH-PATHOGENIC *Streptococcus agalactiae* STRAINS USING A LABEL-FREE SHOTGUN APPROACH

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## 4.1. ABSTRACT

*Streptococcus agalactiae* (GBS) is a major pathogen of Nile tilapia, a global commodity of the aquaculture sector. The aims of this study were to evaluate protein expression in the main genotypes of GBS isolated from diseased fishes in Brazil using a label-free shotgun liquid chromatography-ultra definition mass spectrometry (LC-UDMS$^E$) approach and to compare the differential abundance of proteins identified in strains isolated from GBS-infected fishes and humans. A total of 1,070 protein clusters were identified by LC-UDMS$^E$ in 5 fish-adapted GBS strains belonging to sequence types ST-260 and ST-927 and the non-typeable (NT) lineage and 1 human GBS strain (ST-23). A total of 1,065 protein clusters corresponded to the pan-proteome of fish-adapted GBS strains; 989 of these were identified in all fish-adapted GBS strains (core proteome), and 62 were shared by at least two strains (accessory proteome). Proteins involved in the stress response and in the regulation of gene expression, metabolism and virulence were detected, reflecting the adaptive ability of fish-adapted GBS strains in response to stressor factors that affect bacterial survival in the aquatic environment and bacterial survival and multiplication inside the host cell. Measurement of protein abundance among different hosts showed that 5 and 26 proteins were exclusively found in the human- and fish-adapted GBS strains, respectively; the proteins exclusively identified in fish isolates were mainly related to virulence factors. Furthermore, 215 and 269 proteins were up- and down-regulated, respectively, in the fish-adapted GBS strains, revealing a reduced catabolic capacity of these strains in comparison to the human isolate. Our study showed that the core proteome of fish-adapted GBS strains is conserved and demonstrated high similarity of the proteins expressed by fish-adapted strains to the proteome of the human strain. Despite the similarity in protein content, the global protein abundance in the human GBS strain differed from that in the fish-adapted strains, suggesting that the bacterium has undergone distinct adaptations to mammalian and fish hosts at the regulatory level.

***Keywords***: comparative proteomics, *Streptococcus agalactiae*, genotypes, fish, human

## 4.2. INTRODUCTION

*Streptococcus agalactiae* (Lancefield's group B Streptococcus, GBS) is a major bacterial species of the genus *Streptococcus* and has medical and veterinary importance, affecting mainly humans (MAIONE *et al.*, 2005; JOHRI *et al.*, 2006), cattle (KEEFE, 1997) and fish (MIAN *et al.*, 2009). GBS is the most important pathogen of Nile tilapia, a global commodity of the aquaculture sector, causing outbreaks of septicemia and meningoencephalitis (HERNÁNDEZ *et al.*, 2009; MIAN *et al.*, 2009).

The multilocus sequence typing (MLST) technique, which is considered the reference tool for genotyping GBS, allows the grouping of different strains according to the similarity of their allelic profiles (sequence typing – ST) and ancestry (clonal complex – CC) (JONES *et al.*, 2003). The strains belonging to CC1, CC17 and CC19 are generally human clinical isolates, whereas CC61 and CC67 consist exclusively of bovine isolates (SPRINGMAN *et al.*, 2014; BERGAL *et al.*, 2015). The strains belonging to CC260, CC261, ST-257 and one non-typeable (NT) group lineage

have been considered to be specialized for infect aquatic animal hosts (BARONY *et al.*, 2017). These fish-adapted genotypic groups are genetically related based on the fact that their MLST profiles have been shown to share at least five identical alleles (BARONY *et al.*, 2017). CC260 has been identified in GBS isolated from diseased fish in Brazil, Colombia, Costa Rica, Honduras and the USA (EVANS *et al.*, 2008; DELANNOY *et al.*, 2013; GODOY *et al.*, 2013; ROSINSKI-CHUPIN *et al.*, 2013; BARATO *et al.*, 2015), and CC261 has a worldwide distribution, having been detected in Israel, Australia, Belgium, the USA, Ghana, Indonesia and China (EVANS *et al.*, 2008; ROSINSKI-CHUPIN *et al.*, 2013; LUSIASTUTI *et al.*, 2014; BOWATER, 2015; DENG *et al.*, 2017; VERNER-JEFFREYS *et al.*, 2018), whereas the other genetic group composed of the ST-257 and NT strains occurs only in Brazil (EVANS *et al.*, 2008; BARONY *et al.*, 2017). In previous studies that classified seventy-five Brazilian GBS fish isolates into different MLST types, it was found that approximately 97 % of the isolates belonged to the CC260 and NT strains (GODOY *et al.*, 2013; BARONY *et al.*, 2017). Considering the evolutionary relationship between these genotypes and the main GBS lineages that infect fishes in Brazil, it is necessary to understand the specific metabolic, adaptive and pathogenic characteristics of these genetic groups and their relationships to their aquatic hosts.

Proteomic studies make it possible to identify and quantify sets of proteins that are expressed by microorganisms under specific culture conditions (ZHANG *et al.*, 2014). Protein expression studies using GBS strains have highlighted the evaluation of surface proteins (HUGHES *et al.*, 2002; DORO *et al.*, 2009; LIU *et al.*, 2013a; LI *et al.*, 2016), secretory proteins (PAPASERGI *et al.*, 2013; LI *et al.*, 2016) and the comparative proteome (LI *et al.*, 2014). These studies were conducted using isolates obtained from human (PAPASERGI *et al.*, 2013) or fish hosts (LI *et al.*, 2016); to date, no comparative proteomic studies of human and fish-adapted GBS strains or of GBS strains belonging to different genotypes have been performed. Pan-proteomics analysis, an alternative strategy that can be used to conduct comparative proteomic studies, seeks to compare the qualitative and quantitative proteome across strains, allowing interpretation of bacterial physiology and promoting knowledge of the genetic variation of each isolate (BROADBENT *et al.*, 2016). Pan-proteomic analysis was previously used to determine the core and pan-proteome of four epidemic *Salmonella* Paratyphi A strains (ZHANG *et al.*, 2014) and to compare the protein expression patterns of *Mycobacterium tuberculosis* strains with different virulence traits (JHINGAN *et al.*, 2016). Thus, a pan-proteomic study of GBS strains that infect fishes would permit the analysis of protein variability within the strains belonging to the main Brazilian genotypes, increase scientific knowledge about the adaptation and pathogenesis of this bacterium in fishes, and make it possible to characterize its host-related adaptations. In addition, this approach would allow the identification of conserved antigenic proteins that can be used as targets in vaccine design.

This study aimed to evaluate the global abundance of proteins produced by the main genotypes of GBS isolated from fishes in Brazil using a label-free shotgun nano-liquid chromatography-ultra definition mass spectrometry (nanoLC-UDMS$^{E}$) approach and to compare the differential expression of proteins identified in isolates obtained from human and fishes.

## 4.3. METHODS

### 4.3.1. BACTERIAL STRAINS

Five GBS strains previously isolated from diseased fish on different farms were selected from the National Reference Laboratory of Aquatic Animal Diseases (AQUACEN) culture collection and used in this study. These strains have whole-genome previously sequenced and belongs to different genotypes by MLST method (BARONY et al., 2017). SA16, SA20 and SA81 are from

a group of NT strains, which have different genetic profiles determined according Godoy et al. (2013*)* through of combination of MLST and the presence/absence of the genes *lmb*, *hylB* and *cylE*, and also from different fish hosts. SA53 is from ST-260 and SA95 is from ST-927. Additionally, the *S. agalactiae* NEM316 strain (ST-23), which was isolated from a human neonate with septicemia, was acquired from the American Type Culture Collection (strain designation ATCC12403) and included in this study to make it possible to compare the protein expression patterns of GBS strains isolated from fish and human hosts. The entire genome of the NEM316 strain has been sequenced and annotated (GenBank accession number NC_004368) (GLASER *et al.*, 2002); its virulence genes have been well characterized, and several studies using transcriptomic and proteomic approaches have been conducted (MEREGHETTI *et al.*, 2008a; b; SITKIEWICZ *et al.*, 2009; PAPASERGI *et al.*, 2013). All strains were stored at -70 °C until use. The characteristics of the strains are listed in Table 1.

### 4.3.2. CULTURE CONDITIONS

*S. agalactiae* strains isolated from fishes were thawed, streaked onto 5 % sheep blood agar and incubated at 28 °C for 48 h according to the method described by Godoy et al. (2013). The NEM316 strain was incubated at 37 °C for 24 h according to the method described by Pereira et al (2010). Each strain was inoculated into BHI broth ("Brain Heart Infusion", Himedia, Mumbai, India) containing 0.05 % (v/v) Tween 80 (BHIT) and cultured at 30 °C with gently agitation. Biological triplicate cultures of each strain were harvested for protein isolation upon reaching absorbances of 0.2 and 0.5 ($OD_{600}$), equivalent to the mid-exponential phase of bacterial growth of the fish-adapted GBS strains (data not shown) and the NEM316 strain (MEREGHETTI *et al.*, 2008b), respectively. The GBS strains were cultured under laboratory conditions at 30 °C; this corresponds to the temperature at which increased outbreaks of streptococcosis normally occur in fishes (MIAN *et al.*, 2009).

### 4.3.3. PROTEIN ISOLATION

Extracts of the whole bacterial lysates from three biological replicates of each strain were prepared. The bacterial cells were harvested by centrifugation at 16,100 x g for 20 min at 4 °C. The bacterial pellets were washed three times with 10 mL of 50 mM Tris-HCl (pH 7.5) and collected by centrifugation after each wash. The bacterial pellets were then resuspended in 1 mL of lysis buffer (7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 12.5 mM Tris-HCl and 1.5 % (w/v) dithiothreitol (DTT)) containing 10 µL of protease inhibitor mix (GE HealthCare, Pittsburgh, USA) and sonicated on ice using an ultrasonic cell disruptor (Unique, Indaiatuba, Brazil) for 20 min in cycles of 1 min at maximum power (495 W) followed by 1 min of rest. The lysates were centrifuged at 21,900 x g for 40 min at 4 °C; the supernatants were collected and subjected to five cycles of centrifugation at 15,000 x g for 30 min at 20 °C using Vivaspin 500 centrifugal concentrators (GE HealthCare) with a cutoff threshold of 3 kDa. Between cycles, the lysis buffer was exchanged for 50 mM ammonium bicarbonate (pH 8.5) to remove detergent from the samples. The extracted proteins were quantified using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, USA) and the Qubit protein assay kit (Molecular Probes, Oregon, USA) according to the manufacturer's instructions.

### 4.3.4. PROTEIN DIGESTION

A volume of 50 µL containing 2 $\mu g.\mu L^{-1}$ protein extract was collected from each replicate and transferred to a tube (1.5 mL) containing 10 µL of 50 mM ammonium bicarbonate. The proteins

in the sample were denatured by the addition of 25 µL of 0.2 % (w/v) *Rapi*GEST SF surfactant (Waters, Manchester, UK) at 80 °C for 15 min. Thiol groups were reduced using 2.5 µL of 100 mM DTT (Sigma Aldrich, Saint Louis, USA) at 60 °C for 30 min and alkylated using 2.5 µL of 300 mM iodoacetamide (Sigma Aldrich) at room temperature for 30 min in a dark chamber. The proteins in the sample were then enzymatically digested by addition of 5 µg of sequencing-grade modified trypsin (Promega, Madison, USA) and incubated at 37 °C for 16 h. Digestion was stopped by the addition of 10 µL of 5 % (v/v) trifluoroacetic acid (Sigma Aldrich) and incubation at 37 °C for 90 min. The resulting peptide extracts were centrifuged at 21,900 x g for 30 min at 6 °C. The supernatants were collected, transferred to Waters Total Recovery vials (Waters), supplemented with 5 µL of 1 N ammonium hydroxide (Sigma Aldrich) and stored at -70 °C until use.

### 4.3.5. MASS SPECTROMETRY

Bidimensional nano ultra-performance liquid chromatography (nanoUPLC) tandem nano electrospray high-definition mass spectrometry (nanoESI-HDMS$^E$) experiments were conducted using a 1-h reverse-phase (RP) gradient from 7 % to 40 % (v/v) acetonitrile (0.1 % v/v formic acid) with a simulated 1D analysis and a delivery of 500 nL.min$^{-1}$ in a nanoACQUITY UPLC 2D Technology system (Waters). A nanoACQUITY UPLC High Strength Silica T3 column (1.8 µm, 100 µm × 10 cm, pH 3) was used in combination with an RP Acquity UPLC Nano Ease XBridge BEH130 C18 column (5 µm, 300 µm × 50 mm nanoflow column, pH 10). Typical on-column sample loads were 500 ng of total protein digest for each of the 5 fractions (500 ng/fraction/load).

For every measurement, the mass spectrometer was operated in resolution mode with a typical *m/z* resolving power of at least 25,000 full width at half-maximum (FWHM), an ion mobility cell that was filled with helium gas, and a cross-section resolving power of at least 40 Ω/Δ Ω. The effective resolution with the conjoined ion mobility was 25,000 FWHM. Analyses were performed using nano-electrospray ionization in positive ion mode nanoESI (+) and a NanoLock-Spray (both from Waters) ionization source. The lock mass channel was sampled every 30 sec. The mass spectrometer was calibrated with the MS/MS spectrum of a solution of human [Glu$^1$]-fibrinopeptide B (Glu-Fib) (100 fmol.µL$^{-1}$) that was delivered through the reference sprayer of the NanoLock-Spray source. The double-charged ion ($[M + 2H]^{2+}$ = 785.8426) was used for initial single-point calibration, and MS/MS fragment ions of Glu-Fib were used to obtain the final instrument calibration.

Multiplexed data-independent acquisition (DIA) scanning with added specificity and selectivity conferred by a non-linear 'T-wave' ion mobility (HDMS$^E$) device was performed on a Synapt G2-Si HDMS mass spectrometer (Waters). The spectrometer was automatically programmed to switch between standard MS (3 eV) and elevated collision energies HDMS$^E$ (19–45 eV) applied to the transfer 'T-wave' collision-induced dissociation cell with nitrogen gas. The trap collision cell was adjusted to 1 eV using a millisecond scan time that was previously adjusted based on the linear velocity of the chromatographic peak that was delivered through a nanoACQUITY UPLC (Waters) to generate a minimum of 20 scan points for each single peak both in low-energy and high-energy transmission at an orthogonal acceleration time-of-flight (*oa*-TOF) and over a mass range of *m/z* 50 to 2,000.

Mass spectrometric analysis of tryptic peptides was performed using a mass spectrometer equipped with a T-Wave-IMS device (Waters) in MS$^E$ and UDMS$^E$ modes according to the method previously described (DISTLER *et al.*, 2014). Stoichiometric measurements based on scouting runs of the integrated total ion account were performed prior to analysis to ensure

standardized molar values across all samples. Based on these values, the tryptic peptides of each strain were injected onto the column in the same amounts. The radio frequency offset (MS profile) was adjusted such that the nanoESI-UDMS$^E$ data were effectively acquired from *m/z* 400 to 2,000 by MassLynx v.4.1 software (Waters), ensuring that any masses that were observed in the high-energy spectra with less than *m/z* 400 arose from dissociations in the collision cell. The MS proteomics data are available at the ProteomeXchange Consortium via the PRIDE (VIZCAÍNO *et al.*, 2016) partner repository under the identifier PXD008744.

### 4.3.6. PROTEIN IDENTIFICATION AND QUANTIFICATION

The UDMS$^E$ raw data were processed using Progenesis QI for Proteomics (QIP) v.2.0 (Nonlinear Dynamics, Newcastle, UK) according to the method previously described by Kuharev et al. (2015). Imported runs were subjected to automatic data processing for protein identification and quantitative information using the following parameters: peak picking limits = 5 and maximum charge retention time limits = 8.

An *in-house* database was created using protein code sequences (CDSs) of the whole genomes of the strains obtained from the GenBank database (BENSON *et al.*, 2013). CD-HIT software version 4.6 (LI e GODZIK, 2006) was used with the -c parameter equal to 1 to create a non-redundant set of CDSs according to the recommendation of Broadbent et al. (2016). The database management tool of the ProteinLynx Global Server (PLGS) v 3.0.2 (Waters) was used to append reversed sequences (to assess the false positive rate during identification) and to create the final fasta file of the used database.

The following parameters were used for peptide identification: digest reagent = trypsin; maximum missed cleavages = 1; maximum protein mass = 600 kDa; modifications: carbamidomethyl of cysteine (fixed), acetyl N-terminal (variable), phosphoryl (variable), and oxidation of methionine (variable); search tolerance parameters: peptide tolerance = 10 ppm, fragment tolerance = 20 ppm, and maximum false discovery rate (FDR) = 4 %. Ion matching requirements used the default parameters (LI *et al.*, 2009), which were fragments per peptide = 1, fragments per protein = 3, and peptides per protein = 1. The protein-level quantitation was performed with relative quantitation using the Hi-N algorithm, which is incorporated in Progenesis QIP. Peptides with scores $\leq 3$, mass errors $\geq 20$ ppm, or sequence length $\leq 6$ amino acids and those found in the decoy reverse database were removed. Proteins identified on the basis of at least two peptides (with $\geq 1$ proteotypic peptide per protein) and that were present in $\geq 2$ of the three biological replicates for each GBS strain were considered.

The variability and quality of the proteomic data were analyzed through principal component analysis (PCA), distribution of peptide precursors and fragment error, peptide match distribution, drift time, number of times that an identified protein appeared in the biological replicates and dynamic range. The PCA biplot was generated using the *ggbiplot* package version 0.55 (VU, 2011) in R software version 3.4.1 (R CORE TEAM, 2013); the other plots were generated from fragment, peptide and protein tables obtained during searching of parameters for peptide identification using TIBCO SpotFire software version 7.0 (TIBCO, Boston, USA). The dynamic range of protein amounts of the identified proteins from all strains was calculated using the average relative abundance of each biological triplicate against protein rank. The data were binned by $\log_{10}$ of their normalized abundance, ordered in decreasing sequence and plotted using TIBCO SpotFire software.

The Progenesis QIP algorithm was used to organize the identified proteins into a list of proteins with statistically significant differences in expression (ANOVA, *p-value* $\leq 0.05$). A protein was

considered to be differentially expressed with respect to NEM316 if there was a ≥ 2-fold change in its expression ($log_2$ ratio ≥ 1 for proteins with higher abundance levels or $log_2$ ratio ≤ -1 for proteins with lower abundance levels). A heat map was generated from normalization of the $log_2$ value of each protein by *z*-score calculation. Clustergrams were created using the unweighted pair group method with the average (UPGMA) approach and Euclidean distance in TIBCO SpotFire software. A similarity matrix was generated according to the agreement between identified proteins of each strain and visualized using the *gplots* package version 3.0.1 in R software (WARNES *et al.*, 2016).

### 4.3.7. BIOINFORMATICS ANALYSIS

The Interactivenn web-based tool (HEBERLE *et al.*, 2015) was used to evaluate the number of proteins identified in each GBS strain through Venn diagrams. The core proteome consisted of the subset of identified proteins in all evaluated strains. The accessory proteome consisted of the subset of identified proteins shared between at least two strains, and the unique proteome consisted of the proteins that were identified exclusively in a particular strain.

Predicted protein clustering (PPC) to indicate the homologous genes between the strains was performed using OrthoMCL software (LI *et al.*, 2003) using the default parameters. In summary, files containing the CDS of each whole-genome were concatenated and adjusted using OrthoMCL scripts. A BLASTp analysis was applied to the resulting concatenated file against itself using an e-value of 10e-20.

To predict orthologous groups by functional category and subcellular localization, the sequences of identified proteins were analyzed using the Cluster of Orthologous Genes (COG) database version 2014db (GALPERIN *et al.*, 2015) and SurfG+ software version 1.0.2 (BARINOV *et al.*, 2009), respectively. The COG database search was performed using an *in-house* script (available at https://github.com/aquacen/blast_cog).

The protein-protein interaction network of identified proteins in the core proteome was built using the STRING tool version 10.5 (SZKLARCZYK *et al.*, 2017) using the *Streptococcus agalactiae* NEM316 strain as the reference and the following settings: meaning of network edges = confidence; active interaction sources = experiments, gene fusion, databases, co-occurrence and co-expression; and minimum required interaction score = 0.980. Predicted interactions were tested for enrichment for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps in STRING.

Prediction of vaccine candidates was performed using the Vaxign webserver (XIANG e HE, 2013). Dynamic Vaxign analysis was performed with the CDS of identified proteins of the core proteome with the subcellular localizations Secreted, Potentially surface-exposed (PSE) or Membrane from SurfG+ analysis. Parameters of gram-positive bacterium and similarity to host proteins of humans were set. Only proteins with adhesion probabilities ≥ 0.51 were included in the result.

## 4.4. RESULTS

### 4.4.1. LABEL-FREE PROTEOMICS RESULTS

The proteomes of GBS strains isolated from fishes ($n$ = 5) and human ($n$ = 1) were determined by LC-UDMS$^E$. A total of 32,145 peptides with a normal distribution of 10 ppm error (~92 % of the peptides were detected with an error of less than 10 ppm) were identified (Supplementary Figure 1A). Approximately 44 % of the peptides were identified from peptide match type data in the first

pass (PepFrag1), whereas ~15 % were obtained in the second pass (PepFrag2); 7 %, 7 % and ~26 % of the peptides were identified as missed trypsin cleavage, in-source fragmentation and variable modifications (VarMod), respectively (Supplementary Figure 1B). Of the total peptides, ~87 % showed a charge state of at least $[M +2H]^{2+}$ (Supplementary Figure 1C), and 90-97 % of the identified proteins were found in 3 of the 3 replicates of each GBS strain (Supplementary Figure 1D). The PCA analysis clustered the GBS isolates based on the proximity of points identified for each strain, demonstrating the reproducibility of the proteomic data among the replicates (Supplementary Figure 1E).

The total number of identified proteins in each biological replicate of the individual strains varied from 1,216 to 1,247, resulting in the detection of 1,273 proteins in at least two of the three replicates. An average of 25 peptides per protein and an FDR of 0.08 % when decoy detection was set at agreement of two of the three replicates were found for each strain. To avoid overestimation of the number of identified proteins based on the pan-proteome database used, protein clustering of highly homologous sequences was performed. The PPC of the genome sequences of the six strains was 2,148 (predicted pan-proteome), and the identified proteome was composed of 1,070 protein clusters. The proteins and clusters identified in this study are shown in Supplementary Table 1.

### 4.4.2. PAN-PROTEOME OF FISH-PATHOGENIC *S. agalactiae* STRAINS

A total of 1,020 proteins were identified in SA16, corresponding to ~62 % of the strain's PPC; 1,036 proteins were identified in SA20 (~63 % of the strain's PPC), 1,023 proteins were identified in SA53 (~62 % of the strain's PPC), 1,051 proteins were identified in SA81 (~64 % of the strain's PPC) and 1,040 proteins were identified in SA95 (~63 % of the strain's PPC). The dynamic range of the quantified proteins of GBS between the most and least abundant proteins in all strains was ~5 log units. The most abundant proteins identified were associated with virulence, metabolism and regulation. Elongation factor Tu, TpiA, RpsJ, Sip and ThrS were among the top 10 most abundant proteins identified in all fish-adapted GBS strains (Figure 1).

To investigate the proteome shared by the isolates, a comparative analysis of the identified proteins in each strain was performed using Venn diagrams. A total of 989 proteins were identified in the core proteome, 62 proteins were present in the accessory proteome, and 1, 2, 2, 4 and 5 proteins were exclusively identified in SA16, SA20, SA53, SA81 and SA95, respectively (Figure 2 and Supplementary Table 1). Therefore, the identification of 1,065 proteins corresponds to a pan-proteome that is representative of the evaluated fish-adapted GBS strains. The fish-adapted GBS strains were closely related (similarity > 95.3 %) in protein content (Figure 3) even among strains isolated from different fish species, considering that SA81 was isolated from Amazon catfish, whereas the other fish-adapted GBS strains were obtained from diseased Nile tilapia. The core proteome represented 92.42 % of the expressed pan-proteome, suggesting that protein expression is conserved among fish-adapted GBS strains.

Approximately 95 % of the pan-proteome ($n$ = 1,018) of the fish-adapted GBS strains was classified into 20 functional categories using COG; the remaining ~5 % of the identified proteins ($n$ = 52) were classified as having unknown functions. The most common categories were translation/ribosomal structure and biogenesis ($n$ = 165), general function prediction only ($n$ = 98), amino acid transport and metabolism ($n$ = 97), cell wall/membrane/envelope biogenesis ($n$ = 91), transcription ($n$ = 86) and carbohydrate transport and metabolism ($n$ = 85) (Figure 4). The main proteins detected in each functional category are shown in Table 2.

According to the subcellular localization analysis, the identified proteins in the core proteome of fish-adapted GBS strains included 880 cytoplasmic proteins, 99 PSE, 57 membrane proteins and 29 secreted proteins. A total of 166 non-cytoplasmic proteins were evaluated as putative vaccine targets, and 38 of these showed an adhesion probability ≥ 0.51 (Table 3). Bacterial virulence proteins related to adhesion, invasion, immune evasion and resistance to cationic antimicrobial peptides were found in fish-adapted GBS strains (Table 4).

### 4.4.3. INTERACTOME ANALYSIS OF THE CORE PROTEOME

To better understand the biological functions of the proteins in the core proteome of fish-adapted GBS strains, a protein-protein interaction (PPI) analysis was conducted. As shown in Figure 5, the greatest numbers of interactions were verified in the proteins related to ribosomal proteins (cluster 1; $n = 62$), ATP synthase (cluster 2; $n = 6$), pyruvate metabolism (cluster 3; $n = 5$), carbohydrate metabolism (cluster 4; $n = 11$), heat shock proteins (cluster 5; $n = 5$), nucleotide metabolism (cluster 6; $n = 17$), aminoacyl-tRNA synthetase (cluster 7; $n = 12$), DNA replication and repair (cluster 8; $n = 15$) and peptidoglycan biosynthesis (cluster 9; $n = 9$).

To determine the metabolic network of GBS that infect fishes, the proteins identified in the core proteome were analyzed using pathway enrichment analysis. The results revealed that a total of 28 pathways showed significant values (FDR < 0.05); the pathways most highly related to the dataset were metabolic pathways (FDR < 6.88e-36), biosynthesis of secondary metabolites (FDR < 2.37e-23), microbial metabolism in diverse environments (FDR < 1.47e-9), and ribosome (FDR < 3.82e-16) (Supplementary Table 2).

### 4.4.4. DIFFERENTIAL EXPRESSION OF PROTEINS AMONG GBS STRAINS

To evaluate the abundance of specific proteins in strains of different host origins, a comparative analysis of the proteome of the NEM316 strain (isolated from a human) and the fish-adapted GBS strains was performed. A total of 1,044 proteins were identified in the NEM316 strain, corresponding to ~53 % of the proteins identified in PPC. Five and 26 proteins were exclusively expressed in the NEM316 strain and the fish-adapted GBS strains, respectively (Supplementary Table 3). The proteins exclusively expressed in the NEM316 strain are involved in transcription ($n = 1$), cell wall/membrane/envelope biogenesis ($n = 1$), nucleotide metabolism ($n = 1$), posttranslational modification, protein turnover, chaperones ($n = 1$) and unknown functions ($n = 1$). On the other hand, the proteins exclusively identified in the fish-adapted GBS strains are involved in putative multidrug resistance (D-alanyl-D-alanine carboxypeptidase, bacteriocin transport accessory protein, bleomycin resistance protein and Pbp2B), hemolysin (cAMP factor), evasins (CpsG and NeuB) and host colonization (Type VII secretion protein EsaA). Two proteins involved in oxidative stress resistance (flavoprotein and phenazine biosynthesis protein) were also identified. Of the identified proteins, five are involved in metabolism (PTS mannose transporter subunit IIB, beta-hexosamidase, 5-formyltetrahydrofolate cyclo-ligase, malate dehydrogenase, gluconate 5-dehydrogenase), two are involved in information storage and processing (ribonuclease HII and 3'-5' exoribonuclease), three are involved in cellular processes and signaling (PhoB, Asp1 and ATPase AAA), and six are poorly characterized (five hypothetical proteins and a membrane protein).

The subset of core proteins decreased slightly from 989 to 978 proteins with the addition of the proteome of the NEM316 strain, and the pan-proteome increased to 1,070 proteins. This strain showed similarity of protein content to that of the fish-adapted GBS strains of 94.3 to 97.3 % (data not shown).

In the expression analysis, only proteins with $p \leq 0.05$ and common to the six strains were considered (*n* = 534). The numbers of DEPs in each strain are presented in Supplementary Table 4. In comparison to the other strains, the NEM316 and SA95 strains are closely related to each other, as shown by the lower number of DEPs between them (*n* = 95). In the NT strains, an average of 307 proteins varied in expression by 2-fold from their expression in the NEM316 strain. The highest variation in the number of DEPs was detected between SA53 and NEM316 (*n* = 358). A total of 215 and 269 proteins were up- and down-regulated, respectively, in fish-adapted GBS strains compared to the human GBS strain (Supplementary Figure 2 and Supplementary Table 5). Of these, 29 and 11 proteins were identified as up- and down-regulated, respectively, in all fish-adapted GBS strains (Supplementary Figure 2 and Table 5). A hierarchical clustering analysis was performed, and the results revealed an association between the regulatory level of proteins and the genotypes of the tested fish-adapted GBS strains (Figure 6). In the COG analysis, twenty-one functional categories were classified as differentially regulated. Translation, ribosomal structure and biogenesis (*n* = 40), cell wall/membrane/envelope biogenesis (*n* = 27), general functions (*n* = 27), carbohydrate metabolism and transport (*n* = 26) and energy production and conversion (*n* = 22) were the most common categories represented by the down-regulated proteins in fish-adapted GBS strains in comparison to the human isolate; amino acid metabolism (*n* = 27), transcription (*n* = 21) and replication, recombination and repair (*n* = 13) were the main functions identified as up-regulated (Figure 7).

## 4.5. DISCUSSION

The fish-adapted GBS strains used in this study were isolated from fishes infected during outbreaks of meningoencephalitis among farm-raised Nile tilapia and Amazon catfish (*Leiarius marmoratus x Pseudoplatystoma fasciatum*) in Brazil on different farms between 2006 and 2010 (Table 1). As mentioned above, the GBS strains belong to serotype Ib, display different genetic profiles (GODOY *et al.*, 2013) and belong to two fish-adapted genotypic groups (the CC260 and NT lineages) that are closely related and that are commonly detected in fishes raised on Brazilian fish farms (BARONY *et al.*, 2017). Although the selected strains display high similarity in genomic content (> 98 %) and are considered very closely related, small variations between isolates with different STs have been verified (BARONY *et al.*, 2017). Based on these considerations, comparison of the protein abundance among strains with different genotypes would demonstrate the pan-proteome of GBS fish-adapted strains.

The LC-UDMS$^E$ label-free proteomic analysis conducted in our study resulted in the identification and quantification of 1,070 proteins expressed by GBS strains. This is the largest number of proteins that have been identified for this bacterial species by proteomics, regardless of strain origin. A limited number (*n* = 65) of proteins was identified in previous studies of comparative proteome of GBS using two-dimensional electrophoresis (2-DE) combined with mass spectrometry (LI *et al.*, 2014; LI *et al.*, 2015). However, use of the LC-UDMS$^E$ technique yielded better results for proteome analysis from whole bacterial lysates, as verified in our study. Of the total identified proteins, 1,065 proteins represented the pan-proteome of the evaluated fish-adapted GBS strains; these proteins constituted approximately 60 % of the predicted proteome of each strain.

The five fish-adapted GBS strains shared ~92 % of their identified proteins, demonstrating that the expression of the core proteome is conserved among strains. Previous studies have also reported conservation of protein expression among isolates of the same bacterial species; in different studies, coverages ranging from 73.1 to 91.4 % of the pan-proteome were reported (ZHANG *et al.*, 2014; JHINGAN *et al.*, 2016; PRAGYA *et al.*, 2017; SILVA *et al.*, 2017).

## 4.5.1. INSIGHTS REGARDING THE PAN-PROTEOME OF FISH-ADAPTED GBS STRAINS

### 4.5.1.1. PROTEINS INVOLVED IN ADAPTATION TO AN AQUATIC ENVIRONMENT

Environmental factors such as the amount of dissolved oxygen, the pH, the osmotic strength, the temperature and the availability of nutrients can modify the expression of proteins in response to changes in these parameters. However, the proteins related to the survival of GBS in the aquatic environment are poorly characterized. It is known that GBS can be transmitted between fish indirectly through the water (MIAN *et al.*, 2009) and that increased expression of proteins involved in the transport of carbohydrates, amino acids and ions increases bacterial survival in this environment (GLASER *et al.*, 2002; ROSINSKI-CHUPIN *et al.*, 2013).

Compounds such as glucose, mannitol, lactose, mannose and pyruvate are used as energy sources by *Streptococcus* species (NEIJSSEL *et al.*, 1997); moreover, GBS has the capacity to utilize a broad range of carbon-containing molecules through the phosphoenolpyruvate-dependent phosphotransferase (PTS) system and ABC transporters (GLASER *et al.*, 2002). Our study identified proteins involved in glycogen synthesis (PgmA), the glycolytic pathway (Eno, Pga, Pgk, and TpiA), the pentose phosphate pathway (AroD), 12 PTS system proteins involved in the transport of ascorbate, glucose, beta-glucoside, lactose, mannose, galactitol and fructose, and 2 sugar-specific ABC transporters (maltose ABC transporter substrate-binding protein and sugar ABC transporter ATP-binding protein). Taken together, the identification of these proteins demonstrated the broad catabolic capacity of GBS strains and corroborated the results obtained from genomic analysis performed by Glaser et al. (2002). Among the proteins involved in carbohydrate metabolism, TpiA and Pgk showed a high number of interactions in PPI analysis; they are also among the more abundant proteins in our proteomic data obtained from fish-adapted GBS strains.

In environments in which glucose or lactose availability is limited, pyruvate is thought to provide an alternative energy source for many bacterial species (MODAK *et al.*, 2002). Proteins involved in pyruvate metabolism (pyruvate dehydrogenase, TPP-dependent acetoin dehydrogenase complex, branched-chain alpha-keto acid dehydrogenase and dihydrolipoyl dehydrogenase) were identified in the core proteome of fish-adapted GBS strains and formed part of an interactive network in PPI analysis. These four proteins make up the pyruvate dehydrogenase complex, which is responsible for the conversion of pyruvate into acetyl-CoA, an important precursor in fatty acid biosynthesis and a metabolic intermediate in acetate production (YAMAMOTO *et al.*, 2006). The degradation of pyruvate by GBS strains generates products such as formate, acetate, acetoin, lactate and ethanol, which serve as carbon substrates for energy production (MICKELSON, 1967; YAMAMOTO *et al.*, 2006; FUCHS *et al.*, 2012). Moreover, acetate kinase (AckA) and L-lactate dehydrogenase (Ldh_2), which were also identified in the core proteome, increase the formation of acetate and lactate, thereby generating more energy in the form of ATP, regenerating NAD from NADH in a reaction catalyzed by the NoxE protein, and permitting bacterial survival in aerobic and oxygen-depleted environments (FUCHS *et al.*, 2012; PRAGYA *et al.*, 2017).

Various amino acids have been considered essential for the growth of GBS strains under aerobic and anaerobic conditions (MILLIGAN *et al.*, 1978). The proteins involved in the metabolic pathways that produce glycine, serine, glutamine, aspartic acid, threonine, alanine and asparagine in the NEM316 strain have already been described through genomic analysis (GLASER *et al.*,

2002). Proteins involved in these metabolic pathways were also identified in our study of fish-adapted GBS strains, as shown in Table 2. In addition, because GBS is an auxotrophic microorganism for the biosynthesis of some amino acids, it is necessary for it to produce the transport proteins and peptidases required to obtain these compounds in a nutrient-rich environment (GLASER *et al.*, 2002; RAJAGOPAL, 2009). Our proteomic data demonstrated that fish-adapted GBS strains express proteins related to the uptake of amino acids, including ABC transporters specific for amino acids and peptides ($n$ = 25), peptidases ($n$ = 29), and proteins involved in arginine, glutamic acid, cysteine and methionine metabolism, all of which are important for survival in aquatic and host environments.

Inorganic and metallic ions are important cofactors that contribute to the biological activities of many bacterial proteins (SCHREUR *et al.*, 2011). These factors must be acquired from the environment (HOHLE *et al.*, 2011). However, in fish-adapted GBS strains, the genes involved in inorganic ion metabolism may be missing or inactivated, affecting ion exchange and reducing the bacterium's ability to maintain homeostasis when exposed to changes in the external environment (ROSINSKI-CHUPIN *et al.*, 2013). The results of our study are inconsistent with previous findings based on the comparative genomic analysis of seven GBS strains isolated from fish and frog hosts, since in our study the expression of 15 ABC transport proteins involved in the mobilization of iron, nickel, ferrichrome, manganese, magnesium, potassium, phosphate and heme, as well as proteins involved in zinc (zinc-binding protein) and copper (CutC) metabolism, was detected. These proteins may be important for the growth and survival of fish-adapted GBS strains in aquatic environments, which often contain a limited supply of essential metal ions. Some of these proteins, such as MscL and TrkA, also participate in bacterial cell osmoregulation. MscL activates the release of cytoplasmic solutes from mechanosensitive channels, decreasing the turgor pressure during changes in osmolarity (LEVINA *et al.*, 1999), and TrkA participates in the uptake of potassium, an important inorganic ion required for the maintenance of constant bacterial internal pH and membrane potential (DI PALO *et al.*, 2013; GRÜNDLING, 2013). TrkA was down-regulated in fish-adapted GBS strains in comparison with NEM316, demonstrating that the strains isolated from fishes have a lower rate of potassium uptake.

Proteins involved in lipid metabolism in GBS have generally been poorly characterized; however, important proteins related to this functional category, including AccD, FabD, FabF, FabG, FabH, FabT and FabZ, were identified in our study. Among these proteins, FabT, a transcriptional regulator of the MarR family, has been shown to be associated with the control of membrane fatty acid composition and survival in low-pH environments in *Streptococcus pneumoniae* (LU e ROCK, 2006). Through the generation of *fabT* mutant strains of *S. pneumoniae*, Lu and RockLU e ROCK (2006) verified that up-regulation of the *fab* gene cluster by the inactivation of FabT leads to a deficiency in unsaturated fatty acids (UFA) and an increase in the proportion of 18-carbon fatty acids in the bacterial membrane, culminating in an acid-sensitive growth phenotype of the pathogen. Loss of UFA also resulted in sensitivity of *Streptococcus mutans* to acidic pH environments (FOZO *et al.*, 2007). Therefore, this behavior seems to be intrinsic to the genus *Streptococcus*. Interestingly, our proteomic data showed that some Fab proteins (FabG and FabZ) were down-regulated in fish-adapted GBS strains in comparison to the human-adapted GBS strain, suggesting a high level of unsaturated fatty acids in the membranes of fish-adapted GBS strains; as a consequence, the membrane becomes more fluid, conferring greater bacterial resistance to acidic environments. It is known that fish-adapted GBS strains are able to grow at a wide range of pH (3 to 11) (LAITH *et al.*, 2017) and that the ability to survive low-pH conditions may be critical for these strains to persist in the aquatic environment. This is especially important considering that the water used in fish farms can sometimes be acidic (pH = 6.3 ± 0.3) (AMAL *et al.*, 2015) and that it may cause fish disease after oral entry and gastrointestinal colonization

(IREGUI *et al.*, 2016) by bacteria that are able to resist the low pH present in the stomach and the high pH present in the gut. In addition, considering that fishes are poikilothermic animals, the higher membrane fluidity of fish-adapted GBS strains may also improve bacterial survival in environments that feature constant thermal variation, such as the water in fish farms and in the host environment.

Because GBS outbreaks usually occur under conditions of high water temperature, water temperature has been considered a predisposing factor for the occurrence of GBS infection in fish (MIAN *et al.*, 2009). The expression of genes and proteins related to thermal adaptation is a universal response observed in prokaryotes, and the transient induction of chaperonin, heat shock and cold shock proteins represents an important mechanism of protection and homeostasis through which such organisms cope with physiological and environmental stress at the cellular level (YURA *et al.*, 1993). One of the thermal adaptation proteins that showed differential expression in our study, ClpP, is involved in the regulation of GBS growth at high temperatures and in bacterial survival under various stress conditions (NAIR *et al.*, 2003). Other thermal shock-associated proteins (DnaK, GroL, GroS, GrpE, Pnp, RNA helicase and cold-shock protein) prevent the inactivation of cellular proteins and assist in the degradation of non-repairable denatured proteins that accumulate during normal growth or under stress conditions (YURA *et al.*, 1993). These proteins were also visualized in our PPI analysis and showed a high number of interactions.

In summary, our pan-proteomic data on metabolic networks suggest that the identified proteins reflect an adaptive ability of fish-adapted GBS strains to response to an aquatic environment. The enhanced expression of these proteins broadens the catabolic capacity for energy generation, increases the diversity of transport system proteins and thereby permits the uptake of carbohydrates, amino acids and ions from water, and modulates the lipid composition of the bacterial membrane. Moreover, the identification of proteins involved in stress responses showed that fish-adapted GBS strains are capable of protecting themselves from a broad range of potential cellular damage that might otherwise be caused by environmental stressors.

#### 4.5.1.2. PROTEINS INVOLVED IN HOST-PATHOGEN INTERACTION

The transition of GBS from the aquatic environment to fish tissues usually requires adaptive changes. One way for this pathogen to monitor and respond to its environment is through the use of proteins that work as part of a two-component signal transduction system (FARALLA *et al.*, 2014). Among the known proteins related to signal transduction, we identified CiaR, CovS/CovR and Stp1/Stk1. CiaR contributes to GBS survival in phagocytic and non-phagocytic cells and to virulence potential in a murine model experimentally infected with wild-type and mutant GBS strains (QUACH *et al.*, 2009). Mutation of the *covS*/*covR* genes in a GBS strain reduced the hemolytic activity of the strain on blood agar and impaired bacterial viability in human serum (LAMY *et al.*, 2004), whereas mutations affecting Stp1/Stk1 impaired GBS growth, cell segregation and virulence in a neonatal rat sepsis model (RAJAGOPAL *et al.*, 2003). The expression of these proteins in fish-adapted GBS strains thus appears to improve bacterial survival and increase their dissemination in fish tissues due to increased bacterial survival in serum.

GBS causes septicemia and meningoencephalitis in fishes (MIAN *et al.*, 2009); however, the pathogenesis of this disease is poorly understood. Although the genome of fish-adapted GBS strains shows the presence of several virulence genes that have already been reported and characterized in human GBS strains, little is known about the participation of these genes in the pathogenesis of the disease in fishes. The primary virulence factors described for GBS are

adhesins, invasins and evasins. We identified some proteins for the first time in fish-adapted GBS strains; these included PavA (adhesion), GapN (adhesion), internalin (invasion), hemolysin A (invasion), several immune evasins (NeuABCD, CpsBCG, RmlABC, and serine protease) and penicillin-binding proteins (PbpX, Pbp1A and Pbp2A). These proteins have not yet been studied in terms of their biological functions in fish-adapted GBS strains, but several them have been very well characterized in human GBS strains (RAJAGOPAL, 2009). The detection of these proteins in fish-adapted GBS strains suggests that their participation in pathogenesis is similar in aquatic hosts and mammalian hosts. An example of this is offered by the identification of the BibA and IagA proteins in our data. These two proteins are involved in GBS invasion and colonization of brain tissue in a murine model and in GBS survival in human blood (DORAN *et al.*, 2005; SANTI *et al.*, 2007). The identification of these proteins in fish-adapted GBS strains suggests their possible association with the clinical manifestations of disease under field conditions in which the diseased fish showed meningoencephalitis and septicemia. However, future research must to be conducted to validate this possibility.

Another identified protein in our study that contributes to bacterial adhesion is elongation factor Tu. This protein was shown to mediate the binding of bacteria to fibronectin, fibrinogen and mucin in studies of *Mycoplasma pneumoniae*, *Listeria monocytogenes* and *Lactobacillus johnsonii*, respectively (GRANATO *et al.*, 2004; SCHAUMBURG *et al.*, 2004; BALASUBRAMANIAN *et al.*, 2008). Elongation factor Tu was previous identified in a proteomic study using fish-adapted GBS strains and shown to be highly expressed in a virulent strain (LI *et al.*, 2014). Similarly, elongation factor Tu was the most abundant protein identified in our work.

Interestingly, some proteins involved in virulence were identified in the SA20-, SA53- and SA95-unique proteomes. These proteins might contribute to the pathogenesis of GBS in fishes. Abortive infection protein, an integrative and conjugative element involved in virulence and metal resistance in GBS (DY *et al.*, 2014), was identified in SA20. Virulence factor EsxA, which was identified in SA53, was shown to contribute to bacterial dissemination and colonization of *Streptococcus suis* in a mouse infection model (LAI *et al.*, 2017) and to induce antibodies in humans infected with *Staphylococcus aureus* (ZHOU *et al.*, 2013). Another virulence protein identified in SA53 was gluconate 5-dehydrogenase, which catalyzes the reversible oxireduction of D-gluconate to 5-keto-D-gluconate (ZHANG *et al.*, 2009). D-gluconate is an important carbon source for prokaryotes and is involved in the colonization, survival and virulence of *E. coli* in streptomycin-treated mice (SWEENEY *et al.*, 1996) and in cell division in *S. suis* (SHI *et al.*, 2014). In the SA95-unique proteome, glycosyl transferase was identified. This protein belongs to a class of enzymes that are responsible for the formation of structural molecules such as glycoproteins, glycolipids, oligosaccharides and of the cell wall and that also act in immune recognition, bacterial evasion, intercellular signaling and biofilm formation (KEENLEYSIDE *et al.*, 2001).

Other proteins identified in our pan-proteome data, such as those involved in nucleotide metabolism and oxidative stress, may also contribute to the pathogenicity of GBS in fishes. A previous study demonstrated that purine and pyrimidine metabolism is essential for the survival and growth of *Escherichia coli*, *Salmonella enterica* and *Bacillus anthracis* in human serum (SAMANT *et al.*, 2008). In GBS, on the other hand, genes involved in purine and pyrimidine metabolism showed significant modification of transcription in response to incubation with human blood, revealing a dynamic metabolic adaptation of this bacterium (MEREGHETTI *et al.*, 2008a). In our PPI analysis, numerous interactions between proteins related to nucleotide metabolism were detected. Therefore, after fish infection, the expression of proteins involved in

nucleotide metabolism may be associated with GBS serum resistance in the fish host, as previously demonstrated by Wang et al. (2016).

During the infection process, bacteria encounter reactive oxygen species (ROS) generated by neutrophils and macrophages of the host as a defense mechanism; these ROS directly damage proteins, nucleic acids and other cellular components (STORZ e IMLAYT, 1999; YAMAMOTO *et al.*, 2006). We identified the expression of proteins involved in ROS detoxification, including SodA, SufB, SufC, SufD, TrxB, thioredoxin and NoxE, that have been previously characterized in GBS strains (GLASER *et al.*, 2002; PEREIRA *et al.*, 2013a). Among these proteins, superoxide dismutase (SodA) is also involved in virulence, contributing to the pathogenicity of GBS by allowing bacterial survival in macrophages and maintaining a high bacterial load in the blood of experimentally infected mice (POYART *et al.*, 2001).

#### 4.5.1.3. PUTATIVE VACCINE TARGETS

Due to the high similarity of the genomic content of the GBS strains used in this work, a predicted vaccine candidate for all strains could reasonably be expected to confer protection against the disease regardless of the circulating genotype in a fish farm.

Eleven of the 38 predicted antigenic proteins were also detected in a previous study of conserved antigenic proteins in GBS strains isolated from human ($n$ = 10), bovine ($n$ = 1) and fish ($n$ = 4) hosts (PEREIRA *et al.*, 2013b) as being shared only by fish-adapted GBS strains. Among these proteins, the immunogenicity and efficacy of a recombinant vaccine against GBS prepared against the cell wall surface anchor protein has already been evaluated in tilapia and turbot that were vaccinated and experimentally infected (LIU *et al.*, 2016). Although the evaluation was performed using high doses ($10^8$ CFU $fish^{-1}$) of a fish-adapted GBS strain, the vaccine provided relatively high percentage survival (RPS) of 72.5 % and 72.7 % for tilapia and turbot, respectively (LIU *et al.*, 2016).

An important putative vaccine target in our study was Sip. This protein is highly conserved among all GBS strains regardless of serotype (MA *et al.*, 2017) and was one of the most abundant proteins in our pan-proteome data (Figure 1). Moreover, Sip has been used in the preparation of vaccines against GBS in tilapia, resulting in an RPS of 41.6 to 95.8 % using DNA or adjuvanted vaccines and conferring high protection in vaccinated fish (HE *et al.*, 2014; MA *et al.*, 2017).

Other predicted proteins with unknown functions (hypothetical proteins) and proteins that have not yet been tested as vaccine targets may be used for vaccine development in further studies aimed at evaluating their potential for the protection of fishes against GBS infection and to determine whether they confer immunity to strains belonging to different clonal complexes.

### 4.5.2. GLOBAL DIFFERENTIAL EXPRESSION OF PROTEINS

To explore changes in protein abundance linked to host adaptation, we performed a comparative proteome analysis of human and fish-adapted GBS strains. This type of comparison was previously performed using a microarray approach; the results obtained using that approach showed that there is a closer genetic relationship between the GBS CF01173 strain isolated from fish (ST-7) and the A909 strain isolated from human (ST-7) and indicated genetic divergence of the strains 2-22 (ST-261) and SS1219 (ST-260) from strain ST-7 at the transcriptional level (ROSINSKI-CHUPIN *et al.*, 2013). However, the proteomic approach is more robust than the microarray technique for evaluating the expression of the functional genome because it measures

the expression of proteins that are directly involved in enzymatic catalysis, molecular signaling, and physical interactions (YATES *et al.*, 2009).

One protein present in the NEM316-unique proteome was associated with specialization of the bacterium to the human host. This transcriptional regulator (GBS_RS10725) is present only in the genome of the NEM316 strain, having been deleted during reductive evolution of the ST260-261 strains (ROSINSKI-CHUPIN *et al.*, 2013). This protein is a positive regulator of resistance to cadmium in some GBS strains (NITSCHKE *et al.*, 2014). On the other hand, the proteins that were exclusively identified in fish-adapted GBS strains are related mainly to virulence factors that have already been discussed in this work and that together may increase the possibility of onset of disease in fish. However, our qualitative proteomic analysis of human- and fish-adapted GBS strains did not indicate the basis for the host specificity of the strains, as highly similar protein content was observed in all of the examined strains.

Although the protein content of human and fish-adapted GBS strains was similar, there was differential expression at the proteome level. An association between the level of expression of specific proteins and the genotype of fish-adapted strains was observed. In all fish-adapted GBS strains, 40, 27, 26 and 22 proteins involved in translation, ribosomal structure and biogenesis, cell wall/membrane/envelope biogenesis, carbohydrate transport and metabolism and energy production and conversion, respectively, were expressed at lower levels than in the NEM316 strain. These results reveal a reduced catabolic capacity of fish-pathogenic *S. agalactiae* in comparison with the human GBS strain. Previous studies using genomic approaches have suggested that the reduction of catabolic capacity in fish-adapted strains could be linked to adaptation of the bacterium to aquatic hosts (LIU *et al.*, 2013b; ROSINSKI-CHUPIN *et al.*, 2013).

Among the proteins identified as DEPs in fish- and human-adapted GBS strains (Table 5), reticulocyte binding protein showed increased expression (its $\log_2$ ratio increased from 1.51 to 5.45) compared with the NEM316 strain. This protein is a serine protease that is homologous to C5a peptidase (ScpB), which facilitates host immune evasion through cleavage and inactivation of complement component C5a and promotes adhesion to host cells (RAJAGOPAL, 2009). In addition, proteins involved in multidrug resistance, such as DltA, antibiotic ABC transporter ATP-binding protein and GNAT family acetyltransferase, were expressed at $\log_2$ ratios 1.02-5.44-fold higher in fish-adapted GBS strains than in the NEM316 strain. The up-regulation of these proteins might modulate host cellular processes, especially the complement cascade and the IFN pathway, both of which are considered effective defenses against bacterial pathogens (CASTRO *et al.*, 2015) and are known to contribute to the adhesion, dissemination, and persistence of GBS in various fish tissues. On the other hand, 11 proteins were down-regulated in fish-adapted GBS strains compared to the NEM316 strain; beta-lactamase ($\log_2$ ratio of -1.32 to -5.62), ThrB (-2.85 to -4.69) and PavA (-1.22 to -3.66) showed higher expression in the latter strain. Despite the identification of virulence proteins that showed differential regulation in the human GBS strain, our results are not consistent with the results of *in vivo* trials previously performed by our group in which it was shown that fish-adapted GBS strains cause mortality in Nile tilapia whereas the NEM316 strain causes a transient infection in which fish do not manifest clinical signs of disease or mortality (PEREIRA *et al.*, 2010). However, some of the differentially expressed proteins, such as PavA, modulate the activity of important virulence factors in *Streptococcus pneumoniae* that are associated with adherence and survival in experimentally infected mice; thus, even a wild-type strain that expresses the *pavA* gene may cause higher mortality than that caused by the isogenic mutant (PRACHT *et al.*, 2005). Therefore, the proteins that are up-regulated in the NEM316 strain might be active in GBS virulence only in the mammalian host and may not contribute to disease in aquatic animals.

## 4.6. CONCLUSIONS

The current study is the first to evaluate the whole proteome of GBS strains by LC-UDMS$^{E}$; it is also the first study to compare the proteome of this pathogen in different, closely related genotypes. Our results demonstrated high similarity of the expressed proteins and showed that the core proteome of fish-adapted GBS strains is conserved. Our comparison of protein expression among isolates with different genotypes belonging to fish-associated clonal complexes provided information about the metabolism, the survival strategy, the adaptation and the pathogenicity of fish-pathogenic GBS strains. The high degree of conservation among strains with different STs suggests that monovalent vaccines may be effective against different genetic variants within clonal complexes. Despite the similarity in protein content, the global protein expression of the NEM316 strain was different from that of the fish-adapted GBS strains, suggesting distinct adaptations to mammalian and fish hosts at the proteome level.

## 4.7. REFERENCES


AMAL, M. N. A.; SAAD, M. Z.; ZAHRAH, A. S. *et al.* Water quality influences the presence of *Streptococcus agalactiae* in cage cultured red hybrid tilapia, *Oreochromis niloticus* × *Oreochromis mossambicus*. *Aquacult. Res.*, v. 46, n. 2, p. 313-323, 2015.

BALASUBRAMANIAN, S.; KANNAN, T. R.; BASEMAN, J. B. The surface-exposed carboxyl region of *Mycoplasma pneumoniae* Elongation Factor Tu interacts with fibronectin. *Infect. Immun.*, v. 76, n. 7, p. 3116-3123, 2008.

BARATO, P.; MARTINS, E. R.; MELO-CRISTINO, J. *et al.* Persistence of a single clone of *Streptococcus agalactiae* causing disease in tilapia (*Oreochromis* sp.) cultured in Colombia over 8 years. *J. Fish Dis.*, v. 38, n. 12, p. 1083-1087, 2015.

BARINOV, A.; LOUX, V.; HAMMANI, A. *et al.* Prediction of surface exposed proteins in *Streptococcus pyogenes*, with a potential application to other Gram-positive bacteria. *Proteomics*, v. 9, n. 1, p. 61-73, 2009.

BARONY, G. M.; TAVARES, G. C.; PEREIRA, F. L. *et al.* Large-scale genomic analyses reveal the population structure and evolutionary trends of *Streptococcus agalactiae* strains in Brazilian fish farms. *Sci. Rep.*, v. 7, n. 1, p. 13538, 2017.

BENSON, D. A.; CAVANAUGH, M.; CLARK, K. *et al.* GenBank. *Nucleic Acids Res.*, v. 41, n. D1, p. D36-D42, 2013.

BERGAL, A.; LOUCIF, L.; BENOUARETH, D. E. *et al.* Molecular epidemiology and distribution of serotypes, genotypes, and antibiotic resistance genes of *Streptococcus agalactiae* clinical isolates from Guelma, Algeria and Marseille, France. *Eur. J. Clin. Microbiol. Infect. Dis.*, v. 34, n. 12, p. 2339-48, 2015.

BOWATER, R. O. Investigation of an emerging bacterial disease in wild Queensland groupers, marine fish and stingrays with production of diagnostic tools to reduce the spread of disease to other states of Australia. 2015. Disponível em: < http://frdc.com.au/research/Final_reports/2010-034-DLD.pdf >. Acesso em: 15 dez. 2017.

BRAUN, L.; COSSART, P. Interactions between *Listeria monocytogenes* and host mammalian cells. *Microbes Infect.*, v. 2, n. 7, p. 803-811, 2000.

BROADBENT, J. A.; BROSZCZAK, D. A.; TENNAKOON, I. U. K. *et al.* Pan-proteomics, a concept for unifying quantitative proteome measurements when comparing closely-related bacterial strains. *Exp. Rev. Proteomics*, v. 13, n. 4, p. 355-365, 2016.

CASTRO, R.; JOUNEAU, L.; TACCHI, L. *et al.* Disparate developmental patterns of immune responses to bacterial and viral infections in fish. *Sci. Rep.*, v. 5, p. 15458, 2015.

DELANNOY, C. M.; CRUMLISH, M.; FONTAINE, M. C. *et al.* Human *Streptococcus agalactiae* strains in aquatic mammals and fish. *BMC Microbiol.*, v. 13, n. 1, p. 1-9, 2013.

DENG, Z. B.; ZHANG, Y. W.; GENG, Y. *et al.* A new sequence type (ZST-1) of infectious *Streptococcus agalactiae* from Chinese cyprinid fish, *Schizopygopsis pylzovi*. *Aquaculture*, v. 468, n. Part 1, p. 496-500, 2017.

DI PALO, B.; RIPPA, V.; SANTI, I. *et al.* Adaptive response of Group B *Streptococcus* to high glucose conditions: new insights on the CovRS regulation network. *Plos One*, v. 8, n. 4, p. e61294, 2013.

DISTLER, U.; KUHAREV, J.; NAVARRO, P. *et al.* Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics. *Nat. Meth.*, v. 11, n. 2, p. 167-170, 2014.

DORAN, K. S.; ENGELSON, E. J.; KHOSRAVI, A. *et al.* Blood-brain barrier invasion by Group B *Streptococcus* depends upon proper cell-surface anchoring of lipoteichoic acid. *J. Clin. Investig.*, v. 115, n. 9, p. 2499-2507, 2005.

DORO, F.; LIBERATORI, S.; RODRÍGUEZ-ORTEGA, M. J. *et al.* Surfome analysis as a fast track to vaccine discovery: identification of a novel protective antigen for Group B *Streptococcus* hypervirulent strain COH1. *Mol. Cell. Proteomics*, v. 8, n. 7, p. 1728-1737, 2009.

DY, R. L.; PRZYBILSKI, R.; SEMEIJN, K. *et al.* A widespread bacteriophage abortive infection system functions through a type IV toxin–antitoxin mechanism. *Nucleic Acids Res.*, v. 42, n. 7, p. 4590-4605, 2014.

EVANS, J. J.; BOHNSACK, J. F.; KLESIUS, P. H. *et al.* Phylogenetic relationships among *Streptococcus agalactiae* isolated from piscine, dolphin, bovine and human sources: a dolphin and piscine lineage associated with a fish epidemic in Kuwait is also associated with human neonatal infections in Japan. *J. Med. Microbiol.*, v. 57, n. 11, p. 1369-1376, 2008.

FARALLA, C.; METRUCCIO, M. M.; DE CHIARA, M. *et al.* Analysis of two-component systems in Group B *Streptococcus* shows that RgfAC and the novel FspSR modulate virulence and bacterial fitness. *MBio*, v. 5, n. 3, p. e00870-14, 2014.

FOZO, E. M.; SCOTT-ANNE, K.; KOO, H. *et al.* Role of unsaturated fatty acid biosynthesis in virulence of *Streptococcus mutans*. *Infect. Immun.*, v. 75, n. 3, p. 1537-1539, 2007.

FUCHS, T. M.; EISENREICH, W.; HEESEMANN, J. *et al.* Metabolic adaptation of human pathogenic and related nonpathogenic bacteria to extra- and intracellular habitats. *FEMS Microbiol. Rev.*, v. 36, n. 2, p. 435-462, 2012.

GALPERIN, M. Y.; MAKAROVA, K. S.; WOLF, Y. I. *et al.* Expanded microbial genome coverage and improved protein family annotation in the COG database. *Nucleic Acids Res.*, v. 43, n. D1, p. D261-D269, 2015.

GLASER, P.; RUSNIOK, C.; BUCHRIESER, C. *et al.* Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Mol. Microbiol.*, v. 45, n. 6, p. 1499-1513, 2002.

GODOY, D. T.; CARVALHO-CASTRO, G. A.; LEAL, C. A. G. *et al.* Genetic diversity and new genotyping scheme for fish pathogenic *Streptococcus agalactiae*. *Lett. Appl. Microbiol.*, v. 57, n. 6, p. 476-483, 2013.

GRANATO, D.; BERGONZELLI, G. E.; PRIDMORE, R. D. *et al.* Cell surface-associated Elongation Factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. *Infect. Immun.*, v. 72, n. 4, p. 2160-2169, 2004.

GRÜNDLING, A. Potassium uptake systems in *Staphylococcus aureus*: new stories about ancient systems. *MBio*, v. 4, n. 5, p. e00784-13, 2013.

HE, Y.; WANG, K.-Y.; XIAO, D. *et al.* A recombinant truncated surface immunogenic protein (tSip) plus adjuvant FIA confers active protection against Group B *Streptococcus* infection in tilapia. *Vaccine*, v. 32, n. 51, p. 7025-7032, 2014.

HEBERLE, H.; MEIRELLES, G. V.; DA SILVA, F. R. *et al.* InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics*, v. 16, n. 1, p. 1-7, 2015.

HERNÁNDEZ, E.; FIGUEROA, J.; IREGUI, C. Streptococcosis on a red tilapia, *Oreochromis* sp., farm: a case study. *J. Fish Dis.*, v. 32, n. 3, p. 247-252, 2009.

HOHLE, T. H.; FRANCK, W. L.; STACEY, G. *et al.* Bacterial outer membrane channel for divalent metal ion acquisition. *Proc. Natl. Acad. Sci. USA*, v. 108, n. 37, p. 15390-15395, 2011.

HOLMES, A. R.; MCNAB, R.; MILLSAP, K. W. *et al.* The *pavA* gene of *Streptococcus pneumoniae* encodes a fibronectin-binding protein that is essential for virulence. *Mol. Microbiol.*, v. 41, n. 6, p. 1395-1408, 2001.

HUGHES, M. J. G.; MOORE, J. C.; LANE, J. D. *et al.* Identification of major outer surface proteins of *Streptococcus agalactiae*. *Infect. Immun.*, v. 70, n. 3, p. 1254-1259, 2002.

IREGUI, C. A.; COMAS, J.; VÁSQUEZ, G. M. *et al.* Experimental early pathogenesis of *Streptococcus agalactiae* infection in red tilapia *Oreochromis* spp. *J. Fish Dis.*, v. 39, n. 2, p. 205-215, 2016.

JHINGAN, G. D.; KUMARI, S.; JAMWAL, S. V. *et al.* Comparative proteomic analyses of avirulent, virulent, and clinical strains of *Mycobacterium tuberculosis* identify strain-specific patterns. *J. Biolog. Chem.*, v. 291, n. 27, p. 14257-14273, 2016.

JOHRI, A. K.; PAOLETTI, L. C.; GLASER, P. *et al.* Group B *Streptococcus*: global incidence and vaccine development. *Nat. Rev. Microbiol.*, v. 4, n. 12, p. 932-942, 2006.

JONES, N.; BOHNSACK, J. F.; TAKAHASHI, S. *et al.* Multilocus sequence typing system for Group B *Streptococcus*. *J. Clin. Microbiol.*, v. 41, n. 6, p. 2530-2536, 2003.

KEEFE, G. P. *Streptococcus agalactiae* mastitis: a review. *Can. Vet. J.*, v. 38, n. 7, p. 429-437, 1997.

KEENLEYSIDE, W. J.; CLARKE, A. J.; WHITFIELD, C. Identification of residues involved in catalytic activity of the inverting glycosyl transferase WbbE from *Salmonella enterica* serovar Borreze. *J. Bacteriol.*, v. 183, n. 1, p. 77-85, 2001.

KUHAREV, J.; NAVARRO, P.; DISTLER, U. *et al.* In-depth evaluation of software tools for data-independent acquisition based label-free quantification. *Proteomics*, v. 15, n. 18, p. 3140-3151, 2015.

LAI, L.; DAI, J.; TANG, H. *et al. Streptococcus suis* serotype 9 strain GZ0565 contains a type VII secretion system putative substrate EsxA that contributes to bacterial virulence and a vanZ-like gene that confers resistance to teicoplanin and dalbavancin in *Streptococcus agalactiae*. *Vet. Microbiol.*, v. 205, p. 26-33, 2017.

LAITH, A. A.; AMBAK, M. A.; HASSAN, M. *et al.* Molecular identification and histopathological study of natural *Streptococcus agalactiae* infection in hybrid tilapia (*Oreochromis niloticus*). *Vet. World*, v. 10, n. 1, p. 101-111, 2017.

LAMY, M.-C.; ZOUINE, M.; FERT, J. *et al.* CovS/CovR of Group B *Streptococcus*: a two-component global regulatory system involved in virulence. *Mol. Microbiol.*, v. 54, n. 5, p. 1250-1268, 2004.

LATA, K.; PAUL, K.; CHATTOPADHYAY, K. Functional characterization of *Helicobacter pylori* TlyA: pore-forming hemolytic activity and cytotoxic property of the protein. *Biochem. Biophys. Res. Commun.*, v. 444, n. 2, p. 153-157, 2014.

LEVINA, N.; TÖTEMEYER, S.; STOKES, N. R. *et al.* Protection of *Escherichia coli* cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: identification of genes required for MscS activity. *EMBO J.*, v. 18, n. 7, p. 1730-1737, 1999.

LI, G.-Z.; VISSERS, J. P. C.; SILVA, J. C. *et al.* Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. *Proteomics*, v. 9, n. 6, p. 1696-1719, 2009.

LI, L.; SHI, Y.; WANG, R. *et al.* Proteomic analysis of tilapia *Oreochromis niloticus Streptococcus agalactiae* strains with different genotypes and serotypes. *J. Fish Biol.*, v. 86, n. 2, p. 615-636, 2015.

LI, L.; STOECKERT, C. J.; ROOS, D. S. OrthoMCL: Identification of ortholog groups for eukaryotic genomes. *Genome Res.*, v. 13, n. 9, p. 2178-2189, 2003.

LI, W.; GODZIK, A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics*, v. 22, n. 13, p. 1658-1659, 2006.

LI, W.; SU, Y. L.; MAI, Y. Z. *et al.* Comparative proteome analysis of two *Streptococcus agalactiae* strains from cultured tilapia with different virulence. *Vet. Microbiol.*, v. 170, n. 1-2, p. 135-143, 2014.

LI, W.; WANG, H.-Q.; HE, R.-Z. *et al.* Major surfome and secretome profile of *Streptococcus agalactiae* from Nile tilapia (*Oreochromis niloticus*): insight into vaccine development. *Fish Shellfish Immunol.*, v. 55, p. 737-746, 2016.

LIU, G.; ZHANG, W.; LU, C. Identification of immunoreactive proteins of *Streptococcus agalactiae* isolated from cultured tilapia in China. *Pathog. Dis.*, v. 69, n. 3, p. 223-231, 2013a.

LIU, G. J.; ZHANG, W.; LU, C. P. Comparative genomics analysis of *Streptococcus agalactiae* reveals that isolates from cultured tilapia in China are closely related to the human strain A909. *BMC Genomics*, v. 14, p. 10, 2013b.

LIU, H.; ZHANG, S.; SHEN, Z. *et al.* Development of a vaccine against *Streptococcus agalactiae* in fish based on truncated cell wall surface anchor proteins. *Vet. Rec.*, v. 179, n. 14, p. 359, 2016.

LU, Y.-J.; ROCK, C. O. Transcriptional regulation of fatty acid biosynthesis in *Streptococcus pneumoniae*. *Mol. Microbiol.*, v. 59, n. 2, p. 551-566, 2006.

LUSIASTUTI, A. M.; TEXTOR, M.; SEEGER, H. *et al.* The occurrence of *Streptococcus agalactiae* sequence type 261 from fish disease outbreaks of tilapia *Oreochromis niloticus* in Indonesia. *Aquacult. Res.*, v. 45, n. 7, p. 1260-1263, 2014.

MA, Y.-P.; KE, H.; LIANG, Z.-L. *et al.* Protective efficacy of cationic-PLGA microspheres loaded with DNA vaccine encoding the sip gene of *Streptococcus agalactiae* in tilapia. *Fish Shellfish Immunol.*, v. 66, n. Supplement C, p. 345-353, 2017.

MAIONE, D.; MARGARIT, I.; RINAUDO, C. D. *et al.* Identification of a universal Group B *Streptococcus* vaccine by multiple genome screen. *Science*, v. 309, n. 5731, p. 148-150, 2005.

MEREGHETTI, L.; SITKIEWICZ, I.; GREEN, N. M. *et al.* Extensive adaptive changes occur in the transcriptome of *Streptococcus agalactiae* (Group B *Streptococcus*) in response to incubation with human blood. *Plos One*, v. 3, n. 9, p. e3143, 2008a.

MEREGHETTI, L.; SITKIEWICZ, I.; GREEN, N. M. *et al.* Remodeling of the *Streptococcus agalactiae* transcriptome in response to growth temperature. *Plos One*, v. 3, n. 7, p. e2785, 2008b.

MIAN, G. F.; GODOY, D. T.; LEAL, C. A. G. *et al.* Aspects of the natural history and virulence of *S. agalactiae* infection in Nile tilapia. *Vet. Microbiol.*, v. 136, n. 1–2, p. 180-183, 2009.

MICKELSON, M. N. Aerobic metabolism of *Streptococcus agalactiae*. *J. Bacteriol.*, v. 94, n. 1, p. 184-191, 1967.

MILLIGAN, T. W.; DORAN, T. I.; STRAUS, D. C. *et al.* Growth and amino acid requirements of various strains of Group B streptococci. *J. Clin. Microbiol.*, v. 7, n. 1, p. 28-33, 1978.

MODAK, J.; DECKWER, W.-D.; ZENG, A.-P. Metabolic control analysis of eucaryotic pyruvate dehydrogenase multienzyme complex. *Biotechnol. Prog.*, v. 18, n. 6, p. 1157-1169, 2002.

NAIR, S.; POYART, C.; BERETTI, J.-L. *et al.* Role of the *Streptococcus agalactiae* ClpP serine protease in heat-induced stress defence and growth arrest. *Microbiol.*, v. 149, n. 2, p. 407-417, 2003.

NEIJSSEL, O. M.; SNOEP, J. L.; TEIXEIRA DE MATTOS, M. J. Regulation of energy source metabolism in streptococci. *J. Appl. Microbiol.*, v. 83, n. S1, p. 12S-19S, 1997.

NITSCHKE, H.; SLICKERS, P.; MÜLLER, E. *et al.* DNA microarray-based typing of *Streptococcus agalactiae* isolates. *J. Clin. Microbiol.*, v. 52, n. 11, p. 3933-3943, 2014.

PAPASERGI, S.; GALBO, R.; LANZA-CARICCIO, V. *et al.* Analysis of the *Streptococcus agalactiae* exoproteome. *J. Proteomics*, v. 89, p. 154-164, 2013.

PEREIRA, U. P.; MIAN, G. F.; OLIVEIRA, I. C. M. *et al.* Genotyping of *Streptococcus agalactiae* strains isolated from fish, human and cattle and their virulence potential in Nile tilapia. *Vet. Microbiol.*, v. 140, n. 1–2, p. 186-192, 2010.

PEREIRA, U. P.; SANTOS, A. R.; HASSAN, S. S. *et al.* Complete genome sequence of *Streptococcus agalactiae* strain SA20-06, a fish pathogen associated to meningoencephalitis outbreaks. *Stand. Genomic Sci.*, v. 8, n. 2, p. 188-97, 2013a.

PEREIRA, U. P.; SOARES, S. C.; BLOM, J. *et al.* In silico prediction of conserved vaccine targets in *Streptococcus agalactiae* strains isolated from fish, cattle, and human samples. *Genet. Mol. Res.*, v. 12, n. 3, p. 2902-12, 2013b.

POYART, C.; PELLEGRINI, E.; GAILLOT, O. *et al.* Contribution of Mn-cofactored superoxide dismutase (SodA) to the virulence of *Streptococcus agalactiae*. *Infect. Immun.*, v. 69, n. 8, p. 5098-5106, 2001.

PRACHT, D.; ELM, C.; GERBER, J. *et al.* PavA of *Streptococcus pneumoniae* modulates adherence, invasion, and meningeal inflammation. *Infect. Immun.*, v. 73, n. 5, p. 2680-2689, 2005.

PRAGYA, P.; KAUR, G.; ALI, S. A. *et al.* High-resolution mass spectrometry-based global proteomic analysis of probiotic strains *Lactobacillus fermentum* NCDC 400 and RS2. *J. Proteomics*, v. 152, p. 121-130, 2017.

QUACH, D.; VAN SORGE, N. M.; KRISTIAN, S. A. *et al.* The CiaR response regulator in Group B *Streptococcus* promotes intracellular survival and resistance to innate immune defenses. *J. Bacteriol.*, v. 191, n. 7, p. 2023-2032, 2009.

R CORE TEAM. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing 2013.

RAJAGOPAL, L. Understanding the regulation of Group B streptococcal virulence factors. *Future Microbiol.*, v. 4, n. 2, p. 201-221, 2009.

RAJAGOPAL, L.; CLANCY, A.; RUBENS, C. E. A eukaryotic type serine/threonine kinase and phosphatase in *Streptococcus agalactiae* reversibly phosphorylate an inorganic pyrophosphatase and affect growth, cell segregation, and virulence. *J. Biolog. Chem.*, v. 278, n. 16, p. 14429-14441, 2003.

ROSINSKI-CHUPIN, I.; SAUVAGE, E.; MAIREY, B. *et al.* Reductive evolution in *Streptococcus agalactiae* and the emergence of a host adapted lineage. *BMC Genomics*, v. 14, n. 1, p. 1-15, 2013.

SAMANT, S.; LEE, H.; GHASSEMI, M. *et al.* Nucleotide biosynthesis is critical for growth of bacteria in human blood. *Plos Pathog.*, v. 4, n. 2, p. e37, 2008.

SANTI, I.; SCARSELLI, M.; MARIANI, M. *et al.* BibA: a novel immunogenic bacterial adhesin contributing to Group B *Streptococcus* survival in human blood. *Mol. Microbiol.*, v. 63, n. 3, p. 754-767, 2007.

SCHAUMBURG, J.; DIEKMANN, O.; HAGENDORFF, P. *et al.* The cell wall subproteome of *Listeria monocytogenes*. *Proteomics*, v. 4, n. 10, p. 2991-3006, 2004.

SCHREUR, P. J. W.; REBEL, J. M. J.; SMITS, M. A. *et al.* TroA of *Streptococcus suis* is required for manganese acquisition and full virulence. *J. Bacteriol.*, v. 193, n. 19, p. 5073-5080, 2011.

SEIFERT, K. N.; MCARTHUR, W. P.; BLEIWEIS, A. S. *et al.* Characterization of Group B streptococcal glyceraldehyde-3-phosphate dehydrogenase: surface localization, enzymatic activity, and protein–protein interactions. *Can. J. Microbiol.*, v. 49, n. 5, p. 350-356, 2003.

SHI, Z.; XUAN, C.; HAN, H. *et al.* Gluconate 5-dehydrogenase (Ga5DH) participates in *Streptococcus suis* cell division. *Protein Cell*, v. 5, n. 10, p. 761-769, 2014.

SILVA, W. M.; FOLADOR, E. L.; SOARES, S. C. *et al.* Label-free quantitative proteomics of *Corynebacterium pseudotuberculosis* isolates reveals differences between biovars ovis and equi strains. *BMC Genomics*, v. 18, n. 1, p. 451, 2017.

SITKIEWICZ, I.; GREEN, N. M.; GUO, N. *et al.* Transcriptome adaptation of Group B *Streptococcus* to growth in human amniotic fluid. *Plos One*, v. 4, n. 7, p. e6114, 2009.

SPRINGMAN, A. C.; LACHER, D. W.; WAYMIRE, E. A. *et al.* Pilus distribution among lineages of Group B *Streptococcus*: an evolutionary and clinical perspective. *BMC Microbiol.*, v. 14, n. 1, p. 1-11, 2014.

STORZ, G.; IMLAYT, J. A. Oxidative stress. *Curr. Opin. Microbiol.*, v. 2, n. 2, p. 188-194, 1999.

SWEENEY, N. J.; LAUX, D. C.; COHEN, P. S. *Escherichia coli* F-18 and *E. coli* K-12 eda mutants do not colonize the streptomycin-treated mouse large intestine. *Infect. Immun.*, v. 64, n. 9, p. 3504-11, 1996.

SZKLARCZYK, D.; MORRIS, J. H.; COOK, H. *et al.* The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. *Nucleic Acids Res.*, v. 45, n. Database issue, p. D362-D368, 2017.

VERNER-JEFFREYS, D. W.; WALLIS, T. J.; CANO CEJAS, I. *et al. Streptococcus agalactiae* multilocus sequence type 261 is associated with mortalities in the emerging Ghanaian tilapia industry. *J. Fish Dis.*, v. 41, n. 1, p. 175-179, 2018.

VIZCAÍNO, J. A.; CSORDAS, A.; DEL-TORO, N. *et al.* 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.*, v. 44, n. D1, p. D447-D456, 2016.

VU, V. Q. *ggbiplot*: A *ggplot2* Based Biplot. R package version 0.55. 2011. Disponível em: < http://github.com/vqv/ggbiplot >. Acesso em: 15 dez. 2017.

WANG, Z.; LI, M.-Y.; PENG, B. *et al.* GC–MS-based metabolome and metabolite regulation in serum-resistant *Streptococcus agalactiae*. *J. Proteome Res.*, v. 15, n. 7, p. 2246-2253, 2016.

WARNES, G. R.; BOLKER, B.; BONEBAKKER, L. *et al.* Various R programming tools for plotting data. R package version 3.0.1. 2016. Disponível em: <https://cran.r-project.org/web/packages/gplots/index.html>. Acesso em: 15 dez. 2017.

XIANG, Z.; HE, Y. Genome-wide prediction of vaccine targets for human herpes simplex viruses using Vaxign reverse vaccinology. *BMC Bioinformatics*, v. 14, n. 4, p. S2, 2013.

YAMAMOTO, Y.; PARGADE, V.; LAMBERET, G. *et al.* The Group B *Streptococcus* NADH oxidase Nox-2 is involved in fatty acid biosynthesis during aerobic growth and contributes to virulence. *Mol. Microbiol.*, v. 62, n. 3, p. 772-785, 2006.

YATES, J. R.; RUSE, C. I.; NAKORCHEVSKY, A. Proteomics by mass spectrometry: approaches, advances, and applications. *Annu. Rev. Biomed. Eng.*, v. 11, p. 49-79, 2009.

YURA, T.; NAGAI, H.; MORI, H. Regulation of the heat-shock response in bacteria. *Annu. Rev. Microbiol.*, v. 47, n. 1, p. 321-350, 1993.

ZHANG, L.; XIAO, D.; PANG, B. *et al.* The core proteome and pan proteome of *Salmonella* Paratyphi A epidemic strains. *Plos One*, v. 9, n. 2, p. e89197, 2014.

ZHANG, Q.; PENG, H.; GAO, F. *et al.* Structural insight into the catalytic mechanism of gluconate 5-dehydrogenase from *Streptococcus suis*: crystal structures of the substrate-free and quaternary complex enzymes. *Protein Sci.*, v. 18, n. 2, p. 294-303, 2009.

ZHOU, H.; DU, H.; ZHANG, H. *et al.* EsxA might as a virulence factor induce antibodies in patients with *Staphylococcus aureus* infection. *Braz. J. Microbiol.*, v. 44, n. 1, p. 267-271, 2013.

TABLES

FIGURE LEGENDS

Figure 1. Dynamic range of the protein abundances found in fish-adapted GBS strains. The data are binned according to the log10 of their normalized abundance. SA16 (blue), SA20 (green), SA53 (red), SA81 (yellow), and SA95 (purple).

Figure 2. Venn diagram showing the number of unique and shared proteins in fish-adapted GBS strains.

Figure 3. Proteome similarity matrix of fish-adapted GBS strains. The numbers inside the frames indicate the percentage of similarities among strains.

Figure 4. Prediction of COG functional categories of the proteins identified in the pan-proteome of fish-adapted GBS strains. The total number of proteins in the functional categories does not correspond to the final number of proteins in the pan-proteome because some proteins are associated with more than one COG category.

Figure 5. Interaction networks of identified core proteins of fish-adapted GBS strains. Thicker lines denote interactions with score ≥ 0.980. (1) Ribosomal proteins; (2) ATP synthase; (3) Pyruvate metabolism; (4) Carbohydrate metabolism; (5) Heat shock proteins; (6) Nucleotide metabolism; (7) Aminoacyl-tRNA synthetase; (8) DNA replication and repair; (9) Peptidoglycan biosynthesis.

Figure 6. Heat map analysis of proteins that were significantly up- and down-regulated in fish-adapted GBS strains in comparison to the NEM316 strain.

Figure 7. COG functional categories of the proteins that were differentially expressed in human- and fish-adapted GBS strains. The blue bars represent up-regulated proteins in fish-adapted GBS strains with respect to the NEM316 strain; the red bars represent down-regulated proteins.

Supplementary Figure 1. Quality control of the proteins identified by LC-MS. A: normal distribution of 10 ppm error of the total identified peptides. B: peptide detection type. PepFrag1 and PepFrag2 correspond to the peptide matches obtained by comparison to the database by Progenesis QIP, VarMod corresponds to variable modifications, InSource corresponds to fragmentation that occurred at the ionization source, and MissedCleavage corresponds to missed trypsin cleavage. C: drift time for ions of 1+ (blue), 2+ (green), 3+ (red), 4+ (yellow) and 5+ (purple) charge states. D: repeat rate indicating the number of times that an identified protein appears in the replicates: 3 of 3 (blue) and 2 of 3 (red). E: principal component analysis, biological replicates ($n$ = 3) of GBS strains; NEM316 (red), SA16 (blue), SA20 (yellow), SA53 (gray), SA81 (green) and SA95 (black).

Supplementary Figure 2. Venn diagram showing the number of proteins up- and down-regulated in fish-adapted GBS strains in comparison to the NEM316 strain.

Table 1. Characteristics of the *Streptococcus agalactiae* strains evaluated in this study.

| Isolate codes[1] | Host | Country/State | Year of Isolation | Capsular serotype | ST | NCBI accession No. | Predicted Proteins / Protein clusters | Ref. |
|---|---|---|---|---|---|---|---|---|
| SA16 | Nile tilapia | Brazil / São Paulo | 2006 | Ib | NT[2] | CP019807.1 | 1690/1652 | BARONY *et al*., 2017 |
| SA20 | Nile tilapia | Brazil / Paraná | 2006 | Ib | NT | CP003919.2 | 1679/1643 | BARONY *et al*., 2017 |
| SA53 | Nile tilapia | Brazil / Ceará | 2007 | Ib | 260 | CP019802.1 | 1700/1655 | BARONY *et al*., 2017 |
| SA81 | Amazon catfish | Brazil / Mato Grosso | 2009 | Ib | NT | CP019810.1 | 1687/1650 | BARONY *et al*., 2017 |
| SA95 | Nile tilapia | Brazil / Alagoas | 2010 | Ib | 927 | CP019812.1 | 1707/1662 | BARONY *et al*., 2017 |
| NEM316 | Human | Unknown | 1975 | III | 23 | NC_004368.1 | 2127/1968 | GLASER *et al*., 2002 |

[1] Strains refers to the identifier from AQUACEN culture collection.
[2] Non-typeable

Table 2. Main proteins identified in the pan-proteome of fish-adapted GBS strains and their classification into different COG functional categories.

| COG Category | Function | Proteins |
|---|---|---|
| **Cellular processes and signaling** | | |
| Cell cycle control, cell division, chromosome partitioning | Cell division | FtsL, FtsE, FtsZ and DivIVA |
| Cell wall/membrane/envelope biogenesis | Capsular polysaccharide biosynthesis | CpsB, CpsC, CpsD, CpsG, NeuB, NeuC |
| | Peptidoglycan biosynthesis | MurA, MurD, MurE, MurF, MurG |
| | Group B antigen | RmlA, RmlB, RmlC |
| | Multidrug resistance | PbpX, Pbp1A, Pbp2A, DltB |
| | Immunoreactive antigen | PcsB, Sip |
| Cell motility | Transport | Asp1, CglA |
| Post-translational modification, protein turnover, and chaperones | Heat shock | GroL, GroS, GrpE, DnaK, DnaJ, CplX, CplP, HslO |
| | Oxidative stress resistance | Trx, TrxB, Thioredoxin, SufB, SufC |
| Signal transduction mechanisms | Two-component system | CiaR, CovS/CovR, Stp1/Stk1 |
| | Stress response | Universal stress protein |
| Intracellular trafficking, secretion, and vesicular transport | Protein translocation | SecA, SecY |
| Defense mechanisms | Multidrug resistance | EcsA, Beta-lactamase |
| **Information storage and processing** | | |
| Translation, ribosomal structure and biogenesis | 30S ribosomal proteins | RpsJ, RpsS, RpsC, RpsQ, RpsH, RpsE, RpsM, RpsK, RpsO, RpsI, RpsT, RpsA, RpsP, RpsU, RpsR, RpsF, RpsN, RpsG, RpsL, RpsB, RpsD |
| | 50S ribosomal proteins | RplC, RplD, RplW, RplB, RplV, RplP, RplN, RplX, RplE, RplF, RplR, RplO, RplQ, RplM, RplS, RplU, RplT, RplA, RplK, RpmC, RpmD, RpmB, RpmL, RpmG |
| Transcription | RNA polymerase | RpoA, RpoB, RpoC, RpoE, RpoZ |
| | Transcriptional regulators | MutR, GntR, LysR, LacI, MarR, MerR, DeoR, TetR |
| Replication, recombination and repair | DNA replication | DnaA, DnaN, GyrA, GyrB |
| | DNA mismatch repair | MutL, MutS |
| **Metabolism** | | |
| Energy production and conversion | Proton transporting | AtpA, AtpC, AtpD, AtpF, AtpG |
| | Pyruvate metabolism | pdhC, TPP-dependent acetoin dehydrogenase complex, Branched-chain alpha-keto acid dehydrogenase subunit E2, Dihydrolipoyl dehydrogenase |
| Amino acid transport and metabolism | Arginine metabolism | ArcA, ArcB, ArcC, ArcD, ArgF, ArgG, ArgH, ArgR, ArgS |
| | Threonine metabolism | Hom, ThrB, ThrC |
| | Glycine metabolism | GlyA |
| | Glutamic acid metabolism | AlaT |
| | Glutamine metabolism | GlnA |
| | Serine metabolism | SdhA, SdhB, SerB, SerC |
| | Aspartic acid metabolism | AsnA |
| | Cysteine metabolism | Cysteine desulfurase |
| | Methionine metabolism | MetK, MetN |
| Nucleotide transport and metabolism | Purine biosynthesis | GuaC, PurA, PurB, PurC, PurD, PurE, PurF, PurH, PurK, PurM, PurN, PurR |
| | Pyrimidine biosynthesis | PyrC, PyrD, PyrE, PyrG, PyrH, PyrR |
| Carbohydrate transport and metabolism | Glycogen synthesis | PgmA |
| | Glycolytic pathway | Eno, Pga, Pgk, TpiA |
| | Pentose phosphate pathway | AroD |
| | PTS system | 12 proteins involved in transport of ascorbate, glucose, beta-glucoside, lactose, mannose, galactitol and fructose |
| Coenzyme transport and metabolism | Riboflavin biosynthesis | RibBA, RibD, RibE, RibH |
| Lipid transport and metabolism | Fatty acid biosynthesis | AccD, FabD, FabF, FabG, FabH, FabT, FabZ |
| Inorganic ion transport and metabolism | Transporter | Iron, Nickel, Ferrichrome, Manganese, Magnesium, Potassium, Phosphate, Heme |
| | Zinc metabolism | Zinc-binding protein |
| | Copper metabolism | CutC |

| | | |
|---|---|---|
| Secondary metabolites biosynthesis, transport, and catabolism | Multidrug resistance | DltA, Bleomycin resistance protein |
| **Poorly characterized** | | |
| General function prediction only | Phenazine biosynthesis | PhzF |
| Mobilome | Prophages | Phage repressor protein |
| | Transposons | Transposase |
| Function unknown | Stress response | Gls24, General stress protein |

Table 3. Putative vaccine targets for fish-adapted GBS strains identified by reverse vaccinology strategy using the expressed core proteome.

| Accession | Cluster | Protein | Adhesin Probability |
|---|---|---|---|
| SaSA20_0144 | Cluster0008 | ABC transporter substrate-binding protein | 0.552 |
| SaSA20_0016 | Cluster0032 | PcsB protein | 0.746 |
| SaSA20_0031 | Cluster0046 | sip Group B streptococcal surface immunogenic protein | 0.695 |
| SaSA20_0155 | Cluster0160 | Membrane protein | 0.545 |
| SaSA20_0222 | Cluster0210 | Glycine/betaine ABC transporter substrate-binding protein | 0.536 |
| SaSA20_0262 | Cluster0242 | Amino acid ABC transporter substrate-binding protein | 0.521 |
| SaSA20_0270 | Cluster0249 | Penicillin-binding protein 1A | 0.699 |
| SaSA20_0443 | Cluster0363 | Peptidase S16 | 0.548 |
| SaSA20_0646 | Cluster0517 | Cell wall surface anchor protein | 0.548 |
| SaSA20_0682 | Cluster0548 | Foldase protein PrsA | 0.613 |
| SaSA20_0761 | Cluster0612 | Hypothetical protein | 0.586 |
| SaSA20_0810 | Cluster0648 | Hypothetical protein | 0.561 |
| SaSA20_0950 | Cluster0759 | Hypothetical protein | 0.581 |
| SaSA20_0971 | Cluster0779 | Hypothetical protein | 0.541 |
| SaSA20_1003 | Cluster0802 | Zn-dependent protease | 0.666 |
| SaSA20_1090 | Cluster0876 | Choline binding protein D | 0.538 |
| SaSA20_1101 | Cluster0885 | ABC transporter substrate-binding protein | 0.594 |
| SaSA20_1126 | Cluster0908 | Membrane protein | 0.638 |
| SaSA20_1161 | Cluster0932 | Hypothetical protein | 0.576 |
| SaSA20_1201 | Cluster0960 | Glutamine ABC transporter permease | 0.614 |
| SaSA20_1259 | Cluster1012 | Manganese ABC transporter substrate-binding protein | 0.57 |
| SaSA20_1283 | Cluster1027 | Acyltransferase | 0.54 |
| SaSA20_1328 | Cluster1069 | ABC transporter substrate-binding protein | 0.562 |
| SaSA20_1357 | Cluster1094 | Lipoprotein | 0.528 |
| SaSA20_1358 | Cluster1095 | Amino acid ABC transporter substrate-binding protein | 0.575 |
| SaSA20_1625 | Cluster1313 | Phosphate-binding protein PstS 2 | 0.53 |
| SaSA20_1659 | Cluster1339 | cAMP factor | 0.61 |
| SaSA20_1679 | Cluster1357 | Hypothetical protein | 0.553 |
| SaSA20_1731 | Cluster1400 | Membrane protein | 0.669 |
| SaSA20_1745 | Cluster1413 | Peptidoglycan-binding protein LysM | 0.713 |
| GBS_RS02350 | Cluster1590 | Cell wall surface anchor protein | 0.573 |
| SaSA20_0112 | Cluster1592 | rbsB D-ribose-binding protein | 0.568 |
| SaSA16_1063 | Cluster1614 | Hypothetical protein | 0.571 |
| SaSA53_1173 | Cluster1726 | fhuD Ferrichrome ABC transporter substrate-binding protein | 0.527 |
| GBS_RS11195 | Cluster1752 | Cell surface protein | 0.527 |
| GBS_RS03585 | Cluster1754 | Cell wall surface anchor protein | 0.604 |
| GBS_RS03520 | Cluster1888 | Acid phosphatase/phosphotransferase | 0.596 |
| GBS_RS05110 | Cluster1920 | BMP family ABC transporter substrate-binding protein | 0.597 |

Table 4. Proteins potentially involved in virulence identified in the pan-proteome of fish-adapted GBS strains.

| Accession | Description | H[1] | F[2] | Function | Reference[3] |
|---|---|---|---|---|---|
| **Adhesion** | | | | | |
| SaSA20_0637 | Elongation factor Tu | X | X | Mediate bacterium binding to fibronectin, fibrinogen and mucin | GRANATO *et al.*, 2004; SCHAUMBURG *et al.*, 2004; BALASUBRAMANIAN *et al*., 2008 |
| SaSA20_0646 | Cell wall surface anchor protein | X | X | Cell adhesion | LI *et al*., 2016 |
| SaSA20_0697 | gapN Glyceraldehyde-3-phosphate dehydrogenase | X | | GBS adhesion to extracellular matrix and cytoskeletal proteins of host cells | SEIFERT *et al*., 2003 |
| SaSA20_1009 | Dihydroorotate dehydrogenase (PavA) | X | | Interacts with cell surface fibronectin | HOLMES *et al.*, 2001 |
| SaSA20_1475 | gap Glyceraldehyde-3-phosphate dehydrogenase | X | X | GBS adhesion to extracellular matrix and cytoskeletal proteins of host cells | SEIFERT *et al*., 2003 |
| SaSA20_1675 | Hypothetical protein (BibA) | X | X | GBS adhesion to human epithelial cells and binding to complement regulatory protein C4bp, acting as anti-phagocytic factor | SANTI *et al.*, 2007 |
| **Evasion** | | | | | |
| GBS_RS07805 | Nucleotide sugar dehydratase | X | X | Prevents deposition of complement factor C3b and inhibits the opsonophagocytosis | GLASER *et al.*, 2002 |
| SaSA16_1205 | Family 2 glycosyltransferase | | | Acts in immune recognition, bacterial evasion, intercellular signaling and biofilm formation | KEENLEYSIDE *et al.*, 2001 |
| SaSA20_0016 | PcsB protein | X | | Peptidoglycan hydrolase | LI *et al*., 2016 |
| SaSA20_0031 | sip Group B streptococcal surface immunogenic protein | X | X | Protective antigen and vaccine target | MA *et al*., 2017 |
| SaSA20_0382 | Reticulocyte binding protein | X | | Carbohydrate binding | LI *et al*., 2016 |
| SaSA20_0980 | neuA N-acylneuraminate cytidylyltransferase | X | | Prevents deposition of complement factor C3b and inhibits the opsonophagocytosis | GLASER *et al*., 2002 |
| SaSA20_0981 | NeuD protein | X | | Prevents deposition of complement factor C3b and inhibits the opsonophagocytosis | GLASER *et al.*, 2002 |
| SaSA20_0982 | UDP-N-acetylglucosamine-2-epimerase NeuC | X | | Prevents deposition of complement factor C3b and inhibits the opsonophagocytosis | GLASER *et al*., 2002 |
| SaSA20_0983 | N-acetyl neuramic acid synthetase NeuB | X | | Prevents deposition of complement factor C3b and inhibits the opsonophagocytosis | GLASER *et al*., 2002 |
| SaSA20_0987 | Capsular polysaccharide biosynthesis protein | X | | Prevents deposition of complement factor C3b and inhibits the opsonophagocytosis | GLASER *et al*., 2002 |
| SaSA20_0990 | UDP-N-acetylglucosamine:LPS N-acetylglucosamine transferase | X | X | Prevents deposition of complement factor C3b and inhibits the opsonophagocytosis | GLASER *et al*., 2002 |
| SaSA20_0992 | Tyrosine-protein kinase CpsD | X | X | Prevents deposition of complement factor C3b and inhibits the opsonophagocytosis | GLASER *et al*., 2002 |
| SaSA20_0993 | Capsular polysaccharide biosynthesis protein CpsC | X | | Prevents deposition of complement factor C3b and inhibits the opsonophagocytosis | GLASER *et al*., 2002 |
| SaSA20_0994 | Tyrosine-protein phosphatase CpsB | X | | Prevents deposition of complement factor C3b and inhibits the opsonophagocytosis | GLASER *et al*., 2002 |
| SaSA20_1017 | rmlB dTDP-glucose 4,6-dehydratase | X | | Biosynthesis of group B antigen | GLASER *et al*., 2002 |
| SaSA20_1018 | rmlC dTDP-4-dehydrorhamnose 3,5-epimerase | X | | Biosynthesis of group B antigen | GLASER *et al*., 2002 |
| SaSA20_1019 | rmlA Glucose-1-phosphate thymidylyltransferase | X | | Biosynthesis of group B antigen | GLASER *et al*., 2002 |
| SaSA20_1438 | Peptidase | X | X | Prevention of complement factor C5a deposition and phagocytosis from host cells | RAJAGOPAL, 2009 |
| **Invasin** | | | | | |
| GBS_RS03175 | TlyA family rRNA (cytidine-2'-O)-methyltransferase | X | | Hemolytic activity in different pathogenic bacteria | LATA *et al*., 2014 |

| | | | | | |
|---|---|---|---|---|---|
| SaSA20_0534 | eno Enolase | X | X | Binding or activation of plasminogen by GBS during incubation in human blood | MEREGHETTI *et al.*, 2008a |
| SaSA20_0586 | Glycosyl transferase family 1 (IagA) | X | X | GBS blood-brain barrier penetration in neonates | DORAN *et al.*, 2005 |
| SaSA20_1659 | cAMP fator | X | X | Forms pore on membrane of the host cells | RAJAGOPAL, 2009 |
| SaSA53_0799 | Internalin | | | Required by *Listeria monocytogenes* for invasion of different nonphagocytic mammalian cell lines | BRAUM e COSSART, 2000 |
| **Multidrug Resistance** | | | | | |
| SaSA20_0259 | pbpX Penicillin-binding protein 2x | X | | Mediate GBS resistance to AMPs | RAJAGOPAL, 2009 |
| SaSA20_0270 | Penicillin-binding protein 1A | X | | Mediate GBS resistance to AMPs | RAJAGOPAL, 2009 |
| SaSA20_1495 | D-alanyl-lipoteichoic acid biosynthesis protein DltD | X | X | Resist AMPs is to decrease the charge on their cell surface | RAJAGOPAL, 2009 |
| SaSA20_1499 | dltA D-alanine--poly(phosphoribitol) ligase subunit 1 | X | X | Resist AMPs is to decrease the charge on their cell surface | RAJAGOPAL, 2009 |
| SaSA20_1677 | pbp2A Penicillin-binding protein 2A | X | | Mediate GBS resistance to AMPs | RAJAGOPAL, 2009 |

[1]The letter X in this column represents that the virulence protein was previously identified in human GBS strains.
[2]The letter X in this column represents that the virulence protein was previously identified in fish GBS strains.
[3]Reference based on function of each virulence protein.

Table 5. List of differentially regulated proteins identified in all fish-adapted GBS strains compared to the NEM316 strain.

| Up/Down | Accession | Product | SA16 ratio | SA20 ratio | SA53 ratio | SA81 ratio | SA95 ratio |
|---|---|---|---|---|---|---|---|
| Down | GBS_RS00535 | 50S ribosomal protein L6 | -4.57 | -3.45 | -4.69 | -4.37 | -1.78 |
| Down | SaSA20_0140 | Fe-S assembly protein NifU | -4.23 | -2.33 | -3.59 | -1.40 | -1.12 |
| Down | GBS_RS01110 | Acetate kinase | -2.95 | -1.73 | -2.03 | -1.42 | -1.15 |
| Down | SaSA20_0551 | Beta-lactamase | -5.34 | -5.25 | -4.93 | -5.62 | -1.32 |
| Down | SaSA20_0944 | thrB Homoserine kinase | -4.67 | -3.52 | -2.85 | -3.46 | -4.69 |
| Down | SaSA20_1545 | Peptidase S66 | -1.84 | -1.06 | -1.83 | -1.02 | -1.16 |
| Down | GBS_RS09900 | Proline--tRNA ligase | -3.45 | -3.01 | -3.36 | -2.68 | -2.93 |
| Down | SaSA20_1738 | DHH family phosphoesterase | -3.99 | -2.65 | -2.69 | -1.84 | -2.15 |
| Down | SaSA20_1009 | Dihydroorotate dehydrogenase | -2.62 | -2.13 | -3.66 | -1.22 | -1.58 |
| Down | SaSA53_1431 | 3-hydroxybutyryl-CoA dehydrogenase | -1.30 | -2.33 | -1.71 | -1.64 | -1.21 |
| Down | GBS_RS01730 | Hypothetical protein | -4.30 | -4.42 | -3.84 | -4.19 | -1.16 |
| Up | GBS_RS07220 | DNA (cytosine-5-)-methyltransferase | 6.76 | 2.53 | 7.20 | 5.40 | 1.05 |
| Up | GBS_RS07415 | ABC transporter ATP-binding protein | 2.06 | 3.34 | 1.40 | 3.30 | 2.38 |
| Up | SaSA20_0474 | mlcE | 1.55 | 2.47 | 2.62 | 2.06 | 1.37 |
| Up | SaSA20_0529 | gyrB DNA gyrase subunit B | 5.10 | 6.07 | 5.44 | 5.55 | 2.71 |
| Up | SaSA20_1579 | galE_2 UDP-glucose 4-epimerase | 1.07 | 1.52 | 1.45 | 1.69 | 1.02 |
| Up | SaSA20_0382 | Reticulocyte binding protein | 4.98 | 4.52 | 5.45 | 4.21 | 1.51 |
| Up | SaSA20_0123 | argH Argininosuccinate lyase | 1.74 | 2.78 | 2.58 | 2.05 | 1.97 |
| Up | SaSA95_0249 | cglA Competence protein | 6.16 | 7.67 | 6.80 | 6.31 | 5.25 |
| Up | SaSA20_0173 | Thioredoxin | 3.14 | 3.43 | 3.52 | 3.71 | 1.03 |
| Up | SaSA53_0400 | Hypothetical protein | 3.86 | 3.72 | 7.25 | 4.21 | 1.78 |
| Up | SaSA20_0423 | GNAT family acetyltransferase | 3.36 | 5.04 | 5.44 | 5.31 | 1.94 |
| Up | SaSA20_0437 | asnA Aspartate--ammonia ligase | 4.12 | 3.94 | 3.28 | 3.90 | 1.46 |
| Up | SaSA20_0674 | nagB Glucosamine-6-phosphate deaminase | 4.61 | 5.11 | 6.03 | 3.37 | 2.54 |
| Up | SaSA20_0995 | LytR family transcriptional regulator | 2.58 | 2.06 | 1.94 | 2.97 | 1.15 |
| Up | SaSA20_1068 | fni Isopentenyl-diphosphate delta-isomerase | 4.28 | 4.82 | 4.21 | 5.04 | 1.77 |
| Up | SaSA20_1080 | Antibiotic ABC transporter ATP-binding protein | 2.07 | 2.42 | 1.73 | 2.42 | 1.02 |
| Up | SaSA20_1112 | thiI tRNA sulfurtransferase | 3.29 | 2.89 | 2.73 | 3.08 | 1.29 |
| Up | SaSA20_1168 | sigA RNA polymerase sigma factor | 3.52 | 5.44 | 4.82 | 4.98 | 1.77 |
| Up | SaSA20_1173 | Amino acid ABC transporter substrate-binding protein | 1.43 | 2.94 | 1.35 | 1.86 | 1.41 |
| Up | SaSA20_1287 | 3-phosphoglycerate dehydrogenase | 1.03 | 2.35 | 2.37 | 1.31 | 1.27 |
| Up | SaSA20_1317 | Non-canonical purine NTP pyrophosphatase | 2.09 | 2.20 | 3.83 | 2.47 | 1.38 |
| Up | SaSA53_1361 | Non-canonical purine NTP pyrophosphatase | 2.09 | 2.20 | 3.83 | 2.47 | 1.38 |
| Up | SaSA20_1402 | scrK Fructokinase | 2.78 | 1.32 | 2.16 | 3.81 | 2.31 |
| Up | SaSA20_1492 | hypothetical protein | 3.04 | 2.26 | 3.15 | 3.02 | 1.29 |
| Up | SaSA20_1499 | dltA | 2.74 | 3.09 | 2.94 | 3.38 | 1.09 |
| Up | SaSA20_1688 | ABC transporter ATP-binding protein | 8.13 | 7.87 | 7.46 | 7.26 | 3.66 |
| Up | SaSA20_1692 | Glyoxalase | 2.32 | 3.16 | 2.77 | 3.06 | 1.43 |
| Up | SaSA20_1762 | arcB Ornithine carbamoyltransferase | 2.85 | 2.82 | 2.71 | 3.01 | 1.09 |
| Up | SaSA16_1675 | Hypothetical protein | 1.91 | 2.40 | 2.35 | 2.81 | 3.39 |

Supplementary Table 1. Complete list of proteins identified by LC-UDMS$^{E}$.

(digital content only)

Supplementary Table 2. Enrichment analysis using KEGG pathways in the STRING web tool.

| **Pathway description** | **Nº of proteins** | **FDR** |
|---|---|---|
| Metabolic pathways | 232 | 6.88e-36 |
| Biosynthesis of secondary metabolites | 104 | 2.37e-23 |
| Ribosome | 51 | 3.82e-16 |
| Purine metabolism | 48 | 1.03e-10 |
| Carbon metabolism | 43 | 8.92e-10 |
| Biosynthesis of amino acids | 48 | 8.92e-10 |
| Aminoacyl-tRNA biosynthesis | 25 | 1.2e-09 |
| Microbial metabolism in diverse environments | 61 | 1.47e-09 |
| Amino sugar and nucleotide sugar metabolism | 27 | 2.7e-06 |
| Glycolysis / Gluconeogenesis | 23 | 3.4e-06 |
| Pyrimidine metabolism | 36 | 5.81e-06 |
| Glycine, serine and threonine metabolism | 16 | 3.2e-05 |
| Methane metabolism | 13 | 0.000392 |
| Peptidoglycan biosynthesis | 18 | 0.000423 |
| Pyruvate metabolism | 18 | 0.000423 |
| Arginine and proline metabolism | 12 | 0.000748 |
| RNA degradation | 8 | 0.00341 |
| Starch and sucrose metabolism | 14 | 0.00585 |
| Mismatch repair | 14 | 0.00585 |
| Streptomycin biosynthesis | 7 | 0.00727 |
| Pentose phosphate pathway | 16 | 0.00792 |
| One carbon pool by folate | 8 | 0.0153 |
| Riboflavin metabolism | 6 | 0.0158 |
| Homologous recombination | 14 | 0.0236 |
| Nicotinate and nicotinamide metabolism | 7 | 0.0303 |
| Cysteine and methionine metabolism | 12 | 0.0346 |
| RNA polymerase | 5 | 0.0346 |
| Bacterial secretion system | 9 | 0.0484 |

Supplementary Table 3. Exclusive proteins identified in human and fish-adapted GBS strains.

| Accession | Cluster | Description | Host Exclusive in |
|---|---|---|---|
| SaSA20_0369 | Cluster0329 | 5-formyltetrahydrofolate cyclo-ligase | Fish |
| SaSA53_0506 | Cluster0420 | Hypothetical protein | Fish |
| GBS_RS05625 | Cluster0685 | Ribonuclease HII | Fish |
| SaSA20_0983 | Cluster0788 | N-acetyl neuramic acid synthetase NeuB | Fish |
| SaSA20_0989 | Cluster0793 | Polysaccharide biosynthesis protein CpsG | Fish |
| SaSA20_1480 | Cluster1201 | 3'-5' exoribonuclease | Fish |
| SaSA20_1574 | Cluster1277 | Bacteriocin transport accessory protein | Fish |
| GBS_RS09930 | Cluster1278 | Malate dehydrogenase | Fish |
| SaSA20_1598 | Cluster1294 | Flavoprotein | Fish |
| SaSA20_1606 | Cluster1302 | PTS mannose transporter subunit IIB | Fish |
| SaSA20_1629 | Cluster1316 | Hypothetical protein | Fish |
| SaSA16_0843 | Cluster1475 | PhoB family transcriptional regulator | Fish |
| SaSA20_0863 | Cluster1562 | Hypothetical protein | Fish |
| SaSA20_1453 | Cluster1580 | Phenazine biosynthesis protein PhzF | Fish |
| SaSA53_0595 | Cluster1713 | Gluconate 5-dehydrogenase | Fish |
| SaSA16_1239 | Cluster1728 | Accessory Sec system protein Asp1 | Fish |
| GBS_RS07210 | Cluster1749 | Hypothetical protein | Fish |
| GBS_RS06055 | Cluster1759 | ATPase AAA | Fish |
| SaSA20_1265 | Cluster1780 | Bleomycin resistance protein | Fish |
| GBS_RS01380 | Cluster1823 | Hypothetical protein | Fish |
| GBS_RS03805 | Cluster1906 | Beta-hexosamidase | Fish |
| SaSA20_0640 | Cluster0512 | Penicillin-binding protein 2B | Fish |
| SaSA53_1568 | Cluster1236 | Membrane protein | Fish |
| GBS_RS05750 | Cluster1939 | Type VII secretion protein EsaA | Fish |
| SaSA20_0091 | Cluster0101 | D-alanyl-D-alanine carboxypeptidase | Fish |
| SaSA16_1707 | Cluster1339 | cAMP factor | Fish |
| GBS_RS00775 | Cluster0115 | CTP synthetase | Human |
| SaSA20_0108 | Cluster0118 | DNA repair protein radA | Human |
| SaSA20_0127 | Cluster0135 | Hypothetical protein | Human |
| GBS_RS07805 | Cluster0929 | Nucleotide sugar dehydratase | Human |
| GBS_RS10725 | Cluster2091 | Transcriptional regulator | Human |

Supplementary Table 4. Number of proteins differentially expressed in fish-adapted GBS strains and the NEM316 strain.

| Strain | Up-regulated | Down-regulated | Total |
|---|---|---|---|
| SA16 | 127 | 203 | 330 |
| SA20 | 134 | 186 | 320 |
| SA53 | 159 | 199 | 358 |
| SA81 | 119 | 153 | 272 |
| SA95 | 58 | 35 | 90 |

Supplementary Table 5. Complete list of proteins that were up-regulated and down-regulated in fish- and human-adapted GBS strains.

(digital content only)

Figure 1. Dynamic range of the protein abundances found in fish-adapted GBS strains. The data are binned according to the log10 of their normalized abundance. SA16 (blue), SA20 (green), SA53 (red), SA81 (yellow), and SA95 (purple).

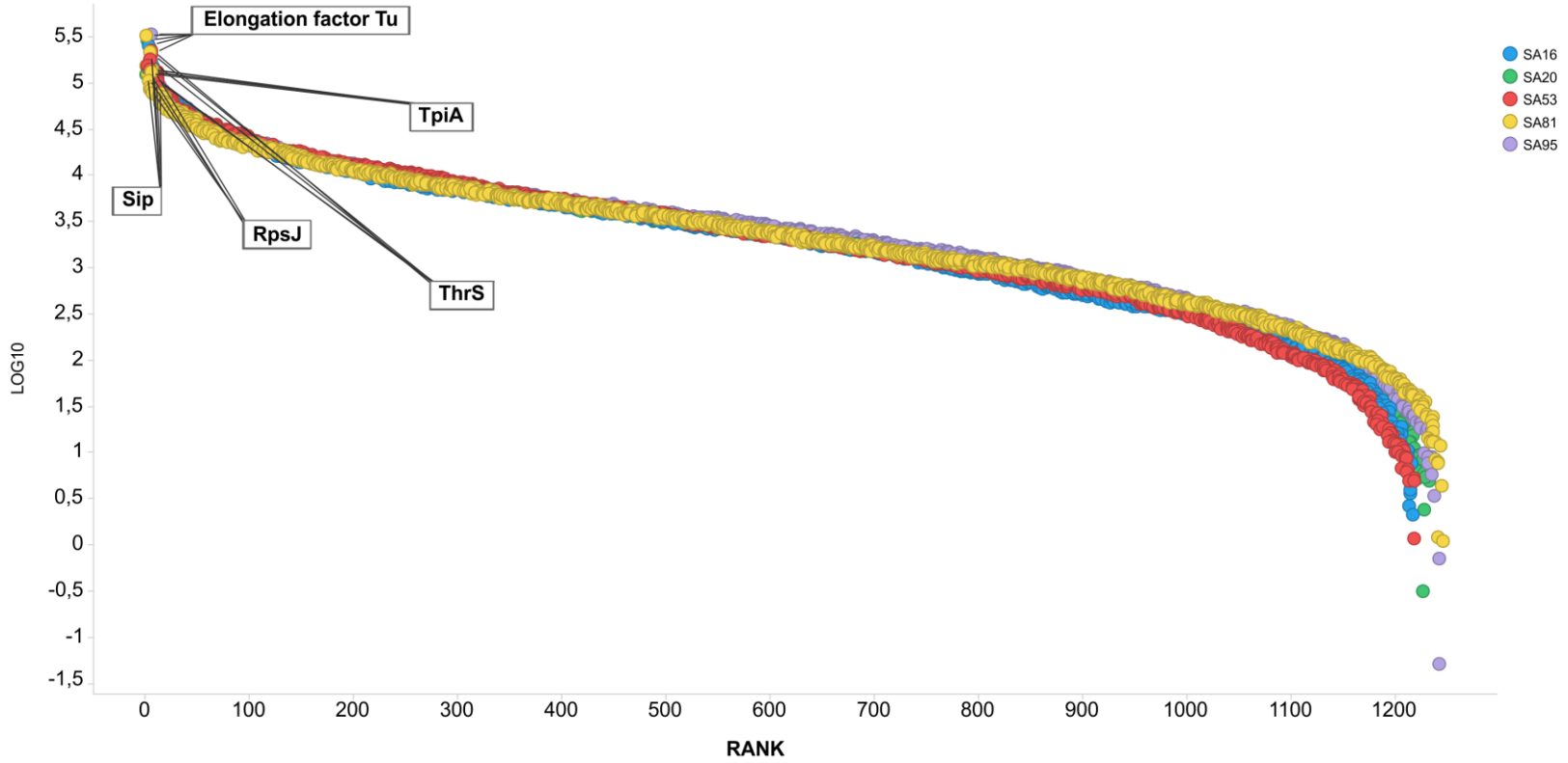


Figure 2. Venn diagram showing the number of unique and shared proteins in fish-adapted GBS strains.

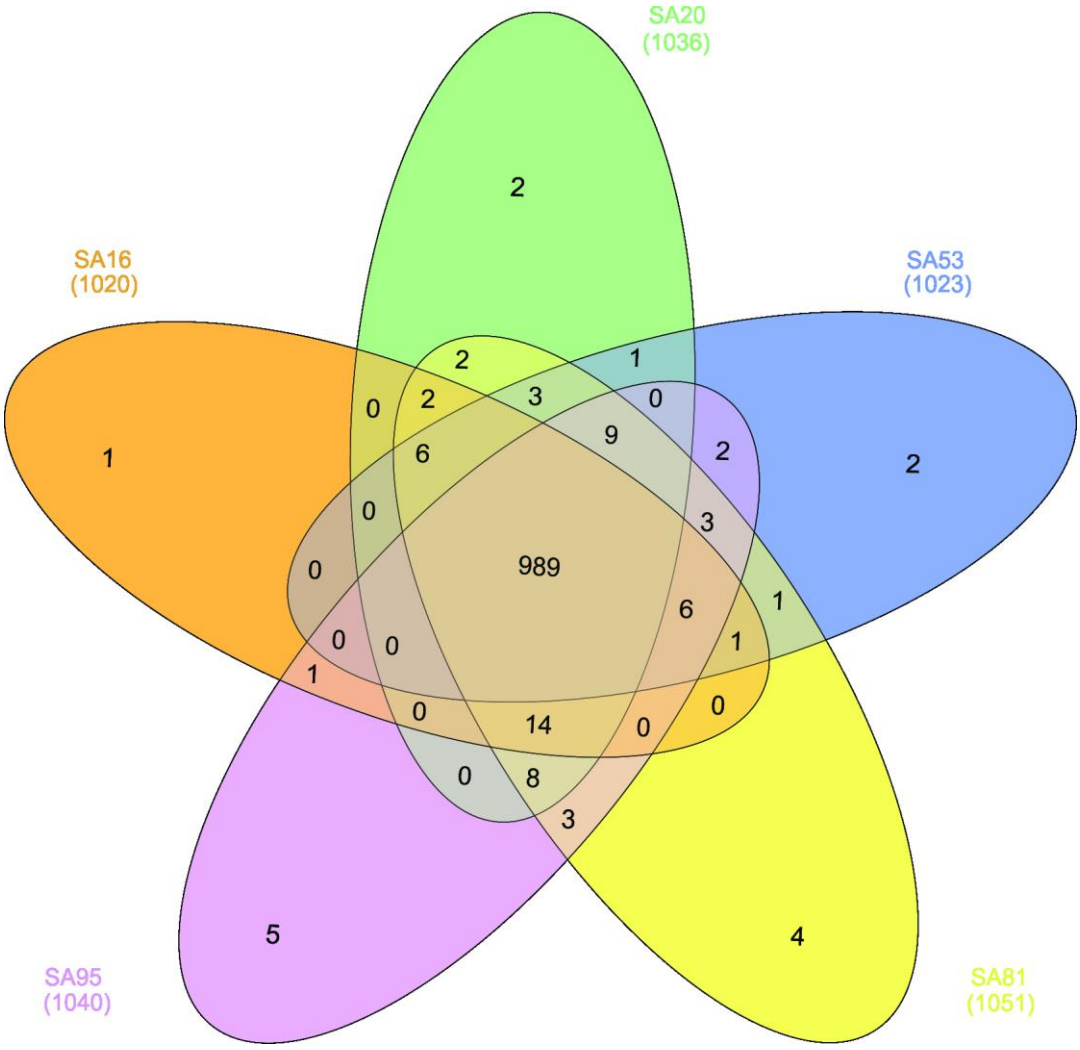


Figure 3. Proteome similarity matrix of fish-adapted GBS strains. The numbers inside the frames indicate the percentage of similarities among strains.

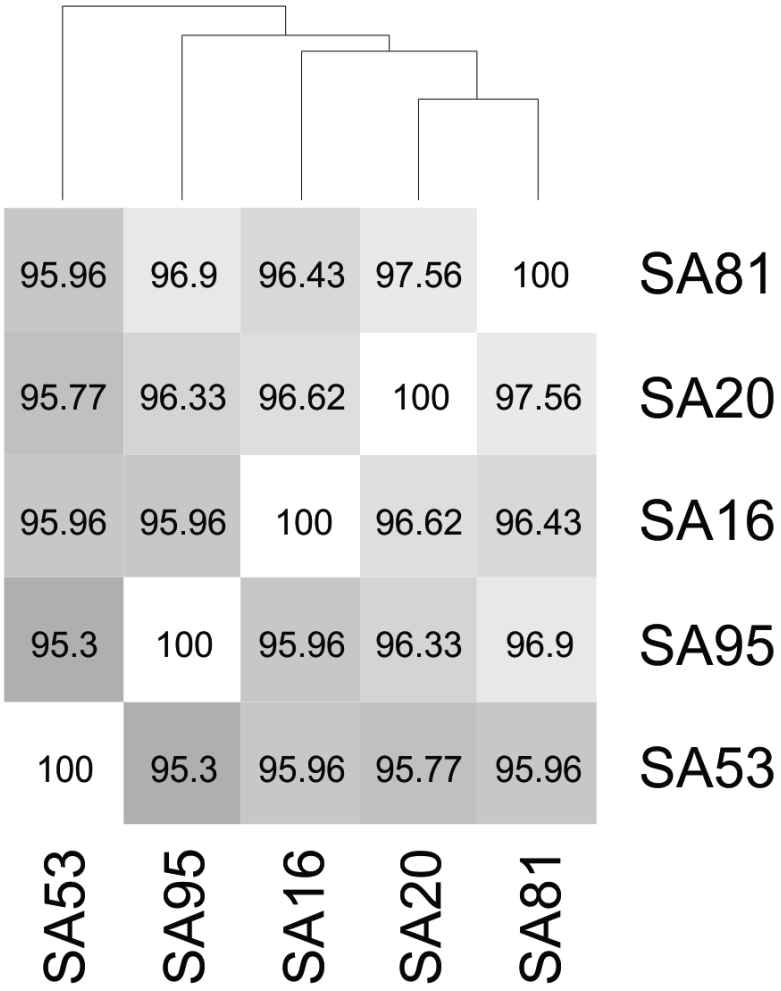


Figure 4. Prediction of COG functional categories of the proteins identified in the pan-proteome of fish-adapted GBS strains. The total number of proteins in the functional categories does not correspond to the final number of proteins in the pan-proteome because some proteins are associated with more than one COG category.

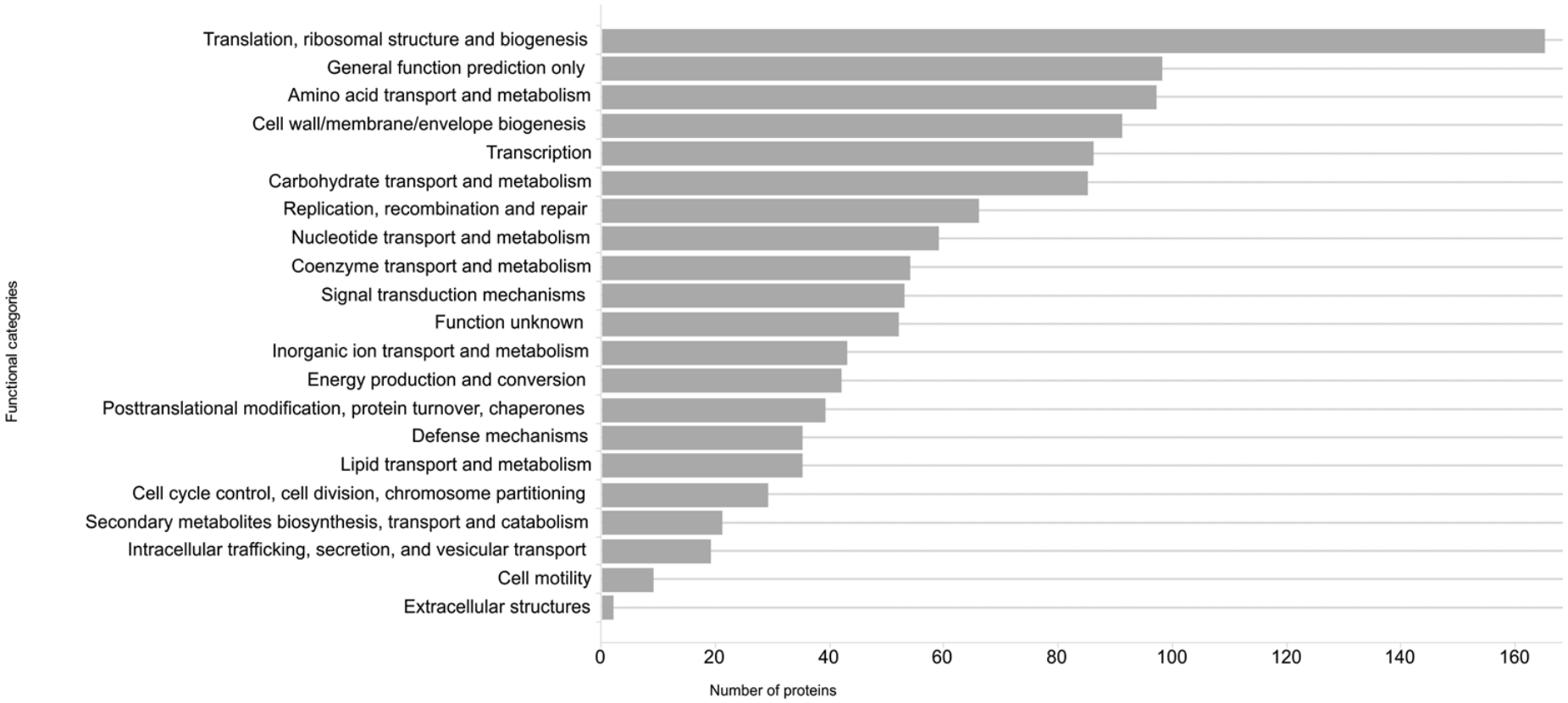


Figure 5. Interaction networks of identified core proteins of fish-adapted GBS strains. Thicker lines denote interactions with score ≥ 0.980. (1) Ribosomal proteins; (2) ATP synthase; (3) Pyruvate metabolism; (4) Carbohydrate metabolism; (5) Heat shock proteins; (6) Nucleotide metabolism; (7) Aminoacyl-tRNA synthetase; (8) DNA replication and repair; (9) Peptidoglycan biosynthesis.

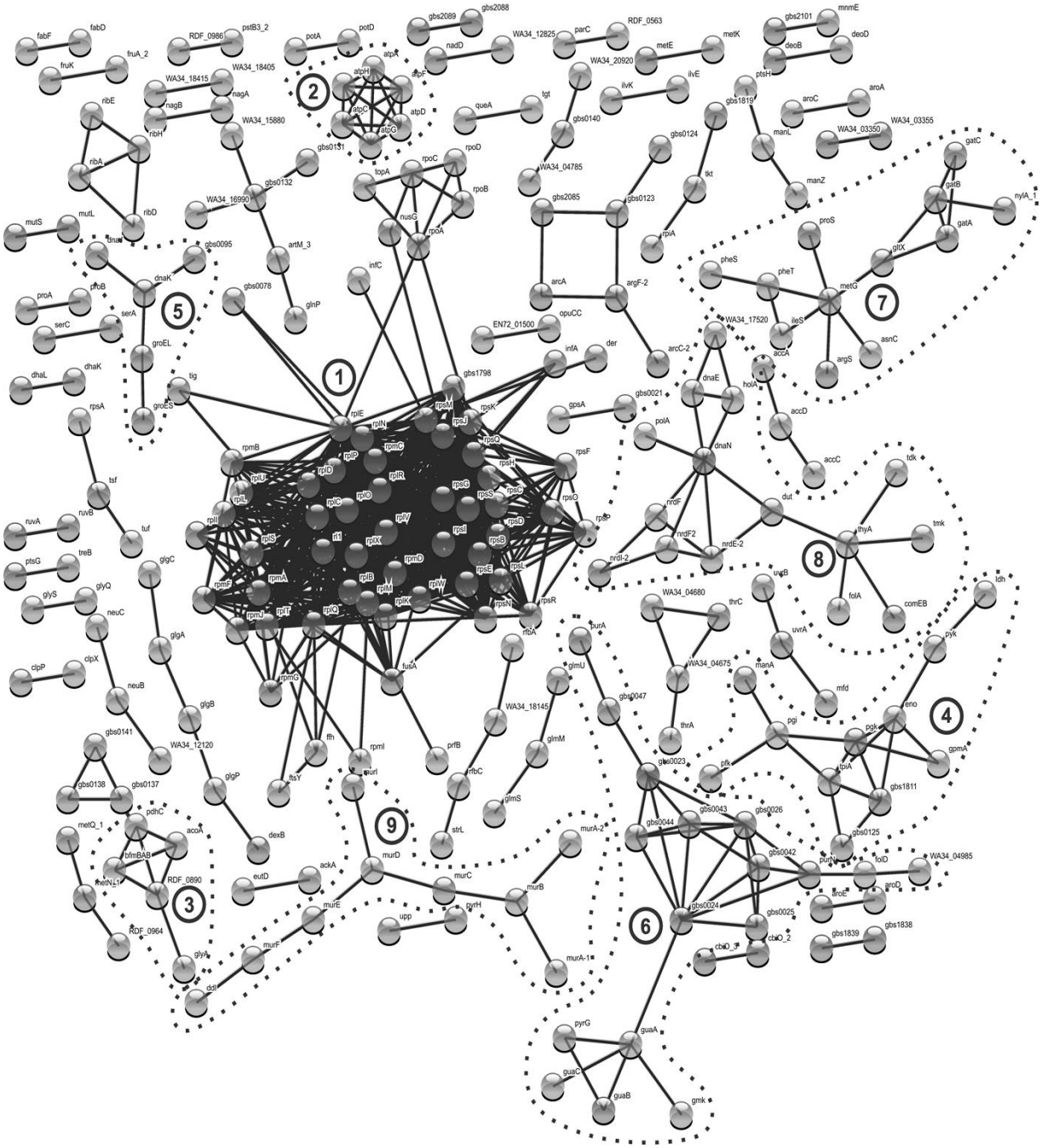


Figure 6. Heat map analysis of proteins that were significantly up- and down-regulated in fish-adapted GBS strains in comparison to the NEM316 strain.

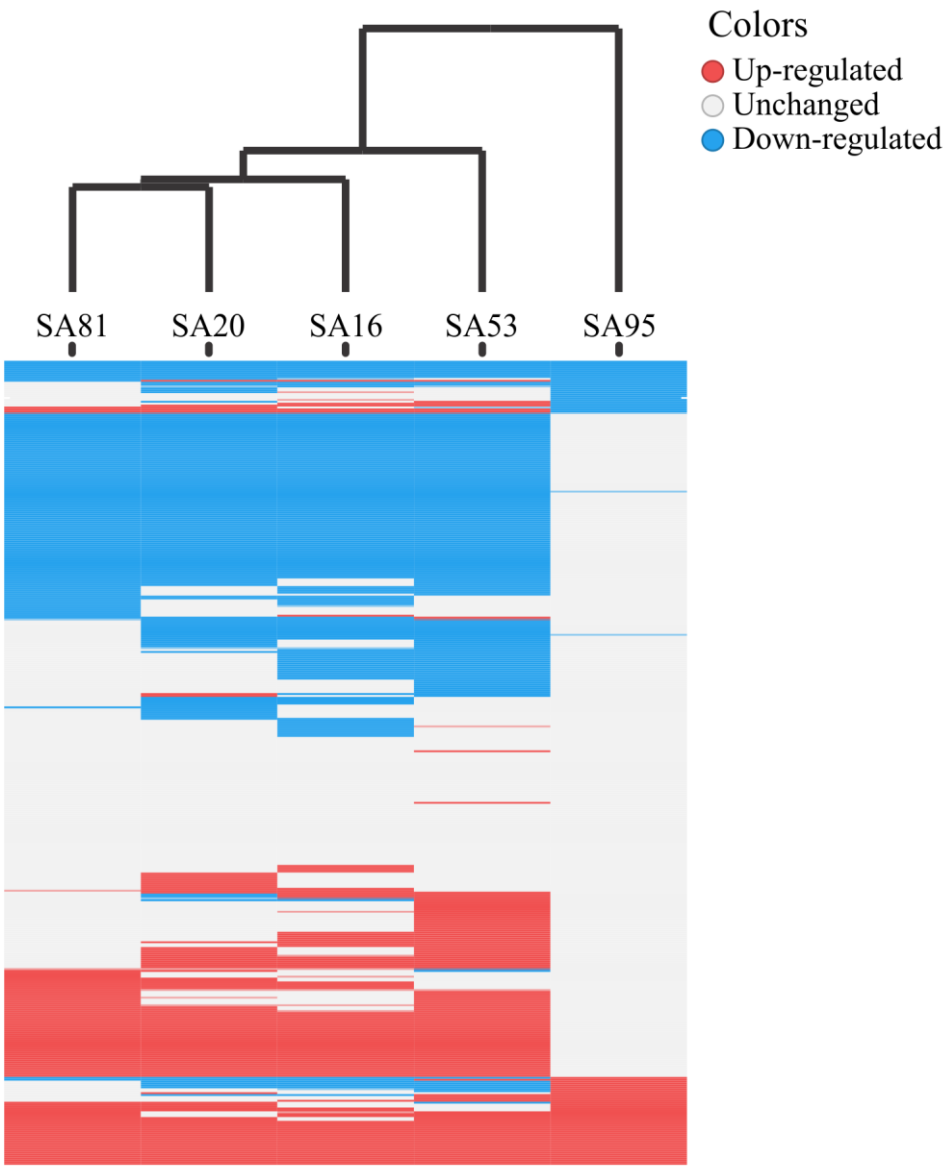
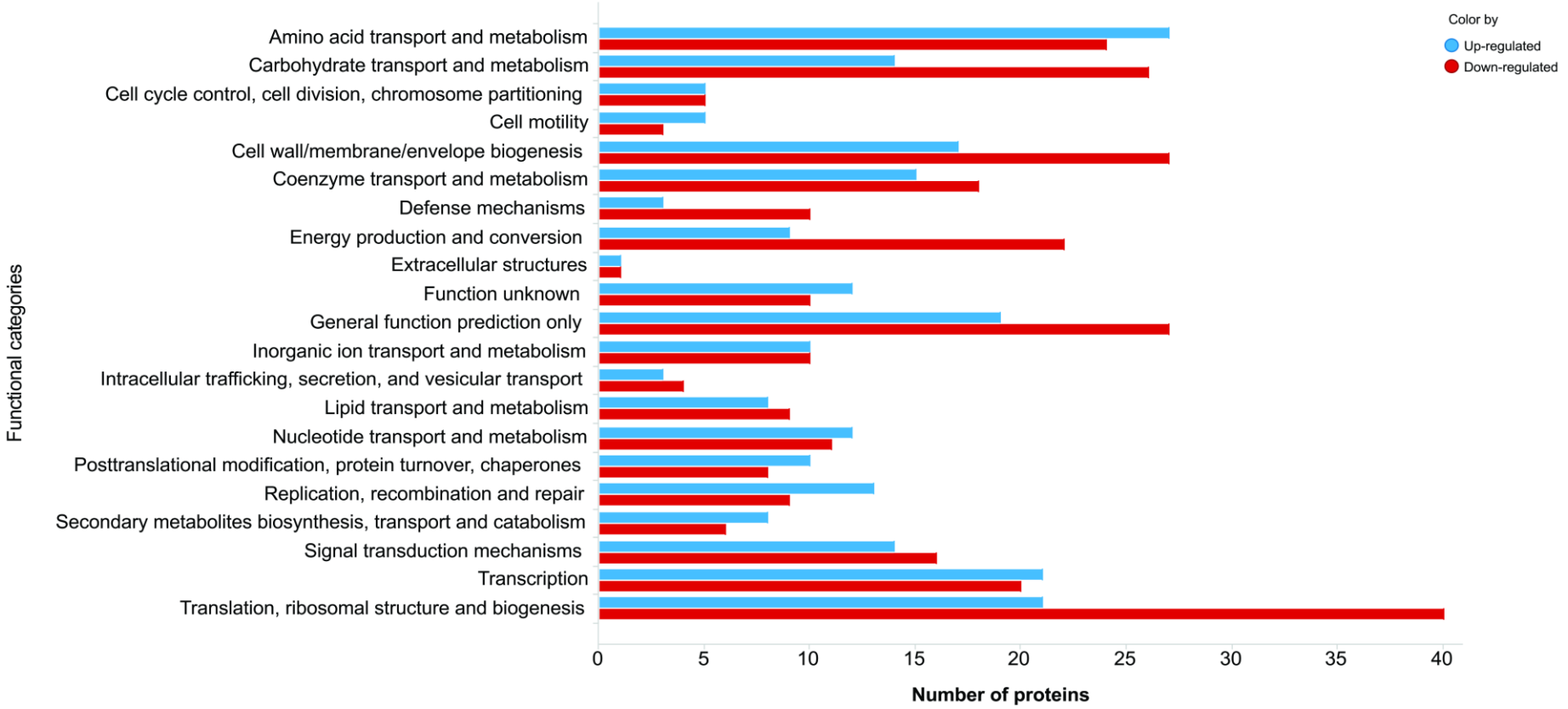
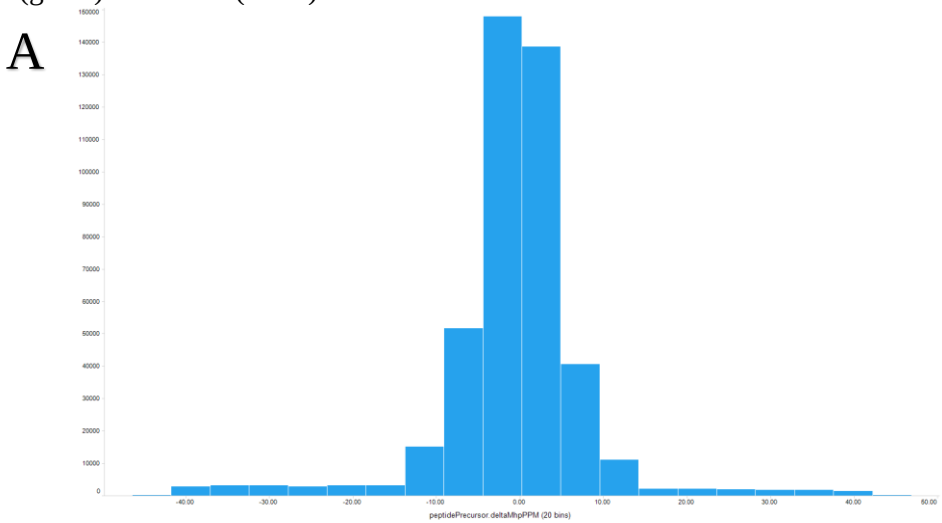
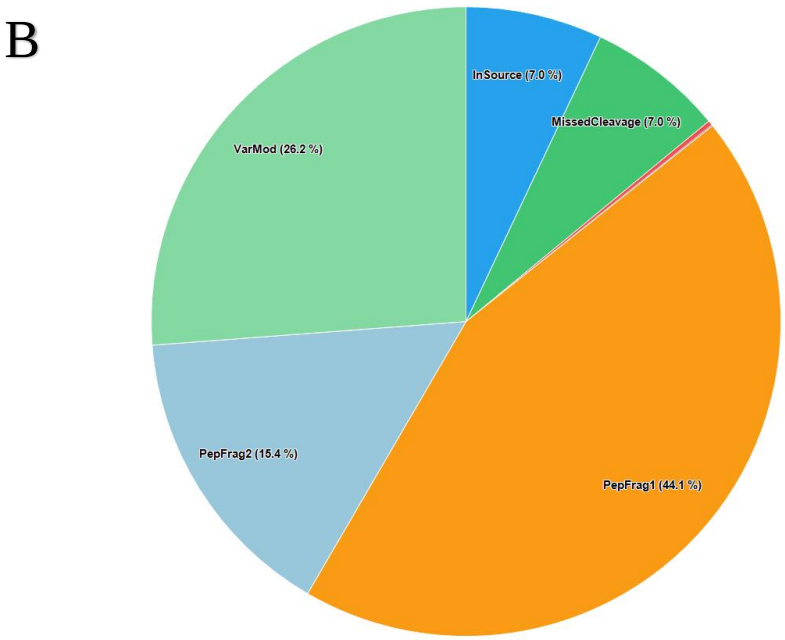


Figure 7. COG functional categories of the proteins that were differentially expressed in human- and fish-adapted GBS strains. The blue bars represent up-regulated proteins in fish-adapted GBS strains with respect to the NEM316 strain; the red bars represent down-regulated proteins

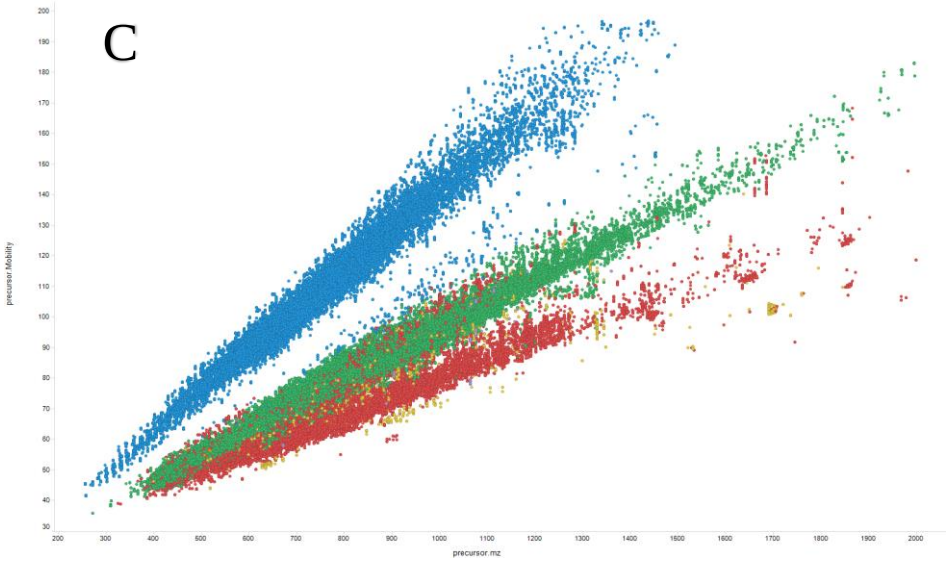


Supplementary Figure 1. Quality control of the proteins identified by LC-MS. A: normal distribution of 10 ppm error of the total identified peptides. B: peptide detection type. PepFrag1 and PepFrag2 correspond to the peptide matches obtained by comparison to the database by Progenesis QIP, VarMod corresponds to variable modifications, InSource corresponds to fragmentation that occurred at the ionization source, and MissedCleavage corresponds to missed trypsin cleavage. C: drift time for ions of 1+ (blue), 2+ (green), 3+ (red), 4+ (yellow) and 5+ (purple) charge states. D: repeat rate indicating the number of times that an identified protein appears in the replicates: 3 of 3 (blue) and 2 of 3 (red). E: principal component analysis, biological replicates (n = 3) of GBS strains; NEM316 (red), SA16 (blue), SA20 (yellow), SA53 (gray), SA81 (green) and SA95 (black).

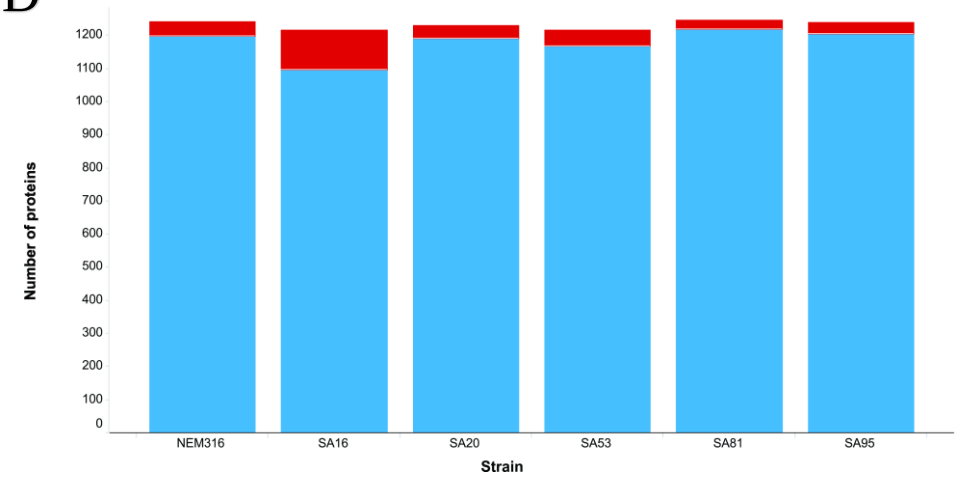


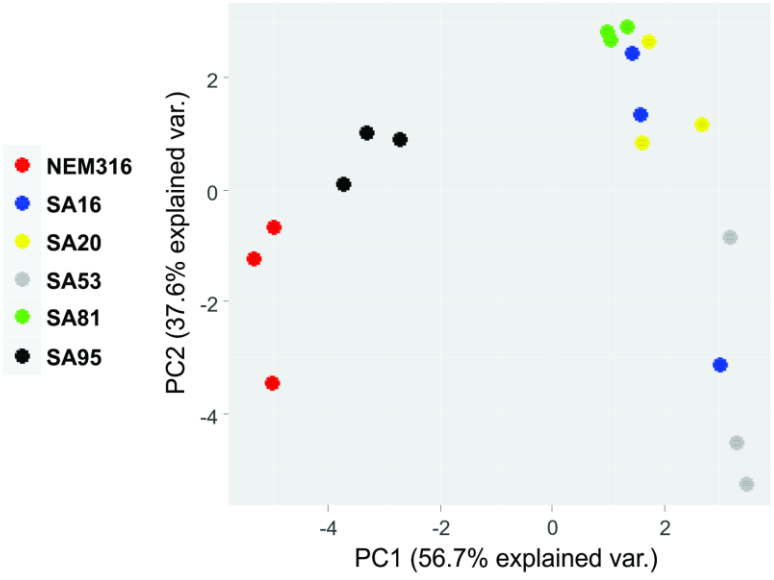
C


precursor Mobility
precursor.mz

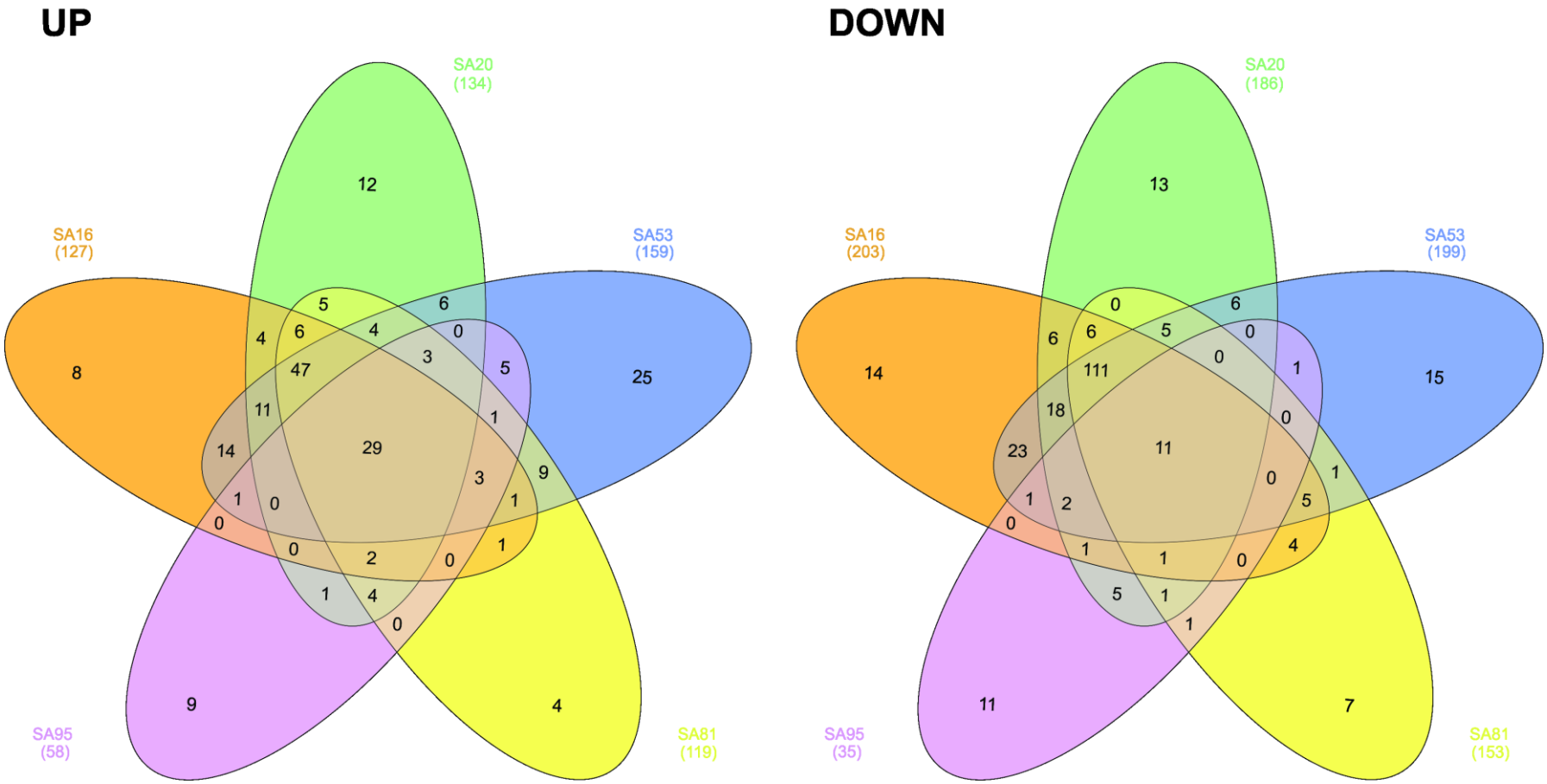
D


Number of proteins
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
NEM316
SA16
SA20
SA53
SA81
SA95
Strain

E


NEM316
SA16
SA20
SA53
SA81
SA95
PC2 (37.6% explained var.)
PC1 (56.7% explained var.)
-4
-2
0
2

Supplementary Figure 2. Venn diagram showing the number of proteins up- and down-regulated in fish-adapted GBS strains in comparison to the NEM316 strain.



# 5. CHAPTER 3. TRANSCRIPTOME AND PROTEOME OF FISH-PATHOGENIC *Streptococcus agalactiae* ARE MODULATED BY TEMPERATURE CHANGE

Original manuscript submitted to the Journal "Frontiers in Microbiology".

## 5.1. ABSTRACT

*Streptococcus agalactiae* is one of the most important pathogens associated with outbreaks of streptococcis in Nile tilapia farms around the world. High water temperature (above 27°C) have been described as factor predisposing for disease in fish. On the other hand, at low temperature (below 25°C) fish mortalities are no usually observed in farms. The temperature variation can modulate the expression of genes and proteins involved with metabolism, adaptation and bacterial pathogenicity, increasing or decreasing the host susceptibility to infection. The aim of this study was to evaluate the transcriptome and proteome of fish-pathogenic *S. agalactiae* strain (SA53) submitted to *in vitro* growth under different temperatures using microarray and label-free shotgun LC-HDMS$^{E}$ approach, and to compare the expression trends of proteins shared among GBS strains from different hosts (SA53 and NEM316). Biological triplicates of isolates were cultured in BHIT broth at 22°C or 32°C for RNA and protein isolation and submitted to transcriptomic and proteomic analysis. Total of 1730 transcripts were identified in SA53, being 107 genes differentially expressed among the temperature evaluated. A higher number of genes related with metabolism were detected as up-regulated proteins at 32°C, mainly PTS system and ABC transport system. In proteome analysis, 1046 proteins were identified in SA53 strain, being 81 proteins differentially regulated at 22 and 32ºC. Proteins involved in Defense mechanisms (V), Lipid transport and metabolism (I), and Nucleotide transport and metabolism (F) were up-regulated at 32ºC. A higher number of interactions was observed in the category F. The induction of genes/proteins involved in virulence were detected in both temperatures evaluated. A low correlation between transcriptome and proteome datasets was observed. And there is a distinct adaptation between fish and human GBS strains at the proteome level. Our study showed that the transcriptome and proteome of fish-adapted GBS strain are modulated by temperature, especially regulating the differential expression of genes/proteins involved with metabolism, adaptation and virulence, and revealing a host specificity at proteome regulation for human and fish hosts.

***Keywords***: GBS, temperature, fish, microarray, label-free shotgun proteome

## 5.2. INTRODUCTION

*Streptococcus agalactiae* (Lancefield's Group B *Streptococcus*, GBS) is one of the most important pathogens associated with outbreaks in farm-raised Nile tilapia in Brazil (SALVADOR *et al.*, 2005; MIAN *et al.*, 2009; CHIDEROLI *et al.*, 2017), being responsible for significant economic losses annually (MIAN *et al.*, 2009). This pathogen causes septicemia and meningoencephalitis in different fish species from freshwater, estuarine and marine environments in the world (EVANS *et al.*, 2002), and commonly affects adult fish (MIAN *et al.*, 2009).

High water temperature, intensive husbandry and high stock densities are considered risk factors to streptococcis in tilapia (ZAMRI-SAAD *et al.*, 2014). A higher number of GBS outbreaks have been observed during summer season, when the water temperature is greater than 27 ºC and a higher thermal amplitude is observed during the day (MIAN *et al.*, 2009; KAYANSAMRUAJ *et al.*, 2014) and fish mortalities resulting from the infection by this pathogen are no usually observed when the water temperature is below than 25 ºC (RODKHUM *et al.*, 2011; MARCUSSO *et al.*, 2015; CHIDEROLI *et al.*, 2017).

In aquatic environment, fish are often exposed to spatial and temporal variations in temperature that affect the physiological traits and survival of aquatic host (BOLTAÑA *et al.*, 2017). In addition, it is known that the variation on the water temperature can influence the fish immune response against bacterial infection, as well as modify the morphology, metabolism and pathogenicity of bacteria, increasing or decreasing the host susceptibility to infection (MEREGHETTI *et al.*, 2008b; KAYANSAMRUAJ *et al.*, 2014; ZHAO *et al.*, 2015).

In this context, the evaluation of global changes in gene expression and protein levels, in consequence to an adaptation to a particular niche in the host or environment condition could be performed through transcriptomic and proteomic approaches using different methods (TIAN *et al.*, 2013; SILVA *et al.*, 2014). The microarray approach allows to analyze the global gene expression of a microorganism under a given experimental condition (TIAN *et al.*, 2013), while the proteomic studies through use of liquid chromatography-mass spectrometry (LC-MS) allow to evaluate the global expression of the functional genome of a bacterial pathogen at the protein level, identifying and quantifying a set of proteins under a given experimental condition (SILVA *et al.*, 2014).

Regarding to thermal stress, small temperature variation represents a challenge to survival of pathogen. These effects, when associated, can block the cell cycle, culminating in the stagnation of bacterial growth and proliferation, or depending on the severity heat stress, can result in the death of the bacterium (RICHTER *et al.*, 2010). On the other hand, the cold shock proteins can alters the bacterial membrane fatty acid composition and global protein profile, avoiding the decrease of membrane fluidity, reduction of protein synthesis, inefficient protein folding and changes in nucleic acids structures (PHADTARE e SEVERINOV, 2010). All microorganisms respond to temperature variation through of increase of heat shock or cold shock protein expression, which acts as activators or repressors of these kind of proteins (EHIRA *et al.*, 2009). The evaluation of temperature-induced transcriptome using microarray technology have been reported in *Yersinia pestis* (HAN *et al.*, 2004), *Escherichia coli* (GADGIL *et al.*, 2005), *Streptococcus pyogenes* (SMOOT *et al.*, 2001) and GBS (MEREGHETTI *et al.*, 2008b). Whereas the label-free proteomics analysis was used to evaluate the differential expression of proteins induced by temperature in *E. coli* (KOCHARUNCHITT *et al.*, 2012), *Ochrobactrum anthropi* (VARANO *et al.*, 2016) and *Bacillus weihenstephanensis* (STELDER *et al.*, 2015).

Previous study demonstrated that at 35 ºC a fast growth, higher hemolytic activity, and a higher viability in tilapia whole blood were observed in GBS, when compared at 28 ºC. In addition, several virulence genes were up-regulated in this temperature, inducing a higher mortality rate in infected fish (KAYANSAMRUAJ *et al.*, 2014). However, there are no studies which demonstrate the global expression of the functional genome of fish-adapted GBS strains at the transcript and protein level under low or high temperature conditions.

Thus, the aim of this study was to evaluate the transcriptome and proteome of *Streptococcus agalactiae*, isolated from diseased fish, submitted to *in vitro* growth under different temperatures using microarray and liquid chromatography-mass spectrometry label-free shotgun (LC-HDMS$^E$) approaches. In addition, this study also aimed to compare the expression trends of orthologous proteins shared among fish and human GBS strains.

## 5.3. MATERIAL AND METHODS

### 5.3.1. BACTERIAL STRAINS AND GROWTH CONDITIONS

The *S. agalactiae* SA53 (ST-260) isolated from diseased fish was used in this study. This strain was selected from culture collection from the National Reference Laboratory for Aquatic Animal Diseases (AQUACEN) to belong to the most identified genotype in Latin America (EVANS *et al.*, 2008; GODOY *et al.*, 2013; BARATO *et al.*, 2015) and to have whole genome previously sequenced (GenBank accession number CP019802.1) (BARONY *et al.*, 2017). The strain was

streaked onto 5 % sheep blood agar and incubated at 28 ºC for 48 h. Then, colonies were pickep up, inoculated in triplicate culture of 100 mL BHI broth ("Brain Heart Infusion", Himedia, Mumbai, India) with 0.05 % (v/v) Tween 80 (BHIT), incubated at 32 ºC or 22 ºC under low agitation and harvested at the mid-exponential phase of bacterial growth ($OD_{600}$ = 0.2). Two aliquots of 50 mL of each biological replicate were harvested for RNA and protein extractions. In addition, The *S. agalactiae* strain NEM316 (ATCC12403) isolated from human invasive infection and acquired from American Type Culture Collection was cultured under these same conditions and the bacterial cells was harvested for protein extraction.

### 5.3.2. TRANSCRIPTOMIC APPROACH

#### 5.3.2.1. RNA EXTRACTION

For RNA extraction, a culture volume of 50 mL of each biological triplicate was immediately centrifuged at 12,000 x g for 30 min at 4 ºC. Bacterial pellets obtained were resuspended in 2 mL of RNAlater (Life Technologies, Carlsbad, USA), incubated at room temperature for 5 min, and posteriorly stored at -70 ºC overnight. Then, the mixture was centrifuged at 12,000 x g for 10 min at 4 ºC and the pellets were lysed mechanically using pestle. Total RNA was extracted using TRIzol RNA Isolation Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The extracted RNA of each replicate was treated with Turbo DNA-free kit (Ambion, Carlsbad, USA) and 1 µL was submitted to GBS-specific PCR (MATA *et al.*, 2004) to determine the absence of genomic DNA. The extracted RNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, USA) and its quality and integrity was evaluated using TapeStation 2200 (Agilent Technologies, Santa Clara, USA). RIN values ranging from 7.7 to 9.1 were used.

#### 5.3.2.2. RNA LABELING AND cRNA SYNTHESIS

A total of 50 ng of RNA of each biological replicate was amplified and Cy3-labeled using the Agilent Quick Amp Labeling kit (Agilent Technologies) along with RNA Spike-in controls according to Agilent One-Color Microarray-Based Expression Analysis protocol (Agilent Technologies). The resulting cRNA of each replicate was purified using RNAeasy Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions, and its concentration measured using a NanoDrop spectrophotometer.

#### 5.3.2.3. HYBRIDIZATION AND MICROARRAY ANALYSIS

The cRNA fragmentation and hybridization were performed using Agilent Gene Expression Hybridization kit according to the manufacturer's instructions. For this, 600 ng of Cy3-labeled cRNA, with specific activity ≥ 16.2 pmol Cy3/ng was fragmented at 60 ºC for 30 min in a mix composed by 5 µL of 10X Blocking agent, 1 µL of 25X Fragmentation Buffer and nuclease-free water to reach a final volume of 25 µL. After fragmentation, 25 µL of 2X GEx Hybridization Buffer were added in each replicate, which were centrifuged at 21,900 x g for 2 min. Next, 45 µL of hybridization solution were dispensed into a custom-made Agilent slide (8 x 60K) formulated based on the library of 4673 non-redundant genes (Agilent.SingleColor.72627) of 9 fish GBS strains (SA07, SA20, SA53, SA288, SA289, SA320, 138P, GD201008_001, ZQ0910) for microarray-based gene expression analysis. Each microarray was created with 2 probes for each gene. This oligonucleotide microarray slide was designed using the eArray server (https://earray.chem.agilent.com/earray/) with the objective of to perform comparative transcriptomics studies with different fish GBS strains. The slide was incubated at 65 ºC for 18 h at 10 rpm in a hybridization oven (Agilent Technologies). Then, the slides were washed in two buffers (Gene Expression Wash Buffer 1 and 2, both Agilent Technologies) and scanned using

Agilent DNA Microarray Scanner (Agilent Technologies). The data obtained from array images were extracted using Agilent Features Extraction software version 11.5 (Agilent Technologies).

Data analysis was performed with GeneSpring GX version 11.0.2 software (Agilent Technologies) using find differentially expressed genes as workflow type. The processed raw signal intensity for all probes was adjusted with percentile shift normalization (percentile target = 75). Sample quality was assessed by box plot to compare the intensity distributions of all replicates, correlation matrix to compare the reproducibility across the biological replicates, principal component analysis (PCA) to assess the variability among replicates among temperatures, and hierarchical clustering analysis to arrange replicates into groups based on the gene expression level. These quality assessments of transcriptomic data were performed in workflow analysis, which is incorporated in GeneSpring. Then, normalized data were filtered to retain probesets with present (acceptable flag) signal intensity values at least two of the three biological replicates in any one out of two conditions. Statistical analyses were performed on filtered data using an unpaired t-test. Genes with $p$ values $< 0.05$ were considered to be statistically significant. A fold-change cut-off of 2.0 was used to consider a gene as differentially expressed with statistical significance between the two temperatures evaluated. All microarray data were deposited to Gene Expression Omnibus Database under the accession number GSE112416.

#### 5.3.2.4. qPCR VALIDATION ANALYSIS

Four differentially expressed genes (DEGs) were selected to further validation by qPCR (Table 1). The primers were designed using Primer Express 3.0 software (Life Technologies) and synthetized by Integrated DNA Technologies (IDT, Coralville, USA). The same three biological replicates of treated RNA used for the microarrays analysis for both temperatures were reverse transcribed into cDNA using the SuperScript III reverse transcriptase kit (Invitrogen), according to the manufacturer's instructions. The qPCR reactions were performed using a GoTaq qPCR Master Mix (Promega) intercalating dye kit in a final volume of 20 µL containing 10 µL of 1x Master Mix, 0.5 µM of primers (Table 1), 0.2 µL of CXR reference dye, and 50 ng of RNA template. The qPCR assay was performed using a ViiA 7 Real-Time PCR System (Life Technologies) with the following cycle protocol: an initial step at 50 ºC for 2 min followed by 1 cycle of 95 ºC for 10 min and 40 cycles of 95 ºC for 15 s, 60 ºC for 1 min. The relative mRNA expression of genes evaluated were normalized with the gyrA and recA genes, which has been considered reference genes for *S. agalactiae* (FLORINDO *et al.*, 2012; FARALLA *et al.*, 2014) using the ΔΔCt method (LIVAK e SCHMITTGEN, 2001). Data acquisition and analysis were performed using the ViiA 7 software v.1.2.3 (Life Technologies). The Cohen Kappa test was used to measure concordance between microarray and qPCR results (VIERA e GARRETT, 2005).

### 5.3.3. PROTEOMIC APPROACH

#### 5.3.3.1. PROTEIN EXTRACTION AND TRYPTIC DIGESTION

For protein extraction, a culture volume of 50 mL of each biological triplicate were immediately centrifuged at 16,100 x g for 20 min at 4 ºC and were washed three time with 50 mM Tris-HCl pH 7.5. Then, the pellets were resuspended in 1 mL of lysis buffer (42 % (w/v) urea, 15 % thiourea, 4 % sodium deoxycholate (SDC), 12.5 mM Tris-HCl pH 7.5, 1.5 % dithiothreitol (DTT)) containing 1 % of a protease inhibitor mix (GE Healthcare, Pittsburgh, USA). Afterwards, the samples were incubated on ice for 15 min and sonicated on ice using cell ultrasonic disruptor (Unique, Indaiatuba, Brazil) for 20 min, worked for 1 min in maximum power (495 W) and stopped for 1 min in a cycle. The lysates were centrifugated at 21,900 x g for 40 min at 4 ºC. The supernatant was collected, load into a Vivaspin 500 column with a threshold of 10 kDa (GE HealthCare), concentrated and washed five times with 50 mM $NH_4HCO_3$. After washing, the

concentrated samples were collected, quantified on Qubit 2.0 fluorometer (Invitrogen, Oregon, USA) using a Qubit protein assay kit (Molecular Probes, Oregon, USA).

For tryptic digestion, 50 µL (100 µg) of each protein extract were mixed with 10 µL of 50 mM $NH_4HCO_3$ and denatured with 25 µL 0.2 % RapiGest SF surfactant (Waters, Milford, USA) through incubation at 80 ºC for 15 min. After this, 2.5 µL of 100 mM DTT were added and heated at 60 ºC for 30 min. Afterwards, 2.5 µL of 300 mM iodoacetamine were added and the samples were kept at room temperature in a dark chamber for 30 min. Then, the proteins were enzymatically digested with 0.5 µg/µL trypsin (Promega, Madison, USA) through incubation at 37 ºC for 18 h. Following this incubation, 10 µL of 5 % Trifluoroacetic acid (TFA) (Sigma Aldrich, Saint Louis, USA) were added in each sample and incubated at 37 ºC for 90 min. The resulting peptide extracts were centrifuged at 21,900 x g for 30 min at 6 ºC. Next, the removal of SDC was performed by two-phase solvent extraction with ethyl acetate (Sigma Aldrich) (2:1) followed by the addition of 0.5 % TFA and centrifuged at 15,000 x g for 5 min at 20 ºC. After centrifugation, the aqueous phase was collected and desalted using C18 MacroSpin Columns (Harvard Apparatus, Holliston, USA), according to the manufacturer's instructions. The samples were dried under vacuum in Vacufuge Concentrator (Eppendorf, Hamburg, Germany), resuspended in 100 µL of 20 mM ammonium formate (Sigma Aldrich), transferred for Waters Total Recovery vials (Waters), and stored at -70 ºC until use.

### 5.3.3.2. 2D NANOUPLC ANALYSIS

The biological replicates were analyzed by LC-MS using nanoACQUITY ultra-performance liquid chromatography (UPLC) system coupled to a Synapt G2Si HDMS mass spectrometer (Waters). Bidimensional nanoUPLC tandem nano electrospray high definition mass spectrometry (nanoESI-HDMS$^E$) (multiplexed DIA – data-independent acquisition) experiments were conducted using both a 1-h reverse-phase gradient from 7 % to 40 % (v/v) acetonitrile (0.1 % v/v formic acid) and a 500 $nL.min^{-1}$ nanoACQUITY UPLC 2D Technology system. Stoichiometric measurements based on scouting runs of the integrated total ion account (TIC) prior to analysis were performed to ensure standardized molar values across all samples. Typical on-column sample loads were 500 ng of total protein digests for each of the 5 fractions (500 ng per fraction/load).

For every measurement, the mass spectrometer was operated in resolution mode with a typical *m/z* resolving power of at least 25,000 full width at half maximum (FWHM) and an ion mobility cell that was filled with helium gas and a cross-section resolving power at least 40 Ω/Δ Ω. Analyses were performed using nano-electrospray ionization in positive ion mode nanoESI (+) and a NanoLock-Spray ionization source. Mass spectrometric analysis of tryptic peptides was performed using a mass spectrometer equipped with a T-Wave-IMS device in MS$^E$ and HDMS$^E$ modes as previously described (DISTLER *et al.*, 2014). The radio frequency offset (MS profile) was adjusted such that the nanoESI-HDMS$^E$ data were effectively acquired from *m/z* 400 to 2,000 by MassLynx v.4.1 software (Waters), ensuring that any masses that were observed in the high energy spectra with less than *m/z* 400 arose from dissociations in the collision cell.

### 5.3.3.3. PROTEIN IDENTIFICATION AND QUANTITATION

HDMS$^E$ raw data of samples were processed using Progenesis QI for Proteomics (QIP) v.2.0 (Nonlinear Dynamics, Newcastle, UK), following methods previously described (KUHAREV *et al.*, 2015). Imported runs were submitted to data automatic processing for protein identifications and quantitative information using the dedicated algorithms in Progenesis QIP. The following parameters were used: peak picking limits = 5; maximum charge retention time limits = 8.

For peptide identification, data from the three biological replicates of each strain in two conditions were searched against *S. agalactiae in-house* database compiled from the annotated protein .fasta files for each respective strain (i.e., one database for SA53 and other for NEM316). Using the software ProteinLynx Global Server (PLGS) v.3.0.2 (Waters) in database management tool, the sequence of each protein was reversed during the database queries and appended to the original database to assess the false positive rate during identification. The searches were divided into separate experiments, with each experiment consisting of the raw files corresponding two conditions evaluated per strain. The following parameters were used for peptide identification: digest reagent = trypsin; maximum missed cleavage = one; maximum protein mass = 600 kDa; modifications: carbamidomethyl of cysteine (fixed), acetyl N-terminal (variable), phosphoryl (variable), oxidation of methionine (variable); search tolerance parameters: peptide tolerance = 10 ppm, fragment tolerance = 20 ppm, maximum false discovery rate (FDR) = 4 %; ion matching requirements used default parameters (LI *et al.*, 2009): fragments per peptide = 1, fragments per protein = 3, peptide per protein = 1.

The protein-level quantitation was performed with relative quantitation using Hi-N algorithm, which is incorporated in Progenesis. Peptides identified with score ≤ 3, mass error ≥ 20 ppm, and sequence length ≤ 6 amino acids were removed. Protein identified with at least two peptides (with ≥ 1 proteotypic peptide per protein) and presents in at least two of the three biological replicates for GBS strain were considered. To obtain a general overview of protein expression among strains in the different conditions, PCA and hierarchical clustering analyses were performed using the *ggbiplot* version 0.55 (VU, 2011) and *gplots* version 3.0.1 (WARNES *et al.*, 2016) packages, respectively, in R software version 3.4.1 (R CORE TEAM, 2013). A protein was considered to be differentially expressed at 32 ºC in relation to 22 ºC if there was a significant ($p < 0,05$, ANOVA) change in expression ≥ 2-fold ($\log_2$ ratio ≥ 1.0). The MS proteomics data are available at the ProteomeXchange Consortium via the PRIDE (VIZCAÍNO *et al.*, 2016) partner repository under the identifier PXD009330.

### 5.3.4. BIOINFORMATIC ANALYSES

The transcripts and proteins identified for SA53 under both temperature conditions were analyzed using the prediction tools SurfG+ version 1.0.2 (BARINOV *et al.*, 2009) and Cluster of Orthologous Genes (COG) version 2014db (GALPERIN *et al.*, 2015) to predict subcellular localization and orthologous group by functional category, respectively. The COG database search was performed using an *in-house* script (available at: https://github.com/aquacen/blast_cog).

The interaction among genes/proteins identified as differentially expressed at 32 ºC were analyzed by STRING web tool version 10.5 (SZKLARCZYK *et al.*, 2017) using *Streptococcus agalactiae* NEM316 as reference, with experimental confidences and interaction score ≥ 0.700 (high confidence) allowed. The interaction networks obtained were visualized using Cytoscape version 3.5.1 (SHANNON *et al.*, 2003).

The Interactivenn web-based tool (HEBERLE *et al.*, 2015) was used to evaluate the number of proteins identified at 22 ºC or 32 ºC between SA53 and NEM316 strains through venn diagrams.

And an *in-house* application was developed (available at https://www.github.com/aquacen/dynamic_compare) to compare the abundance of orthologous proteins shared between two dynamic ranges. This application permits the visualization of protein alignment through a threshold previously determined for the percentage of differential abundance among conditions or strains. We used this application to identify the behavior of protein abundance between SA53 and NEM316 strains in both temperatures tested using a threshold of 25% and 50%.

### 5.3.5. CORRELATION ANALYSIS BETWEEN TRANSCRIPTOMIC AND PROTEOMIC DATA

To determine the correlation between expressed genes and proteins from our results, the expression level of a transcript with statistical significance ($p < 0.05$) were correlated with abundance of the corresponding protein (also $p < 0.05$) present in proteomic dataset. For this, the Pearson's correlation was calculated using the R software version 3.4.1 (R CORE TEAM, 2013).

## 5.4. RESULTS

### 5.4.1. TRANSCRIPTOME ANALYSIS

The effect of temperature in the GBS transcriptome was evaluated using whole-genome DNA microarray. We identified a total of 1,730 transcripts in SA53, with at least 98 % identify matches with probes of the array, characterizing 94.9 % of predicted genome of strain. The same transcripts were identified in both temperature, but transcriptome differences at intensity values were observed during bacterial grown at 22 ºC and 32 ºC. The quality of transcriptomic data showed small variations among replicates at intensity values (Supplementary Figure 1A) with a correlation coefficient greater than 95% (Supplementary Figure 1B), demonstrating a high reproducibility of transcriptome profile. And, in the PCA analysis the data were discriminated between 22 ºC and 32 ºC (Supplementary Figure 1C).

The 1,730 transcripts originally identified in two out of three replicates were narrowed down to 579 transcripts with $p \leq 0.05$. The hierarchical clustering of these transcripts showed that the strain has distinct transcriptomic pattern influenced by temperature, demonstrating a relationship in the gene expression profiling across replicates into temperature condition (Figure 1A). Among these transcripts, 75 were detected as being up-regulated at 32 ºC, while 32 were down-regulated at 32 ºC (Table 2). These differentially expressed genes represented ~6 % of the genome of SA53 strain.

According with the subcellular localization analysis these DEGs were classified as cytoplasmic ($n = 73$), membrane ($n = 22$), potentially surface-exposed ($n = 9$) and secreted ($n = 3$), being that ~34% and ~30% are of bacterial surface when down- and up-regulated at 32 ºC respectively (Table 2). The DEGs were classified using COG on 18 functional categories (Table 2 and Figure 2A). A higher number of genes related with metabolism were detected as up-regulated at 32 ºC, except those involved in Nucleotide metabolism (F) and Coenzyme transport and metabolism (H) which were exclusively detected as down-regulated at 32 ºC. On the other hand, genes involved in Cell cycle control (D), Cell wall biogenesis (M), Defense mechanisms (V), Replication (L) and Secondary metabolites biosynthesis (Q) were exclusively detected as up-regulated at 32 ºC.

The putative virulence genes cAMP factor, *lmb* and *noxE* were detected as down-regulated at 32 ºC, while stress protein genes (*hrcA*, *grpE*, Universal stress protein and *trx*) were up-regulated (Table 3).

To better understand the biological functions of the transcripts identified as differentially expressed at 32ºC, a interactome analysis was conducted, revealing 97 interactions (Fig 3A). The greatest number of interactions were verified in the proteins related to phosphoenolpyruvate-dependent phosphotransferase system (PTS) (cluster 1), ABC transport system (cluster 2), ascorbate and aldarate metabolism (cluster 3), purine metabolism (cluster 4) and metabolic pathways (cluster 5), both down- and up-regulated at 32 ºC (Figure 3A).

In validation analysis, we observed a perfect agreement (kappa coefficient = 1) between microarray and qPCR results at regulatory direction level (down- and up-regulation) for all genes evaluated (Table 1). Therefore, this result demonstrated that the microarray data are valid.

## 5.4.2. PROTEOME ANALYSIS

The effect of temperature on the GBS proteome was evaluated using LC-HDMS$^E$ approach. A total of 29,790 peptides with a normal distribution of 10 ppm error were identified (Supplementary Figure 2A). Peptides as source fragments, with charge state of at least $[M+2H]^{2+}$, and absence of decoys were considered to increased data quality. Therefore, our proteomics analysis allowed the identification and quantitation of 1046 proteins for SA53, with an average of 28 peptides per protein and a calculated FDR = 0 % when decoy detection was set at agreement of 2 of the 3 replicates. This result characterized ~62 % of the predicted proteome of SA53 strain. A total of 1,043 proteins were present under two temperatures tested. In relation of protein content, more than 99 % of the identified proteins were found in 3 of the 3 replicates of each temperature, showing a high reproducibility among the biological replicates (Supplementary Figure 2B). In PCA analysis there was no a clear variability in the protein expression among the temperatures evaluated (Supplementary Figure 2C). The dynamic range of the identified proteins reached ~4.5 logs orders of magnitude between the most and least abundant proteins in each temperature tested, moreover an extensive proteome differences during the bacterial grown at 22 ºC and 32ºC was observed in proteins involved with thermal stress and virulence (Figure 4).

The label-free quantification was applied to evaluate the relative abundance of the proteome of the strain at low and high temperature conditions. In summary, 163 proteins had $p \leq 0.05$, and 81 (4.7 % of the predicted proteome) of them showed difference in level of expression in SA53, being 37 and 44 proteins down- and up-regulated at 32ºC, respectively (Table 4). Beyond that, 2 proteins were exclusively expressed at 32 ºC, and 1 protein was exclusively expressed at 22 ºC (Table 4). The hierarchical clustering of significant proteins ($n$ = 163) demonstrated the arrangement of biological replicates by temperature condition (Figure 1B), indicating that the strain had distinct proteomic pattern influenced by temperature.

A total of 67, 7, 5 and 2 differentially expressed proteins (DEPs) were predicted as cytoplasmic, membrane, PSE and secreted, respectively, being that ~21% and ~13% are of bacterial surface when down- and up-regulated at 32 ºC respectively (Table 4). According COG analysis, the DEPs were classified into 18 categories (Table 4 and Figure 2B). Proteins involved in Translation, ribosomal structure and biogenesis (J) and Inorganic ion metabolism (P) were the most abundant down-regulated at 32ºC in relation to up-regulated proteins. On the other hand, proteins involved in defense mechanisms (V), lipid metabolism (I), nucleotide metabolism and secondary metabolites biosynthesis (Q) were exclusively expressed as up-regulated at 32ºC.

Among the known virulence factors for GBS we identified Reticulocyte binding protein as down-regulated at 32 ºC, while IagA (Family 1 glycosyl transferase), CpsB and cAMP factor were up-regulated (Table 3). In addition, Thioredoxin, which is a protein involved in oxidative stress was also identified as down-regulated at 32 ºC.

In order to study the main interactions among the proteins identified as differentially expressed at 32 ºC, we performed a interactome analysis, which revealed 26 protein-protein interactions. The greatest number of interactions was observed as up-regulated at 32 ºC, being PurH (SaSA53_0180) and PurM (SaSA53_0177) involved in purine metabolism, Alkyl hydroperoxide reductase subunit C (SaSA53_1585) and Thioredoxin (SaSA53_1469) involved in oxidative stress, Pyruvate kinase (SaSA53_0811) related with glycolysis, and Amino acid ABC transporter permease (SaSA53_0815) involved in amino acid metabolism, were identified (Figure 3B).

## 5.4.3. CORRELATION ANALYSIS

The Pearson correlation coefficient between gene expression level and protein abundance for SA53 was 33%, demonstrating that there was a low correlation between transcriptome and

proteome datasets, regarding the genes and proteins that were down- or up-regulated on the same way, for a given condition (low and high temperature).

### 5.4.4. COMPARATIVE PROTEOMICS AMONG GBS STRAINS

To evaluate the expression trends of functional genome of GBS strains from poikilotherm and homeotherm hosts and to verify if there is a distinct adaptation to these hosts at the proteome level, a comparison between the proteome datasets of SA53 and NEM316 was performed. We identified 840 and 841 shared proteins between SA53 and NEM316 at 22 ºC and 32 ºC, respectively, using venn diagram. A variation greater than 25% between the average of protein abundance of three biological replicates at 22 ºC in SA53 and NEM316 strain was observed in 120 proteins (Figure 5A), while that 27 proteins showed a variation higher than 50% (Figure 5B). At 32 ºC, 116 and 23 proteins demonstrated variation greater than 25% and 50% among the strains compared (Figure 5C-D).

## 5.5. DISCUSSION

Fish-adapted GBS strains are exposed to constant and abrupt changes in temperature during adaptation in aquatic environment and during the infection process, considering that fishes are poikilothermic animals and that the environmental water can present fast changes in its temperature in consequence to short-term weather events. To better understand the characteristics of metabolism, adaptation and pathogenicity of this agent in response to temperature variation, we analyzed the transcriptome and the proteome of a fish-adapted GBS strain subjected to two growth temperatures using microarray and LC-HDMS$^E$ approaches.

The isolate selected to perform these analyses was the SA53 strain. This strain was previously isolated from diseased Nile tilapia in Brazil (MIAN *et al.*, 2009) and belongs to the ST-260, one of the most identified genotype in Latin America (EVANS *et al.*, 2008; DELANNOY *et al.*, 2013; GODOY *et al.*, 2013; BARATO *et al.*, 2015; BARONY *et al.*, 2017). SA53 strain belongs to a group of fish-adapted genotypes that descend from a single branch, unlike what occurs with human GBS strains, and that is going through a reductive genome evolution (BARONY *et al.*, 2017), and, besides that, this strain presented similar expression of protein content (> 95%) with other 4 fish-adapted GBS strains when cultured under one temperature condition (TAVARES *et al.*, 2018, under review). Its $LD_{50}$ was no previously determined, however, isolates obtained from diseased fish have demonstrated to be highly virulent in experimental assays with Nile tilapia, with $LD_{50}$ ranging from $10^1$ to $10^5$ cfu $mL^{-1}$ (MIAN *et al.*, 2009; EVANS *et al.*, 2015). Therefore, due the characteristics above described, the SA53 strain could elucidate the behavior of expression of genes and proteins of fish-adapted GBS strains in low (22 ºC) and high (32 ºC) temperature conditions, which corresponding the temperatures where occurs low and high mortalities rates in fishes, respectively (SALVADOR *et al.*, 2005; MIAN *et al.*, 2009; AMAL *et al.*, 2015; AL-HARBI, 2016; MAINARDI *et al.*, 2016; CHIDEROLI *et al.*, 2017).

For the temperatures analyzed in this study, the necessity of transcriptome and proteome modification was minimal, corroborating with previous study that compared the transcriptional response to temperature for two *Lactococcus garvieae* strains (AGUADO-URDA *et al.*, 2013). This suggests that fish-adapted GBS strains may be physiologically stable in the gene expression and protein synthesis even with rapid variation in the environmental temperature, supporting the idea of possible adaptation of bacteria to the aquatic environment and to the fish host.

Although the alteration of expression of genes and proteins in response to temperature has not been extensive, the heatmap analysis grouped the biological replicates according to temperature tested, regardless of approach used, demonstrating that the temperature is able to modulate the gene expression level and protein abundance in fish-adapted GBS strains.

## 5.5.1. TRANSCRIPTIONAL RESPONSE IS MODULATED BY TEMPERATURE

In transcriptomic analysis we observed that the temperature was able to influence the gene expression involved mainly with cellular metabolism (Supplementary Figure 3A). Down-regulated at 32 ºC, we detected genes involved in purine metabolism as *purD*, *purH*, *purK*, *purM* and *guaC*. These genes grouped in our interactome analysis and they are related to purine biosynthetic pathway, described for Mereghetti et al. (2008b) as responsible for the synthesis of inosine monophosphate, a compound important to satisfy the purine auxotrophic requirements of GBS (RAJAGOPAL *et al.*, 2005). A previous study demonstrated that purine metabolism in GBS is influenced by temperature of incubation, being up-regulated at 40 ºC in comparison 30 ºC (MEREGHETTI *et al.*, 2008b). In our study, the identification of these transcripts occurs at 22 ºC and may be associated with an adaptive condition where the bacterium presumably increases its requirements of adenine and guanine during growth at low temperature in aquatic environment, due the bacterial hydrolytic degradation of nucleic acids and nucleotides or uptake of these nucleotides from environment through the utilization of the purine salvage pathway (RAJAGOPAL *et al.*, 2005). The utilization of this pathway is corroborated by detection of salvage enzymes *apt* (Adenine phosphoribosyltransferase), *xpt* (Xanthine phosphoribosyltransferase), Hypoxanthine phosphoribosyltransferase, Inosise-uride nucleoside N-ribohydrolase and *add* (adenosine deaminase) in the transcriptome dataset.

Four genes involved with PTS mannose transport (subunit IIA, IIB, IIC and IID) related to carbohydrate metabolism were down-regulated at 32 ºC. This PTS system has been considered important to the transport of mannose, fructose and glucose for the species of the genus *Streptococcus* (PELLETIER *et al.*, 1998; ABRANCHES *et al.*, 2003; BIDOSSI *et al.*, 2012), through a mechanism that couples translocation (subunits IIC and IID) with phosphorylation (subunits IIA and IIB) of the carbon sources (GUTKNECHT *et al.*, 1999). The mutation in subunits IIAB in *Streptococcus mutans* led a lower uptake of glucose and mannose in medium containing glucose as the sole carbohydrate source, while the uptake of fructose is not affected (ABRANCHES *et al.*, 2003). Fish-adapted GBS strains need adjust their metabolism in response to nutrient availability in aquatic and host environments, especially in relation to glucose availability. At high temperature, a greater affinity for glucose is required to growth of psychrotolerant and mesophilic bacteria (NEDWELL, 1999). The exposure of GBS strains to glucose permits the modulation of genes involved in cell envelope biogenesis and metabolism and transport of amino acids, ions and other carbohydrates, however, a high availability of this nutrient led the decreasing the expression of genes involved with uptake of carbohydrates and putative virulence factors (DI PALO *et al.*, 2013). In this way, we can speculate the fish-adapted GBS strain have a lower affinity by glucose at 22 ºC, increasing the expression of PTS system involved in transport of mannose, which in turn promotes the increase of the capacity to import glucose for bacterial cell. Nevertheless, future research needs to be conducted to evaluate of PTS mannose activity in fish-adapted GBS strains cultured in medium containing different carbohydrate sources and at different temperatures.

Genes involved in methionine metabolism, such as *metE* and *metF*, were also detected as down-regulated at 32ºC. These genes are involved in methionine synthesis pathway, through of methylation of homocysteine by *metE* in conjunction with *metF*, which after receive the methyl group of *folD* (5-methyltetrahydrofolate), forming the methionine (AFZAL *et al.*, 2016). Genes involved in methionine synthesis have been considered critical for virulence in GBS (SHELVER *et al.*, 2003) and *Brucella melintesis* (LESTRATE *et al.*, 2000), allowing the bacterial survival during infection. Previous study demonstrated that the reduction of growth temperature of *Escherichia coli* strains is directly related to its low capacity to bind methionine, being unable of this in temperature below 8 ºC (GOODRICH e MORITA, 1977). The methionine availability in fish blood, as well as fish body temperature, might influence the growth rate of the GBS, predisposing the expression of virulence factors, and allowing the bacterium resist clearance by

the immune system, as previously verified *in vitro* and *in vivo* assays with a human GBS strain (SHELVER *et al.*, 2003). However, further studies are needed to determine if *metE* and *metF* contribute to GBS virulence in fishes and if they are essentials for the growth of this bacterium in fish blood.

On the other hand, metabolic pathways involved with uptake of cellobiose, ascorbate, N-acetylgalactosamine and amino acids were up-regulated at 32 ºC. GBS strains also use cellobiose, ascorbate and N-acetylgalactosamine as carbon source (GLASER *et al.*, 2002). Cellobiose is a β-glucoside plant-derived carbohydrate acquired by PTS system after degradation of the extracellular matrix or others biopolymers in the host as glycosaminoglycans, which are analogues of cellobiose (SHAFEEQ *et al.*, 2013), or by direct acquisition this compound. Many fish species are able to partially digest cellulose by acid hydrolysis in their stomachs, whereas their intestinal microbiota, especially bacterial cellulases, digest cellulose into cellobiose (ADIN *et al.*, 2008). Fish-adapted GBS strains does not contain cellulase in their genome, therefore, the cellobiose uptake may occurs during infectious process in gastrointestinal epithelium in fishes, once gastrointestinal tract is one of the major routes of entry of GBS in fishes (IREGUI *et al.*, 2016), or during adaptation in aquatic environment, where cellobiose excreted via feces can be found, and used as an alternative energy source. The genes involved with this type of metabolism (PTS cellobiose transporter subunit IIA and IIC) showed a change in expression level of 5- to 7-fold when cultured in high temperature, interacting with each other in our interactome analysis.

Ascorbate can also be used as alternative carbon source for many bacteria species, including *E. coli*, *Klebsiella pneumoniae* and *Streptococcus pneumoniae*, and its entry into the bacterial cells occurs through the ascorbate-specific PTS system (AFZAL *et al.*, 2015). We identified four genes involved with ascorbic acid uptake (*ulaD*, PTS ascorbate transporter subunit IIA, IIB and IIC) in transcriptomic analysis, being that they interact with each other. In fish tissues the concentration of ascorbate vary of 4.89 to 48.06 μg $g^{-1}$ when the diet is not supplemented with this compound (SOLIMAN *et al.*, 1994), and the their content in liver does not change in animals maintained at different temperature (SATO *et al.*, 1983). In this way, during infectious process, the ascorbate contained in fish tissues may be used by GBS strains as energy source for their growth and dissemination in this host, especially in high temperature, as observed in our study.

N-acetylglucosamine is an important component of polysaccharide in the cell wall of GBS strains (PEREIRA *et al.*, 2013), and their catabolism provides organisms with a carbon and nitrogen source (MOYE *et al.*, 2014). The entry of N-acetylglucosamine into bacterial cells occurs through specific PTS system, as previously verified in *E. coli*, or by mannose transporter (ÁLVAREZ-AÑORVE *et al.*, 2005). Corroborating with this statement, we identified the interaction of PTS systems involved in the transport of mannose and N-acetylglucosamine in the interactome analysis. N-acetylglucosamine was described as important for the GBS adhesion to the fish cells (BARATO *et al.*, 2016), thus, the expression of these genes in high temperature may contribute to adhesion and immune evasion of fish-adapted GBS strains in aquatic host.

Also up-regulated at 32 ºC, we identified six genes involved with the transport of peptides, including the Opp transport system (*oppCDF*), an important superfamily of conserved ATP-binding cassette transporters involved in bacterial nutrition, signaling and virulence though of internalization of peptides from the extracellular environment (SILVA *et al.*, 2017). In GBS, the Opp transport system is responsible by the uptake of oligopeptides in a nutritionally rich environment and contribute to infection by stimulate the adherence of pathogen to human cells and modulate the expression of fibrinogen-binding adhesin (SAMEN *et al.*, 2004). However, there are no studies which demonstrate the participation of Opp genes in the pathogenesis of fish-adapted GBS strains.

Variations in temperature during bacterial growth can induce the development of mechanisms of adaptive response to the environment changes, as heat and cold shock proteins. In transcriptome analysis we sole identified *hrcA* and *grpE* as genes related with heat shock, being both up-

regulated at 32 ºC. These genes are required for bacterial growth of *Escherichia coli* or *Streptococcus thermophilus* in both at low and high temperatures, being important to cellular viability and cross-protection at these temperatures conditions (FAYET *et al.*, 1989; SCHULZ e SCHUMANN, 1996; ZHANG e GRIFFITHS, 2003; VARCAMONTI *et al.*, 2006). The detection of these genes in fish-adapted GBS strains suggests that their participation in bacterial resistance to high temperature in both aquatic and host environments.

Genes involved in oxidative stress control was divergently down- and up-regulated at 32ºC. The association between low temperature and the increasing of expression of the genes encoding proteins involved in oxidative stress as superoxide dismutase, thioredoxin reductase and catalase have been verified in *E. coli* (SMIRNOVA *et al.*, 2001), *Listeria monocytogenes* (LIU *et al.*, 2002) and *Pseudomonas fluorescens* (CHATTOPADHYAY *et al.*, 2011). In our study, *noxE* was detected as down-regulated at 32 ºC. NoxE (NADH oxidase) assists in oxygen tolerance in GBS strains, either by decreasing of intracellular NADH/NAD+ ratio or by direct elimination of oxygen, as well as contributes to bacterial infection process in blood, liver and brain of mice (YAMAMOTO *et al.*, 2006). In contrast, the gene *trx* was detected as up-regulated at 32 ºC. Trx and other oxidoreductases proteins are important to the maintenance of thiol state in bacterial cell through reduction of oxidized cysteine residues in cytoplasm. These proteins can repair oxidative damage occurred in other proteins that contain this cysteine residues, and which may be involved in cellular processes, allowing the maintenance of their activity and function (EZRATY *et al.*, 2017). Therefore, the differential expression of *noxE* and *trx* in both temperature evaluated in this study may be associated with the adaptive capacity of fish-adapted GBS strains in response against reactive oxygen species (ROS) in host environment, seeing that the temperature variation results in an increase or decreasing of ROS production in fish species (BANH *et al.*, 2016).

The universal stress protein (*usp*) involved in stress response were also found up-regulated at 32 ºC. This gene has been identified in *E. coli* in a large variety of stress conditions and is involved in oxidative stress resistance, motility and bacterial adhesion (NACHIN *et al.*, 2005), while in GBS, *usp* have been associated with a long term survival during nutrient stress (YANG *et al.*, 2012). Therefore, the expression of *usp* in fish-adapted GBS strain might be essential to its survival during adaptation in aquatic environment, where a nutrient deprivation occurs and can be a major selection pressure for bacteria (SUNDBERG *et al.*, 2014), As this gene was detected at high temperature, we can also speculate that it may to induce a thermal tolerance of fish-adapted GBS strains.

Two putative virulence genes were detected down-regulated at 32 ºC: *cAMP factor* and *adhesion protein*, which is homologous to *lmb* (laminin-binding surface protein). *cAMP factor* is a protein secreted by GBS strains with pore-forming and hemolytic proprieties (RAJAGOPAL, 2009). The high expression of this gene has been described at high temperature conditions (35 ºC) in fish GBS strains (KAYANSAMRUAJ *et al.*, 2014). However, our results contradict this statement, seeing that the higher expression of *cAMP factor* was detected at low temperature. *Lmb* promotes the binding between GBS and host laminin, a glycoprotein of the basement membrane, as well as induce the GBS invasion into brain tissues in human host (AL SAFADI *et al.*, 2010). Few fish-adapted GBS strains showed the presence of the *lmb* gene in our genome when screening by PCR assay (GODOY *et al.*, 2013), however, *lmb* have not yet been studied in terms of their biological functions in these strains. The detection of *lmb* suggests that their participation in pathogenesis is similar in aquatic and human hosts. Nevertheless, future research must to be conducted to validate this possibility.

### 5.5.2. CHANGES IN THE PROTEOME IS MODULATED BY TEMPERATURE

In proteomic analysis we observed that the temperature was also able to influence the abundance of proteins related with metabolic pathway (Supplementary Figure 3B). Down-regulated at 32 ºC we identified proteins involved with carbohydrate metabolism as DexB and Glycerol uptake

permease. DexB (Glucan 1,6-alpha-glucoside) participate of starch and sucrose metabolism pathway and acts releasing glucose from the non-reducing terminus of alpha-1,6-linked dextran or isomaltosaccharides (WHITING *et al.*, 1993). The free glucose units released may be used to fish-adapted GBS strains in gluconeogenesis pathway, even during adaptation to low temperature, where the glucose affinity is decreased (NEDWELL, 1999). Glycerol uptake permease is an aquaporin responsible by conduct water and small hydrophilic solutes, in particular glycerol, into the bacterial cell (LU *et al.*, 2003). The glycerol uptake in low temperature can also contribute to obtaining of an alternative energy source for the bacterial growth in this condition.

Proteins involved in inorganic ion transport and metabolism were detected as down-regulated at 32 ºC, such as PtsAB, Nickel ABC transporter and Cobalt ABC transporter. Genes that encoding these proteins were divergently down- and up-regulated in response to incubation with human blood, demonstrating that they are important to GBS pathogenesis during infection process (MEREGHETTI *et al.*, 2008a). The gene expression of some of these proteins were up-regulated at high temperature (MEREGHETTI *et al.*, 2008b), contracting our findings. In fish-adapted GBS strains, proteins involved in inorganic and metallic ions metabolism were considered important for the growth and survival of pathogen in aquatic environment (TAVARES *et al.*, 2018, under review), therefore, the differential protein abundance of this functional category in low temperature may contribute to this.

A change in regulatory direction was observed in proteins related to purine metabolism and oligopeptide uptake, when compared to transcriptome data. In proteome the OppC protein was down-regulated at 32 ºC, while PurH, PurK and PurM were up-regulated at 32 ºC. This change may have occurred because the samples were collected at the same point time, where transcript and proteins have different half-times, and changes in expression of transcripts just affect protein levels after a certain temporal delay, once the protein synthesis takes time (LIU *et al.*, 2016).

Several proteins related to formation of the ribosomal complex and protein synthesis were identified as down-regulated at 32 ºC. It is known that at low temperature, the bacterial cells require a higher concentration of ribosomes for protein synthesis, however, the increase of number of proteins involved in this functional category do not contribute directly to cell growth, as verified in *E. coli*, where a pool of nontranslating ribosomes were identified, due to defect in translation initiation (FAREWELL e NEIDHARDT, 1998). In this way, although a higher number of proteins related with translation, ribosome structure and biogenesis has been identified at low temperature in the fish-adapted GBS strain, this did not correlate with a rapid bacterial growth in our in vitro assay, since we observed that the SA53 strain take longer time to grow on BHIT broth at 22 ºC relative to 32 ºC, practically the double time (data not shown).

In this context, the pathogen needs to express proteins that could reflect in a better adaptive capacity to environment under low temperature condition. In our study the RNA helicase was identified as down-regulated at 32 ºC and with a change in abundance level of 2.08-fold. RNA helicase is a cold shock protein involved in processing, transport or degradation of mRNA, ribosome biogenesis and translation initiation during cold acclimation (PHADTARE e SEVERINOV, 2010). Similarly, the RNA helicase *deaD* gene was identified as up-regulated at 30 ºC in relative 40 ºC in a previous study using GBS strain during stationary phase of bacterial growth (MEREGHETTI *et al.*, 2008b). In this same study, there was no differential expression of chaperone proteins, such as GroS and ClpL (both heat shock proteins) among the temperatures evaluated. However, in our proteomic dataset GroS and ClpL were identified as down-regulated at 32 ºC. Therefore, the expression of heat and cold shock proteins by fish-adapted GBS strain represents an important mechanism of protection and homeostasis against environmental stressors as temperature, for example.

Proteins involved in oxidative stress control were found up-regulated at 32ºC. Trx and Flavodoxin had a change of abundance level of 6.2- and 3.4-fold respectively. Similarly to Trx, flavodoxin acts in the defense against ROS and contributes to the *Pseudomonas aeruginosa* survival in

macrophagic cells and in *Drosophila melanogaster* (MOYANO *et al.*, 2014). In this way, flavodoxin might also contribute to the survival of fish-adapted GBS strains in fish cells during infection process.

Four proteins related with bacterial pathogenicity showed differential protein abundance between 22 ºC and 32 ºC (Table 3). Reticulocyte binding protein, homologous to C5a peptidase (ScpB), was found down-regulated at 32 ºC, and showed a change of abundance level of 20.8-fold. This protein is a serine protease involved in adhesion and host immune evasion (RAJAGOPAL, 2009). On the other hand, IagA (4.1-fold), CpsB (2-fold) and cAMP factor (2.7-fold) were detected as up-regulated at 32 ºC. IagA contributes to blood-brain barrier invasion by GBS strains (DORAN *et al.*, 2005), while CpsB, a protein related with the capsule synthesis, protects the bacterium thought prevention of complement deposition (factor C3b) and opsonophagocytosis (GLASER *et al.*, 2002). CAMP factor also changed the regulatory direction in comparison to transcriptome analysis, being up-regulated at 32 ºC in proteome analysis. As previously mentioned to *lmb* gene, not all of these virulence factors have been demonstrated as important in fish infection. However, the identification of Reticulocyte binding protein, IagA, CpsB and cAMP factor indicate that these proteins may contribute to the adhesion, dissemination and survival of GBS in fish tissues, with a pathogenesis similar to the human GBS infections.

It is known that GBS can be isolated from diseased fish at low temperature (CHIDEROLI *et al.*, 2017). It is clearly demonstrating that the fishes can become infected under these conditions, and that GBS strains should, therefore, use the virulence proteins, as Reticulocyte binding protein, also under these conditions to cause disease in aquatic host. In field conditions, when the tilapines are cultured in water at high temperature, the GBS load in tissue of infected fish can increase, causing extensive tissue damage with a massive inflammatory response, increasing the expression of several virulence genes, allowing, in this way, that GBS may transcend host-defense mechanism, and raising the number of dead fish (KAYANSAMRUAJ *et al.*, 2014). In our study, we expected to find several proteins related to the pathogen-host interaction being highly expressed at 32 ºC, seeing that in this temperature higher mortality rates are verified. Nevertheless, few proteins were detected with differential expression.

The main trigger to the expression of virulence factors in pathogenic bacteria is temperature (GUIJARRO *et al.*, 2015). In high temperature (37 ºC) *Shigella* spp. (MAURELLI *et al.*, 1984), *Bordetella pertussis* (RAPPUOLI *et al.*, 1992), *Yersinia pestis* (KARLYSHEV *et al.*, 1992) and *Escherichia coli* (FALCONI *et al.*, 1998) had its virulence gene expression stimulated by environmental factor, becoming virulent for the mammal host. On the other hand, in fish-pathogenic bacteria the up-regulation of virulence factors are common verified at low temperature, as observed in *Yersinia ruckeri* (18ºC *vs* 28ºC) (MÉNDEZ *et al.*, 2009), *Flavobacterium psychrophilum* (12ºC *vs* 18ºC) (GOMEZ *et al.*, 2012), *Lactococcus garvieae* (18ºC *vs* 37ºC) (AGUADO-URDA *et al.*, 2013), *Aeromonas hydrophila* (25ºC *vs* 37ºC) (YU *et al.*, 2007) and *Edwardsiella tarda* (25ºC *vs* 37ºC) (SRINIVASA RAO *et al.*, 2004). In our study, the detection of genes/proteins involved in the virulence of GBS was observed at both temperature condition, demonstrating that their expression is not temperature-dependent and keeping the bacterial virulence machinery always active. The fact that the bacterium to be mesophilic may have contributed to this characteristic. In this way, it seems that metabolic adaptations are more marked to fish-adapted GBS strains with the temperature than virulence for the infection process.

Therefore, it is possible that the increase in the number of dead fish at higher temperatures may be associated with the expression of metabolic proteins, or even hypothetical proteins, which may be participating of the growth and dissemination of the bacteria in fish tissues, as well as, may be related to specific failures of host immunity or its metabolic adaptations during acclimatization at high temperature, as demonstrated in a transcriptomic study using tilapia kept at 32ºC or 22ºC and experimentally infected with GBS (WANG *et al.*, 2016). Moreover, the aquatic environment could favor the GBS strains, since acidic environment, observed in some fish farms (AMAL *et*

*al.*, 2015), when associated to a high water temperature allowed the attachment of pathogen to intestinal epithelium of tilapia after oral entry (BARATO *et al.*, 2016; IREGUI *et al.*, 2016) and increasing the bacterial load in this tissue, with further spread through the body of the infected fish.

### 5.5.3. LOW CORRELATION BETWEEN TRANSCRIPTOMIC AND PROTEOMIC DATASETS

A low relationship between transcriptomic and proteomic profiles was observed in our analysis. The correspondence among these datasets have been reported as low for different bacterial pathogen (DRESSAIRE *et al.*, 2010; MARGALEF-CATALÀ *et al.*, 2016), including bacteria of the *Streptococcus* genus (AHN *et al.*, 2017) due to the different regulatory mechanisms involved in gene expression and protein synthesis (HAIDER e PAL, 2013) and technical limitations among the approaches used (LUNDBERG *et al.*, 2010). Among the physiological mechanisms involved in this low correlation between transcriptome and proteome, half-lives of mRNAs and proteins, and post-transcriptional machinery as translation and protein degradation and modification have been described (HAIDER e PAL, 2013; AHN *et al.*, 2017). The half-times of mRNA in bacteria is short, varying from seconds to over an hour (LAALAMI *et al.*, 2014) and is much shorter than their corresponding proteins, which have half-live stable (greater than 20 hours) (DELLER *et al.*, 2016), being possible at detection of different regulation for the same gene/protein at transcript and protein level. In addition, regulatory factors may be responsible for the low correlation between transcriptomic and proteomic datasets, especially of protein nature, where some proteins were no detected in our analysis due of the limitations of capacity detection of method employed.

The lower relationship between transcriptomic and proteomic datasets in fish-adapted GBS fish strain may be related to fast and efficient adaptation in aquatic environment in the presence of different stress factors as temperature, for example, allowing a continuous modification in its repertoire of mRNAs and proteins during acclimation in this environment with constant temperature variation, by increasing of degradation or modification of damaged or unfolded proteins or decreasing the abundance of regulatory proteins, which together could culminate in growth inhibition or bacterial death,

### 5.5.4. EXTENSIVE PROTEOME DIFFERENCES BETWEEN FISH-ADAPTED AND HUMAN GBS STRAINS

The comparison of gene content and transcript expression between fish-adapted and human GBS strains were performed using genomic and transcriptomic analyses, and in both approaches the strains were distantly related (ROSINSKI-CHUPIN *et al.*, 2013). From proteomic analysis, a differential expression of proteins among these strains suggested a distinct adaptation between poikilotherm and homeotherm hosts at regulatory level (TAVARES *et al.*, 2018, under review). However, there are no studies which compare the influence of temperature on protein abundance among fish-adapted and human GBS strains. Here, we observed that the number of orthologous proteins shared between NEM316 and SA53 were similar, regardless of bacterial growth temperature. However, ~14% of these proteins presented difference of expression trends between the two strains with a threshold of 25%. Proteins involved in metabolism, especially those involved in coenzyme transport (H) were the ones that the most contributed to the differentiation of these strains at two temperatures evaluated. However, using the threshold of 50% (~3%), proteins related with replication, recombination and repair (L) and general function prediction only (R) contribute for differentiation of SA53 and NEM316 at 22ºC, while that translation, ribosomal structure and biogenesis (J) contribute at 32ºC. These results showed that despite the high similarity of the protein content, SA53 and NEM316 have different relative abundance for the same group of proteins in response to growth temperature, which may be related to distinct bacterial adaptation for these hosts at the proteome level.

## 5.6. CONCLUSIONS

In conclusion, the transcriptome and proteome of fish-adapted GBS strain are modulated by temperature. A greater variation in the expression of functional genome was observed in transcriptome analysis, especially at 32ºC. Our comparison analysis demonstrated that fish-adapted GBS strain regulates the differential expression of transcripts and proteins involved in metabolism and adaptation to aquatic environment and to fish host at low and high temperature conditions. The temperature does not seem to influence the differential expression of virulence factors, however, fish-adapted GBS strain can produce these factors at low and high temperature conditions, allowing the colonization of the aquatic host in both conditions. The differences in relative abundance for the same group of proteins between fish-adapted and human GBS strains suggest that the proteome regulation is related to the host specificity.

## 5.6. REFERENCES


ABRANCHES, J.; CHEN, Y.-Y. M.; BURNE, R. A. Characterization of *Streptococcus mutans* strains deficient in EIIAB (Man) of the sugar phosphotransferase system. *Appl. Environ. Microbiol.*, v. 69, n. 8, p. 4760-4769, 2003.

ADIN, D. M.; VISICK, K. L.; STABB, E. V. Identification of a cellobiose utilization gene cluster with cryptic β-Galactosidase activity in *Vibrio fischeri*. *Appl. Environ. Microbiol.*, v. 74, n. 13, p. 4059-4069, 2008.

AFZAL, M.; SHAFEEQ, S.; KUIPERS, O. P. Ascorbic acid-dependent gene expression in *Streptococcus pneumoniae* and the activator function of the transcriptional regulator UlaR2. *Front. Microbiol.*, v. 6, p. 72, 2015.

AFZAL, M.; SHAFEEQ, S.; KUIPERS, O. P. Methionine-mediated gene expression and characterization of the CmhR regulon in *Streptococcus pneumoniae*. *Microb. Genomics*, v. 2, n. 10, p. e000091, 2016.

AGUADO-URDA, M.; GIBELLO, A.; BLANCO, M. D. M. *et al.* Global transcriptome analysis of *Lactococcus garvieae* strains in response to temperature. *Plos One*, v. 8, n. 11, p. e79692, 2013.

AHN, S.-J.; GU, T.; KOH, J. *et al.* Remodeling of the *Streptococcus mutans* proteome in response to LrgAB and external stresses. *Sci. Rep.*, v. 7, n. 1, p. 14063, 2017.

AL-HARBI, A. H. Phenotypic and genotypic characterization of *Streptococcus agalactiae* isolated from hybrid tilapia (*Oreochromis niloticus* × *O. aureus*). *Aquaculture*, v. 464, n. Supplement C, p. 515-520, 2016.

AL SAFADI, R.; AMOR, S.; HERY-ARNAUD, G. *et al.* Enhanced expression of *lmb* gene encoding laminin-binding protein in *Streptococcus agalactiae* strains harboring IS1548 in *scpB-lmb* intergenic region. *Plos One*, v. 5, n. 5, p. e10794, 2010.

ÁLVAREZ-AÑORVE, L. I.; CALCAGNO, M. L.; PLUMBRIDGE, J. Why does *Escherichia coli* grow more slowly on glucosamine than on N-acetylglucosamine? Effects of enzyme levels and allosteric activation of GlcN6P deaminase (NagB) on growth rates. *J. Bacteriol.*, v. 187, n. 9, p. 2974-2982, 2005.

AMAL, M. N. A.; SAAD, M. Z.; ZAHRAH, A. S. *et al.* Water quality influences the presence of *Streptococcus agalactiae* in cage cultured red hybrid tilapia, *Oreochromis niloticus* × *Oreochromis mossambicus*. *Aquacult. Res.*, v. 46, n. 2, p. 313-323, 2015.

BANH, S.; WIENS, L.; SOTIRI, E. *et al.* Mitochondrial reactive oxygen species production by fish muscle mitochondria: potential role in acute heat-induced oxidative stress. *Comp. Biochem. Physiol. Part B: Biochem. Mol. Biol.*, v. 191, n. Supplement C, p. 99-107, 2016.

BARATO, P.; MARTINS, E. R.; MELO-CRISTINO, J. *et al.* Persistence of a single clone of *Streptococcus agalactiae* causing disease in tilapia (*Oreochromis* sp.) cultured in Colombia over 8 years. *J. Fish Dis.*, v. 38, n. 12, p. 1083-1087, 2015.

BARATO, P.; MARTINS, E. R.; VASQUEZ, G. M. *et al.* Capsule impairs efficient adherence of *Streptococcus agalactiae* to intestinal epithelium in tilapias *Oreochromis* sp.. *Microb. Pathog.*, v. 100, p. 30-36, 2016.

BARINOV, A.; LOUX, V.; HAMMANI, A. *et al.* Prediction of surface exposed proteins in *Streptococcus pyogenes*, with a potential application to other Gram-positive bacteria. *Proteomics*, v. 9, n. 1, p. 61-73, 2009.

BARONY, G. M.; TAVARES, G. C.; PEREIRA, F. L. *et al.* Large-scale genomic analyses reveal the population structure and evolutionary trends of *Streptococcus agalactiae* strains in Brazilian fish farms. *Sci. Rep.*, v. 7, n. 1, p. 13538, 2017.

BIDOSSI, A.; MULAS, L.; DECOROSI, F. *et al.* A functional genomics approach to establish the complement of carbohydrate transporters in *Streptococcus pneumoniae*. *Plos One*, v. 7, n. 3, p. e33320, 2012.

BOLTAÑA, S.; SANHUEZA, N.; AGUILAR, A. *et al.* Influences of thermal environment on fish growth. *Ecol. Evol.*, v. 7, n. 17, p. 6814-6825, 2017.

CHATTOPADHYAY, M. K.; RAGHU, G.; SHARMA, Y. V. R. K. *et al.* Increase in oxidative stress at low temperature in an Antarctic bacterium. *Curr. Microbiol.*, v. 62, n. 2, p. 544-546, 2011.

CHIDEROLI, R. T.; AMOROSO, N.; MAINARDI, R. M. *et al.* Emergence of a new multidrug-resistant and highly virulent serotype of *Streptococcus agalactiae* in fish farms from Brazil. *Aquaculture*, v. 479, p. 45-51, 2017.

DELANNOY, C. M.; CRUMLISH, M.; FONTAINE, M. C. *et al.* Human *Streptococcus agalactiae* strains in aquatic mammals and fish. *BMC Microbiol.*, v. 13, n. 1, p. 1-9, 2013.

DELLER, M. C.; KONG, L.; RUPP, B. Protein stability: a crystallographer's perspective. *Acta Crystallogr. Sect. F, Struct. Biol. Commun.*, v. 72, n. Pt 2, p. 72-95, 2016.

DI PALO, B.; RIPPA, V.; SANTI, I. *et al.* Adaptive response of Group B *Streptococcus* to high glucose conditions: new insights on the CovRS regulation network. *Plos One*, v. 8, n. 4, p. e61294, 2013.

DISTLER, U.; KUHAREV, J.; NAVARRO, P. *et al.* Drift time-specific collision energies enable deep-coverage data-independent acquisition proteomics. *Nat. Meth.*, v. 11, n. 2, p. 167-170, 2014.

DORAN, K. S.; ENGELSON, E. J.; KHOSRAVI, A. *et al.* Blood-brain barrier invasion by Group B *Streptococcus* depends upon proper cell-surface anchoring of lipoteichoic acid. *J. Clin. Investig.*, v. 115, n. 9, p. 2499-2507, 2005.

DRESSAIRE, C.; LAURENT, B.; LOUBIERE, P. *et al.* Linear covariance models to examine the determinants of protein levels in *Lactococcus lactis*. *Mol. Biosyst.*, v. 6, n. 7, p. 1255-64, 2010.

EHIRA, S.; TERAMOTO, H.; INUI, M. *et al.* Regulation of *Corynebacterium glutamicum* heat shock response by the extracytoplasmic-function sigma factor SigH and transcriptional regulators HspR and HrcA. *J. Bacteriol.*, v. 191, n. 9, p. 2964-2972, 2009.

EVANS, J. J.; BOHNSACK, J. F.; KLESIUS, P. H. *et al.* Phylogenetic relationships among *Streptococcus agalactiae* isolated from piscine, dolphin, bovine and human sources: a dolphin and piscine lineage associated with a fish epidemic in Kuwait is also associated with human neonatal infections in Japan. *J. Med. Microbiol.*, v. 57, n. 11, p. 1369-1376, 2008.

EVANS, J. J.; KLESIUS, P. H.; GILBERT, P. M. *et al.* Characterization of β-haemolytic Group B *Streptococcus agalactiae* in cultured seabream, *Sparus auratus* L., and wild mullet, *Liza klunzingeri* (Day), in Kuwait. *J. Fish Dis.*, v. 25, n. 9, p. 505-513, 2002.

EVANS, J. J.; PASNIK, D. J.; KLESIUS, P. H. Differential pathogenicity of five *Streptococcus agalactiae* isolates of diverse geographic origin in Nile tilapia (*Oreochromis niloticus* L.). *Aquacult. Res.*, v. 46, n. 10, p. 2374-2381, 2015.

EZRATY, B.; GENNARIS, A.; BARRAS, F. *et al.* Oxidative stress, protein damage and repair in bacteria. *Nat. Rev. Microbiol.*, v. 15, n. 7, p. 385-396, 2017.

FALCONI, M.; COLONNA, B.; PROSSEDA, G. *et al.* Thermoregulation of *Shigella* and *Escherichia coli* EIEC pathogenicity. A temperature-dependent structural transition of DNA modulates accessibility of *virF* promoter to transcriptional repressor H-NS. *EMBO J.*, v. 17, n. 23, p. 7033-7043, 1998.

FARALLA, C.; METRUCCIO, M. M.; DE CHIARA, M. *et al.* Analysis of two-component systems in Group B *Streptococcus* shows that RgfAC and the novel FspSR modulate virulence and bacterial fitness. *MBio*, v. 5, n. 3, p. e00870-14, 2014.

FAREWELL, A.; NEIDHARDT, F. C. Effect of temperature on *in vivo* protein synthetic capacity in *Escherichia coli*. *J. Bacteriol.*, v. 180, n. 17, p. 4704-4710, 1998.

FAYET, O.; ZIEGELHOFFER, T.; GEORGOPOULOS, C. The *groES* and *groEL* heat shock gene products of *Escherichia coli* are essential for bacterial growth at all temperatures. *J. Bacteriol.*, v. 171, n. 3, p. 1379-1385, 1989.

FLORINDO, C.; FERREIRA, R.; BORGES, V. *et al.* Selection of reference genes for real-time expression studies in *Streptococcus agalactiae*. *J. Microbiol. Meth.*, v. 90, n. 3, p. 220-227, 2012.

GADGIL, M.; KAPUR, V.; HU, W.-S. Transcriptional response of *Escherichia coli* to temperature shift. *Biotechnol. Prog.*, v. 21, n. 3, p. 689-699, 2005.

GALPERIN, M. Y.; MAKAROVA, K. S.; WOLF, Y. I. *et al.* Expanded microbial genome coverage and improved protein family annotation in the COG database. *Nucleic Acids Res.*, v. 43, n. D1, p. D261-D269, 2015.

GLASER, P.; RUSNIOK, C.; BUCHRIESER, C. *et al.* Genome sequence of *Streptococcus agalactiae*, a pathogen causing invasive neonatal disease. *Mol. Microbiol.*, v. 45, n. 6, p. 1499-1513, 2002.

GODOY, D. T.; CARVALHO-CASTRO, G. A.; LEAL, C. A. G. *et al.* Genetic diversity and new genotyping scheme for fish pathogenic *Streptococcus* aIgalactiae. *Lett. Appl. Microbiol.*, v. 57, n. 6, p. 476-483, 2013.

GOMEZ, E.; PEREZ-PASCUAL, D.; FERNANDEZ, L. *et al.* Construction and validation of a GFP-based vector for promoter expression analysis in the fish pathogen *Flavobacterium psychrophilum*. *Gene*, v. 497, n. 2, p. 263-8, 2012.

GOODRICH, T. D.; MORITA, R. Y. Low temperature inhibition on binding, transport, and incorporation of leucine, arginine, methionine, and histidine in *Escherichia coli*. *Z. Allg. Mikrobiol.*, v. 17, n. 2, p. 91-97, 1977.

GUIJARRO, J. A.; CASCALES, D.; GARCÍA-TORRICO, A. I. *et al.* Temperature-dependent expression of virulence genes in fish-pathogenic bacteria. *Front. Microbiol.*, v. 6, n. 700, 2015.

GUTKNECHT, R.; FLÜKIGER, K.; LANZ, R. *et al.* Mechanism of phosphoryl transfer in the dimeric IIABMan subunit of the *Escherichia coli* mannose transporter. *J. Biolog. Chem.*, v. 274, n. 10, p. 6091-6096, 1999.

HAIDER, S.; PAL, R. Integrated analysis of transcriptomic and proteomic data. *Curr. Genomics*, v. 14, n. 2, p. 91-110, 2013.

HAN, Y.; ZHOU, D.; PANG, X. *et al.* Microarray analysis of temperature-induced transcriptome of *Yersinia pestis*. *Microbiol. Immunol.*, v. 48, n. 11, p. 791-805, 2004.

HEBERLE, H.; MEIRELLES, G. V.; DA SILVA, F. R. *et al.* InteractiVenn: a web-based tool for the analysis of sets through Venn diagrams. *BMC Bioinformatics*, v. 16, n. 1, p. 1-7, 2015.

IREGUI, C. A.; COMAS, J.; VÁSQUEZ, G. M. *et al.* Experimental early pathogenesis of *Streptococcus agalactiae* infection in red tilapia *Oreochromis* spp. *J. Fish Dis.*, v. 39, n. 2, p. 205-215, 2016.

KARLYSHEV, A. V.; GALYOV, E. E.; SMIRNOV, O. *et al.* A new gene of the f1 operon of *Y. pestis* involved in the capsule biogenesis. *FEBS Lett.*, v. 297, n. 1-2, p. 77-80, 1992.

KAYANSAMRUAJ, P.; PIRARAT, N.; HIRONO, I. *et al.* Increasing of temperature induces pathogenicity of *Streptococcus agalactiae* and the up-regulation of inflammatory related genes in infected Nile tilapia (*Oreochromis niloticus*). *Vet. Microbiol.*, v. 172, n. 1–2, p. 265-271, 2014.

KOCHARUNCHITT, C.; KING, T.; GOBIUS, K. *et al.* Integrated transcriptomic and proteomic analysis of the physiological response of *Escherichia coli* O157:H7 sakai to steady-state conditions of cold and water activity stress. *Mol. Cell. Proteomics*, v. 11, n. 1, p. M111.009019, 2012.

KUHAREV, J.; NAVARRO, P.; DISTLER, U. *et al.* In-depth evaluation of software tools for data-independent acquisition based label-free quantification. *Proteomics*, v. 15, n. 18, p. 3140-3151, 2015.

LAALAMI, S.; ZIG, L.; PUTZER, H. Initiation of mRNA decay in bacteria. *Cell. Mol. Life Sci.*, v. 71, n. 10, p. 1799-1828, 2014.

LESTRATE, P.; DELRUE, R. M.; DANESE, I. *et al.* Identification and characterization of *in vivo* attenuated mutants of *Brucella melitensis*. *Mol. Microbiol.*, v. 38, n. 3, p. 543-551, 2000.

LI, G.-Z.; VISSERS, J. P. C.; SILVA, J. C. *et al.* Database searching and accounting of multiplexed precursor and product ion spectra from the data independent analysis of simple and complex peptide mixtures. *Proteomics*, v. 9, n. 6, p. 1696-1719, 2009.

LIU, S.; GRAHAM, J. E.; BIGELOW, L. *et al.* Identification of *Listeria monocytogenes* genes expressed in response to growth at low temperature. *Appl. Environ. Microbiol.*, v. 68, n. 4, p. 1697-1705, 2002.

LIU, Y.; BEYER, A.; AEBERSOLD, R. On the dependency of cellular protein levels on mRNA abundance. *Cell*, v. 165, n. 3, p. 535-50, 2016.

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods*, v. 25, n. 4, p. 402-408, 2001.

LU, D.; GRAYSON, P.; SCHULTEN, K. Glycerol conductance and physical asymmetry of the *Escherichia coli* glycerol facilitator GlpF. *Biophys. J.*, v. 85, n. 5, p. 2977-2987, 2003.

LUNDBERG, E.; FAGERBERG, L.; KLEVEBRING, D. *et al.* Defining the transcriptome and proteome in three functionally different human cell lines. *Mol. Syst. Biol.*, v. 6, p. 450-450, 2010.

MAINARDI, R. M.; LIMA JÚNIOR, E. A.; RIBEIRO JÚNIOR, J. C. *et al.* Complete genome sequence of *Streptococcus agalactiae* strain S25 isolated from peritoneal liquid of Nile tilapia. *Genome Announc.*, v. 4, n. 4, p. e00784-16, 2016.

MARCUSSO, P. F.; AGUINAGA, J. Y.; CLAUDIANO, G. S. *et al.* Influence of temperature on *Streptococcus agalactiae* infection in Nile tilapia. *Braz. J. Vet. Res. Anim. Sci.*, v. 52, n. 1, p. 57-62, 2015.

MARGALEF-CATALÀ, M.; ARAQUE, I.; BORDONS, A. *et al.* Transcriptomic and proteomic analysis of *Oenococcus oeni* adaptation to wine stress conditions. *Front. Microbiol.*, v. 7, p. 1554, 2016.

MATA, A. I.; GIBELLO, A.; CASAMAYOR, A. *et al.* Multiplex PCR assay for detection of bacterial pathogens associated with warm-water streptococcosis in fish. *Appl. Environ. Microbiol.*, v. 70, n. 5, p. 3183-7, 2004.

MAURELLI, A. T.; BLACKMON, B.; CURTISS, R. Temperature-dependent expression of virulence genes in *Shigella* species. *Infect. Immun.*, v. 43, n. 1, p. 195-201, 1984.

MÉNDEZ, J.; FERNÁNDEZ, L.; MENÉNDEZ, A. *et al.* A Chromosomally located *traHIJKCLMN* operon encoding a putative type IV secretion system is involved in the virulence of *Yersinia ruckeri*. *Appl. Environ. Microbiol.*, v. 75, n. 4, p. 937-945, 2009.

MEREGHETTI, L.; SITKIEWICZ, I.; GREEN, N. M. *et al.* Extensive adaptive changes occur in the transcriptome of *Streptococcus agalactiae* (Group B *Streptococcus*) in response to incubation with human blood. *Plos One*, v. 3, n. 9, p. e3143, 2008a.

MEREGHETTI, L.; SITKIEWICZ, I.; GREEN, N. M. *et al.* Remodeling of the *Streptococcus agalactiae* transcriptome in response to growth temperature. *Plos One*, v. 3, n. 7, p. e2785, 2008b.

MIAN, G. F.; GODOY, D. T.; LEAL, C. A. G. *et al.* Aspects of the natural history and virulence of *S. agalactiae* infection in Nile tilapia. *Vet. Microbiol.*, v. 136, n. 1–2, p. 180-183, 2009.

MOYANO, A. J.; TOBARES, R. A.; RIZZI, Y. S. *et al.* A long-chain flavodoxin protects *Pseudomonas aeruginosa* from oxidative stress and host bacterial clearance. *Plos Genet.*, v. 10, n. 2, p. e1004163, 2014.

MOYE, Z. D.; BURNE, R. A.; ZENG, L. Uptake and metabolism of N-acetylglucosamine and glucosamine by *Streptococcus mutans*. *Appl. Environ. Microbiol.*, v. 80, n. 16, p. 5053-5067, 2014.

NACHIN, L.; NANNMARK, U.; NYSTRÖM, T. Differential roles of the universal stress proteins of *Escherichia coli* in oxidative stress resistance, adhesion, and motility. *J. Bacteriol.*, v. 187, n. 18, p. 6265-6272, 2005.

NEDWELL, D. B. Effect of low temperature on microbial growth: lowered affinity for substrates limits growth at low temperature. *FEMS Microbiol. Ecol.*, v. 30, n. 2, p. 101-111, 1999.

PELLETIER, M.; LORTIE, L.-A.; FRENETTE, M. *et al.* The phosphoenolpyruvate:mannose phosphotransferase system of *Streptococcus salivarius*. Functional and biochemical characterization of IIABLMan and IIABHMan. *Biochem.*, v. 37, n. 6, p. 1604-1612, 1998.

PEREIRA, U. P.; SANTOS, A. R.; HASSAN, S. S. *et al.* Complete genome sequence of *Streptococcus agalactiae* strain SA20-06, a fish pathogen associated to meningoencephalitis outbreaks. *Stand. Genomic Sci.*, v. 8, n. 2, p. 188-97, 2013.

PHADTARE, S.; SEVERINOV, K. RNA remodeling and gene regulation by cold shock proteins. *RNA Biol.*, v. 7, n. 6, p. 788-795, 2010.

R CORE TEAM. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing 2013.

RAJAGOPAL, L. Understanding the regulation of Group B streptococcal virulence factors. *Future Microbiol.*, v. 4, n. 2, p. 201-221, 2009.

RAJAGOPAL, L.; VO, A.; SILVESTRONI, A. *et al.* Regulation of purine biosynthesis by a eukaryotic-type kinase in *Streptococcus agalactiae*. *Mol. Microbiol.*, v. 56, n. 5, p. 1329-1346, 2005.

RAPPUOLI, R.; ARICO, B.; SCARLATO, V. Thermoregulation and reversible differentiation in *Bordetella*: a model for pathogenic bacteria. *Mol. Microbiol.*, v. 6, n. 15, p. 2209-11, 1992.

RICHTER, K.; HASLBECK, M.; BUCHNER, J. The heat shock response: life on the verge of death. *Mol. Cell*, v. 40, n. 2, p. 253-266, 2010.

RODKHUM, C.; KAYANSAMRUAJ, P.; PIRARAT, N. Effect of water temperature on susceptibility to *Streptococcus agalactiae* serotype Ia infection in Nile tilapia (*Oreochromis niloticus*). *Thai J. Vet. Med.*, v. 41, n. 3, p. 309-314, 2011.

ROSINSKI-CHUPIN, I.; SAUVAGE, E.; MAIREY, B. *et al.* Reductive evolution in *Streptococcus agalactiae* and the emergence of a host adapted lineage. *BMC Genomics*, v. 14, n. 1, p. 1-15, 2013.

SALVADOR, R.; MULLER, E. E.; FREITAS, J. C. D. *et al.* Isolation and characterization of *Streptococcus* spp. Group B in Nile tilapias (*Oreochromis niloticus*) reared in hapas nets and earth nurseries in the northern region of Parana State, Brazil. *Cien. Rural*, v. 35, p. 1374-1378, 2005.

SAMEN, U.; GOTTSCHALK, B.; EIKMANNS, B. J. *et al.* Relevance of peptide uptake systems to the physiology and virulence of *Streptococcus agalactiae*. *J. Bacteriol.*, v. 186, n. 5, p. 1398-1408, 2004.

SATO, M.; KONDO, T.; YOSHINAKA, R. *et al.* Effect of water temperature on the skeletal deformity in ascorbic acid-deficient rainbow trout (*Salmo gairdnerii*). *Bull. Jap. Soc. Sci. Fish.*, v. 49, n. 3, p. 443-446, 1983.

SCHULZ, A.; SCHUMANN, W. *hrcA*, the first gene of the *Bacillus subtilis dnaK* operon encodes a negative regulator of class I heat shock genes. *J. Bacteriol.*, v. 178, n. 4, p. 1088-93, 1996.

SHAFEEQ, S.; KUIPERS, O. P.; KLOOSTERMAN, T. G. Cellobiose-mediated gene expression in *Streptococcus pneumoniae*: a repressor function of the novel GntR-type regulator BguR. *Plos One*, v. 8, n. 2, p. e57586, 2013.

SHANNON, P.; MARKIEL, A.; OZIER, O. *et al.* Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.*, v. 13, n. 11, p. 2498-2504, 2003.

SHELVER, D.; RAJAGOPAL, L.; HARRIS, T. O. *et al.* MtaR, a regulator of methionine transport, is critical for survival of Group B *Streptococcus in vivo*. *J. Bacteriol.*, v. 185, n. 22, p. 6592-6599, 2003.

SILVA, W. M.; CARVALHO, R. D.; SOARES, S. C. *et al.* Label-free proteomic analysis to confirm the predicted proteome of *Corynebacterium pseudotuberculosis* under nitrosative stress mediated by nitric oxide. *BMC Genomics*, v. 15, n. 1, p. 1065, 2014.

SILVA, W. M.; CARVALHO, R. D. D. O.; DORELLA, F. A. *et al.* Quantitative proteomic analysis reveals changes in the benchmark *Corynebacterium pseudotuberculosis* biovar Equi exoproteome after passage in a murine host. *Front. Cell. Infect. Microbiol.*, v. 7, n. 325, 2017.

SMIRNOVA, G. V.; ZAKIROVA, O. N.; OKTYABRSKII, O. N. The role of antioxidant systems in the cold stress response of *Escherichia coli*. *Microbiol.*, v. 70, n. 1, p. 45-50, 2001.

SMOOT, L. M.; SMOOT, J. C.; GRAHAM, M. R. *et al.* Global differential gene expression in response to growth temperature alteration in Group A *Streptococcus*. *Proc. Natl. Acad. Sci. USA*, v. 98, n. 18, p. 10416-10421, 2001.

SOLIMAN, A. K.; JAUNCEY, K.; ROBERTS, R. J. Water-soluble vitamin requirements of tilapia: ascorbic acid (vitamin C) requirement of Nile tilapia, *Oreochromis niloticus* (L.). *Aquacult. Res.*, v. 25, n. 3, p. 269-278, 1994.

SRINIVASA RAO, P. S.; YAMADA, Y.; TAN, Y. P. *et al.* Use of proteomics to identify novel virulence determinants that are required for *Edwardsiella tarda* pathogenesis. *Mol. Microbiol.*, v. 53, n. 2, p. 573-586, 2004.

STELDER, S. K.; MAHMUD, S. A.; DEKKER, H. L. *et al.* Temperature dependence of the proteome profile of the psychrotolerant pathogenic food spoiler *Bacillus weihenstephanensis* type strain WSBC 10204. *J. Proteome Res.*, v. 14, n. 5, p. 2169-2176, 2015.

SUNDBERG, L.-R.; KUNTTU, H. M. T.; VALTONEN, E. T. Starvation can diversify the population structure and virulence strategies of an environmentally transmitting fish pathogen. *BMC Microbiol.*, v. 14, p. 67-67, 2014.

SZKLARCZYK, D.; MORRIS, J. H.; COOK, H. *et al.* The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. *Nucleic Acids Res.*, v. 45, n. Database issue, p. D362-D368, 2017.

TAVARES, G. C.; PEREIRA, F. L.; BARONY, G. M. *et al.* Delineation of the pan-proteome of fish-pathogenic *Streptococcus agalactiae* strains using a label-free shotgun approach. **BMC Genomics**, 2018, under review.

TIAN, M.; QU, J.; HAN, X. *et al.* Microarray-based identification of differentially expressed genes in intracellular *Brucella abortus* within RAW264.7 cells. *Plos One*, v. 8, n. 8, p. e67014, 2013.

VARANO, M.; GASPARI, M.; QUIRINO, A. *et al.* Temperature-dependent regulation of the *Ochrobactrum anthropi* proteome. *Proteomics*, v. 16, n. 23, p. 3019-3024, 2016.

VARCAMONTI, M.; ARSENIJEVIC, S.; MARTIRANI, L. *et al.* Expression of the heat shock gene *clpL* of *Streptococcus thermophilus* is induced by both heat and cold shock. *Microb. Cell Fact.*, v. 5, p. 6-6, 2006.

VIERA, A. J.; GARRETT, J. M. Understanding interobserver agreement: the kappa statistic. *Fam. Med.*, v. 37, n. 5, p. 360-3, 2005.

VIZCAÍNO, J. A.; CSORDAS, A.; DEL-TORO, N. *et al.* 2016 update of the PRIDE database and its related tools. *Nucleic Acids Res.*, v. 44, n. D1, p. D447-D456, 2016.

VU, V. Q. *ggbiplot*: A *ggplot2* Based Biplot. R package version 0.55. 2011.Disponível em: < http://github.com/vqv/ggbiplot >. Acesso em: 15 dez. 2017.

WANG, L.; LIU, P.; WAN, Z. Y. *et al.* RNA-seq revealed the impairment of immune defence of tilapia against the infection of *Streptococcus agalactiae* with simulated climate warming. *Fish Shellfish Immunol.*, v. 55, p. 679-689, 2016.

WARNES, G. R.; BOLKER, B.; BONEBAKKER, L. *et al.* Various R programming tools for plotting data. R package version 3.0.1. 2016. Disponível em: <https://cran.r-project.org/web/packages/gplots/index.html>. Acesso em: 15 dez. 2017.

WHITING, G. C.; SUTCLIFFE, I. C.; RUSSELL, R. R. B. Metabolism of polysaccharides by the *Streptococcus mutans dexB* gene product. *Microbiol.*, v. 139, n. 9, p. 2019-2026, 1993.

YAMAMOTO, Y.; PARGADE, V.; LAMBERET, G. *et al.* The Group B *Streptococcus* NADH oxidase Nox-2 is involved in fatty acid biosynthesis during aerobic growth and contributes to virulence. *Mol. Microbiol.*, v. 62, n. 3, p. 772-785, 2006.

YANG, Q.; PORTER, A. J.; ZHANG, M. *et al.* The impact of pH and nutrient stress on the growth and survival of *Streptococcus agalactiae*. *Antonie van Leeuwenhoek*, v. 102, n. 2, p. 277-287, 2012.

YU, H. B.; KAUR, R.; LIM, S. *et al.* Characterization of extracellular proteins produced by *Aeromonas hydrophila* AH-1. *Proteomics*, v. 7, n. 3, p. 436-49, 2007.

ZAMRI-SAAD, M.; AMAL, M. N. A.; SITI-ZAHRAH, A. *et al.* Control and prevention of streptococcosis in cultured tilapia in Malaysia: a review. *Pertanika J. Trop. Agric. Sci.*, v. 37, n. 4, p. 389-410, 2014.

ZHANG, Y. I.; GRIFFITHS, M. W. Induced expression of the heat shock protein genes *uspA* and *grpE* during starvation at low temperatures and their influence on thermal resistance of *Escherichia coli* O157:H7. *J. Food Protect.*, v. 66, n. 11, p. 2045-2050, 2003.

ZHAO, X.-L.; HAN, Y.; REN, S.-T. *et al.* l-proline increases survival of tilapias infected by *Streptococcus agalactiae* in higher water temperature. *Fish Shellfish Immunol.*, v. 44, n. 1, p. 33-42, 2015.

TABLES

Table 1. Genes selected and primers used for validation of microarray results.

Table 2. Transcripts identified as differentially regulated at 32 ºC in comparison with 22 ºC.

Table 3. Virulence factors known for GBS strains and their corresponding identification and regulation at 32 ºC in comparison with 22 ºC in transcriptomic and proteomic analysis.

Table 4. Proteins identified as differentially regulated at 32 ºC in comparison with 22 ºC.

FIGURES LEGENDS

Figure 1. Heatmap analysis for biological triplicate of GBS strains tested at 22ºC and 32ºC, as evaluated by microarray (A) and LC-HDMS$^{E}$ (B).

Figure 2. Prediction of COG functional category of the DEGs (A) and DEPs (B) identified in SA53 strain at two temperatures tested. Blue bar represents down-regulation and red bar represents up-regulation.

Figure 3. Interaction networks of DEGs (A) and DEPs (B) identified in SA53 strain between the two temperatures. The red circle represents proteins up-regulated at 32ºC, while blue circles represents proteins down-regulated at 32ºC. (1) PTS systems; (2) ABC transport system; (3) Ascorbate and aldarate metabolism; (4) Purine metabolism; (5) metabolic pathways.

Figure 4. Dynamic range of the protein abundances found in SA53 strain between the two temperatures tested. The data are binned according to the log10 of their normalized abundance. Red circles represent proteins up-regulated at 32ºC. while blue circles represent proteins down-regulated at 32ºC. (A) Thermal proteins resistance; (B) Virulence proteins.

Figure 5. Comparison of protein abundance between SA53 and NEM316 strains at 22 ºC and 32 ºC. (A) at 22ºC with threshold of 25%; (B) at 22ºC with threshold of 50%; (C) at 32ºC with threshold of 25%; (D) at 22ºC with threshold of 50%.

Supplementary Figure 1. Quality assessment of replicates biological used in transcriptome analysis. (A) distributions of the intensities evaluated by box plot; (B) correlation analysis matrix. (C) PCA plot, red circles represent samples growth at 32ºC. while blue circles represent samples at 22ºC.

Supplementary Figure 2. Quality assessment of replicates biological used in proteome analysis. (A) normal distribution of 10 ppm error of the total identified peptides; (B) repeat rate indicating the number of times that an identified protein appears in the replicates: 3 of 3 (blue) and 2 of 3 (red). (C) PCA plot, red circles represent samples growth at 32ºC. while blue circles represent samples at 22ºC.

Supplementary Figure 3. Schematic summarizing the transcriptional (A) and proteomic (B) response of fish-adapted GBS strain to incubation at different temperatures. Red circles indicate up-regulation at 32ºC, while blue circles represent down-regulation at 32ºC.

Table 1. Genes selected and primers used for validation of microarray results.

| Primer | Product | Sequence (5'-3') | Product size (bp) | Microarray[a] | qRT-PCR[b] |
|---|---|---|---|---|---|
| SagaV-20_0110F | Pyridine nucleotide-disulphide oxidoreductase family protein | GAATTAGGGAGCAAAGTGACAG | 100 | 2.23 | 1.09 |
| SagaV-20_0110R | | TCTCTTCTAAATAATCTTGAGCCATCTCT | | | |
| SagaV-20_1663F | Methylenetetrahydrofolate reductase | TTGGGCATGGAATTATTAGGTTTT | 119 | -4.84 | -0.54 |
| SagaV-20_1663R | | GAAAGGAGTTTAAAGGAAGTCACATCA | | | |
| SagaV-53_0507F | Acetoin reductase | GCAATCAATGTCGGAGGAACTAT | 80 | 3.24 | 2.43 |
| SagaV-53_0507R | | TTTCCGCCATGACCTAATTCTC | | | |
| SagaV-53_1711F | 5-methyltetrahydropteroyltriglutamate | TGAAGCAGCCCTTCGAGAAG | 75 | -4.12 | -0.57 |
| SagaV-53_1711R | | GCCGCAACAGCATCATCTAA | | | |
| SagaV-gyrAF | DNA gyrase subunit A | GCAGTGAACGTGAACCTCTTGTT | 99 | Reference gene | |
| SagaV-gyrAR | | GGTGGAATATTTGTTGCCATACCT | | | |
| SagaV-recAF | Protein RecA | AAAACAACGGTTGCCCTTCA | 99 | Reference gene | |
| SagaV-recAR | | CAGCATAGGCTGGGTCAAGAG | | | |

a Genes with log2 ≥ 1 = up-regulated; or ≤ -1 = down-regulated

b ΔΔCt value

Table 2. Transcripts identified as differentially regulated at 32 ºC in comparison with 22 ºC.

| Accession | Gene | Fold Change | Regulation | Functional Category | Subcellular Localization |
|---|---|---|---|---|---|
| SaSA53_0098 | Aspartokinase | -2.53 | Down | E | CYT |
| SaSA53_0175 | Phosphoribosylformylglycinamidine synthase | -2.28 | Down | F | CYT |
| SaSA53_0177 | purM Phosphoribosylformylglycinamidine cyclo-ligase | -2.08 | Down | F | CYT |
| SaSA53_0180 | purH Bifunctional purine biosynthesis protein | -2.02 | Down | F | CYT |
| SaSA53_0194 | purD Phosphoribosylamine--glycine ligase | -2.15 | Down | F | CYT |
| SaSA53_0196 | purK N5-carboxyaminoimidazole ribonucleotide synthase | -2.11 | Down | F | CYT |
| SaSA53_0583 | Phosphate ABC transporter ATP-binding protein | -4.17 | Down | P | CYT |
| SaSA53_0584 | Membrane protein | -4.20 | Down | P | MEM |
| SaSA53_0827 | noxE NADH oxidase | -2.10 | Down | I | CYT |
| SaSA53_0850 | Hypothetical protein | -2.02 | Down | K | CYT |
| SaSA53_0860 | Ribosomal RNA small subunit methyltransferase B | -2.07 | Down | J | MEM |
| SaSA53_0930 | apbE Thiamine biosynthesis protein | -2.09 | Down | H | CYT |
| SaSA53_0931 | NADPH-dependent FMN reductase | -2.11 | Down | C | CYT |
| SaSA53_0932 | NADPH-dependent FMN reductase | -2.12 | Down | S | CYT |
| SaSA53_0936 | guaC GMP reductase | -2.00 | Down | F | CYT |
| SaSA53_1044 | Branched-chain amino acid ABC transporter permease | -2.04 | Down | R | MEM |
| SaSA53_1244 | Glutamine ABC transporter permease | -2.18 | Down | ET | PSE |
| SaSA53_1245 | Peptide ABC transporter ATP-binding protein | -2.30 | Down | E | CYT |
| SaSA53_1300 | Peptidylprolyl isomerase | -2.27 | Down | O | PSE |
| SaSA53_1432 | Isochorismatase | -2.06 | Down | HR | CYT |
| SaSA53_1640 | Adhesion protein | -2.25 | Down | P | PSE |
| SaSA53_1651 | PTS mannose transporter subunit IID | -4.15 | Down | G | MEM |
| SaSA53_1652 | PTS mannose transporter subunit IIC | -4.18 | Down | G | MEM |
| SaSA53_1653 | PTS mannose transporter subunit IIB | -4.77 | Down | G | CYT |
| SaSA53_1654 | PTS mannose transporter subunit IIA | -5.44 | Down | G | CYT |
| SaSA53_1706 | cAMP factor | -2.04 | Down | R | SEC |
| SaSA53_1710 | metF Methylenetetrahydrofolate reductase | -4.84 | Down | E | CYT |
| SaSA53_1711 | metE 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase | -4.04 | Down | E | CYT |
| SaSA53_1735 | ABC transporter ATP-binding protein | -3.81 | Down | R | CYT |
| SaSA53_1736 | ABC transporter permease | -3.74 | Down | R | MEM |
| SaSA53_1737 | ABC transporter substrate-binding protein | -2.96 | Down | R | PSE |
| SaSA53_1745 | nrdD Anaerobic ribonucleoside-triphosphate reductase | -2.16 | Down | F | CYT |
| SaSA53_0015 | Hypothetical protein | 2.05 | Up | Q | CYT |
| SaSA53_0090 | PTS cellobiose transporter subunit IIA | 5.62 | Up | G | CYT |
| SaSA53_0091 | Cytochrome C biogenesis protein CcmE | 5.88 | Up | G | SEC |
| SaSA53_0092 | PTS system. cellobiose-specific IIC component | 7.00 | Up | G | MEM |
| SaSA53_0095 | Competence protein | 2.02 | Up | L | CYT |
| SaSA53_0100 | Enoyl-CoA hydratase | 3.20 | Up | I | CYT |
| SaSA53_0104 | 2-nitropropane dioxygenase | 2.07 | Up | R | CYT |
| SaSA53_0105 | fabD Malonyl CoA-acyl carrier protein transacylase | 2.08 | Up | I | CYT |
| SaSA53_0265 | Single-stranded DNA-binding protein | 2.10 | Up | L | CYT |
| SaSA53_0315 | comX Competence-specific sigma factor | 2.11 | Up | K | CYT |
| SaSA53_0319 | hrcA Heat-inducible transcription repressor | 2.13 | Up | K | CYT |
| SaSA53_0320 | grpE Protein | 2.03 | Up | O | CYT |
| SaSA53_0336 | Pyridine nucleotide-disulfide oxidoreductase family protein | 2.11 | Up | C | CYT |
| SaSA53_0344 | Permease | 2.12 | Up | V | MEM |
| SaSA53_0373 | oppC Oligopeptide transport system permease protein | 2.17 | Up | EP | MEM |
| SaSA53_0374 | oppD Oligopeptide transport ATP-binding protein | 2.17 | Up | EP | CYT |
| SaSA53_0375 | oppF Oligopeptide transport ATP-binding protein | 2.24 | Up | E | CYT |
| SaSA53_0387 | Hypothetical protein | 2.14 | Up | R | CYT |
| SaSA53_0472 | MutT/nudix family protein | 2.75 | Up | V | CYT |
| SaSA53_0477 | Phosphoglucomutase | 2.19 | Up | G | CYT |
| SaSA53_0507 | Acetoin reductase | 2.49 | Up | IQR | CYT |
| SaSA53_0551 | Hypothetical protein | 2.04 | Up | M | CYT |
| SaSA53_0581 | DNA-entry nuclease | 2.57 | Up | L | SEC |
| SaSA53_0589 | Glucuronide permease | 4.04 | Up | G | MEM |
| SaSA53_0590 | 2-dehydro-3-deoxygluconokinase | 2.23 | Up | G | CYT |
| SaSA53_0593 | uxaC Uronate isomerase | 2.98 | Up | G | CYT |
| SaSA53_0594 | uxuA Mannonate dehydratase | 2.01 | Up | G | CYT |
| SaSA53_0660 | MFS transporter | 2.26 | Up | P | MEM |
| SaSA53_0691 | nagB Glucosamine-6-phosphate deaminase | 2.21 | Up | G | CYT |
| SaSA53_0801 | msrB Peptide methionine sulfoxide reductase | 2.07 | Up | O | CYT |
| SaSA53_0919 | Hypothetical protein | 2.09 | Up | P | MEM |
| SaSA53_0954 | Hypothetical protein | 5.49 | Up | I | CYT |

| | | | | | |
|---|---|---|---|---|---|
| SaSA53_0955 | Hypothetical protein | 5.92 | Up | G | MEM |
| SaSA53_0956 | Hypothetical protein | 7.56 | Up | G | MEM |
| SaSA53_0957 | Hypothetical protein | 7.95 | Up | R | CYT |
| SaSA53_0982 | BCCT family transporter | 2.07 | Up | M | MEM |
| SaSA53_1114 | ABC transporter | 2.09 | Up | V | CYT |
| SaSA53_1215 | Ammonium transporter | 2.24 | Up | P | MEM |
| SaSA53_1251 | Cell wall surface anchor protein | 2.04 | Up | - | PSE |
| SaSA53_1268 | Transglutaminase | 2.18 | Up | D | CYT |
| SaSA53_1269 | Hypothetical protein | 3.01 | Up | P | CYT |
| SaSA53_1281 | Hypothetical protein | 2.22 | Up | S | PSE |
| SaSA53_1287 | Hypothetical protein | 3.84 | Up | EP | CYT |
| SaSA53_1288 | Peptide ABC transporter ATP-binding protein | 3.49 | Up | EP | CYT |
| SaSA53_1289 | Peptide ABC transporter permease | 4.11 | Up | EP | MEM |
| SaSA53_1290 | Peptide ABC transporter permease | 4.59 | Up | EP | MEM |
| SaSA53_1291 | Nickel ABC transporter substrate-binding protein | 3.09 | Up | E | PSE |
| SaSA53_1373 | Amidase | 2.26 | Up | J | CYT |
| SaSA53_1406 | Dihydroxyacetone kinase | 2.21 | Up | K | CYT |
| SaSA53_1407 | Dihydroxyacetone kinase subunit K | 2.99 | Up | G | CYT |
| SaSA53_1408 | Hypothetical protein | 2.84 | Up | R | CYT |
| SaSA53_1409 | PTS mannose transporter subunit IID | 2.89 | Up | T | CYT |
| SaSA53_1410 | Glycerol transporter | 2.09 | Up | G | MEM |
| SaSA53_1412 | Hypothetical protein | 2.45 | Up | G | CYT |
| SaSA53_1431 | 3-hydroxybutyryl-CoA dehydrogenase | 2.90 | Up | I | CYT |
| SaSA53_1435 | Universal stress protein | 2.16 | Up | T | CYT |
| SaSA53_1460 | Multidrug MFS transporter | 3.00 | Up | GEPR | MEM |
| SaSA53_1469 | trx Thioredoxin | 2.01 | Up | O | CYT |
| SaSA53_1518 | Acid phosphatase | 3.63 | Up | R | PSE |
| SaSA53_1549 | Glycine/betaine ABC transporter permease | 2.74 | Up | E | PSE |
| SaSA53_1550 | Glycine/betaine ABC transporter ATP-binding protein | 3.33 | Up | E | CYT |
| SaSA53_1561 | tal Transaldolase | 2.30 | Up | G | CYT |
| SaSA53_1564 | ulaD 3-keto-L-gulonate-6-phosphate decarboxylase | 2.93 | Up | G | CYT |
| SaSA53_1565 | PTS ascorbate transporter subunit IIA | 4.00 | Up | GT | CYT |
| SaSA53_1566 | PTS ascorbate transporter subunit IIB | 4.49 | Up | G | CYT |
| SaSA53_1567 | PTS ascorbate transporter subunit IIC | 3.76 | Up | G | MEM |
| SaSA53_1598 | PTS system N-acetylgalactosamine-specific transporter subunit IIC | 2.45 | Up | G | MEM |
| SaSA53_1599 | PTS system N-acetylgalactosamine-specific transporter subunit IIB | 2.12 | Up | G | CYT |
| SaSA53_1600 | Glucuronyl hydrolase | 2.47 | Up | G | CYT |
| SaSA53_1601 | PTS system N-acetylgalactosamine-specific transporter subunit IIA | 2.36 | Up | G | CYT |
| SaSA53_1602 | Gluconate 5-dehydrogenase | 2.93 | Up | IQR | CYT |
| SaSA53_1603 | Hypothetical protein | 3.79 | Up | G | CYT |
| SaSA53_1604 | 2-keto-3-deoxygluconate kinase | 3.87 | Up | G | CYT |
| SaSA53_1605 | 2-dehydro-3-deoxyphosphogluconate aldolase | 2.45 | Up | G | CYT |
| SaSA53_1791 | sdhA L-serine dehydratase. iron-sulfur-dependent. alpha subunit | 2.02 | Up | E | CYT |

Table 3. Virulence factors known for GBS strains and their corresponding identification and regulation at 32 ºC in comparison with 22 ºC in transcriptomic and proteomic analysis.

| Virulence factor | Accession | Transcript detected[a] | Regulation[c] | Protein detected[b] | Regulation[c] |
|---|---|---|---|---|---|
| **Adhesion** | | | | | |
| Elongation factor Tu | SaSA53_0653 | Yes | NS[d] | Yes | NS |
| Cell wall surface anchor protein | SaSA53_0662 | Yes | Unchanged | Yes | NS |
| GapC | SaSA53_1519 | Yes | NS | Yes | NS |
| GapN | SaSA53_0716 | Yes | NS | Yes | Unchanged |
| PavA | SaSA53_1046 | Yes | NS | Yes | NS |
| BibA | SaSA53_1722 | Yes | Unchanged | No | |
| FbsA | SaSA53_0903 | Yes | Unchanged | No | |
| Lmb | SaSA53_1640 | Yes | Down | Yes | NS |
| Pi-2b | SaSA53_1187 | Yes | NS | No | |
| **Invasion** | | | | | |
| CylE | SaSA53_0568 | No | | No | |
| cAMP factor | SaSA53_1706 | Yes | Down | Yes | Up |
| Hemolysin A | SaSA53_0483 | Yes | NS | Yes | NS |
| Eno | SaSA53_0533 | Yes | NS | Yes | NS |
| IagA | SaSA53_0601 | Yes | NS | Yes | Up |
| Internalin | SaSA53_0799 | Yes | NS | No | |
| HylB | SaSA53_1053 | Yes | Unchanged | Yes | NS |
| NoxE | SaSA53_0827 | Yes | Down | Yes | NS |
| **Immune evasion** | | | | | |
| Capsular polysaccharide – CpsG | SaSA53_1029 | Yes | NS | Yes | NS |
| Capsular polysaccharide – CpsF | SaSA53_1030 | Yes | NS | Yes | NS |
| Capsular polysaccharide – CpsE | SaSA53_1031 | Yes | NS | Yes | NS |
| Capsular polysaccharide – CpsD | SaSA53_1032 | Yes | NS | Yes | NS |
| Capsular polysaccharide – CpsC | SaSA53_1033 | Yes | Unchanged | Yes | NS |
| Capsular polysaccharide – CpsB | SaSA53_1034 | Yes | Unchanged | Yes | Up |
| SodA | SaSA53_0678 | Yes | Unchanged | Yes | NS |
| ScpB | SaSA53_0381 | Yes | NS | Yes | Down |
| Group B antigen – RmlB | SaSA53_1054 | Yes | NS | Yes | NS |
| Group B antigen – RmlC | SaSA53_1055 | Yes | NS | Yes | NS |
| Group B antigen – RmlA | SaSA53_1056 | Yes | Unchanged | Yes | NS |
| Sip | SaSA53_0182 | Yes | NS | Yes | NS |
| Serine protease | SaSA53_1720 | Yes | NS | Yes | NS |
| **Multidrug resistance** | | | | | |
| DltD | SaSA53_1540 | Yes | NS | Yes | NS |
| DltB | SaSA53_1542 | Yes | NS | Yes | NS |
| DltA | SaSA53_1543 | Yes | NS | Yes | NS |
| Pbp2A | SaSA53_1724 | Yes | Unchanged | Yes | NS |
| PbpX | SaSA53_0050 | Yes | Unchanged | Yes | NS |
| Pbp1A | SaSA53_0061 | Yes | NS | Yes | NS |
| Pbp2B | SaSA53_0656 | Yes | NS | Yes | NS |
| Beta-lactamase | SaSA53_0562 | Yes | NS | Yes | NS |

[a] Transcripts identified in microarray analysis

[b] Proteins identified in LC-HDMS$^E$ analysis

[c] Transcript or protein with $p < 0.05$ and differential expression (down, up or unchanged) or $p > 0.05$ (considered detected but not significant - NS)

Table 4. Proteins identified as differentially regulated at 32 ºC in comparison with 22 ºC.

| Accession | Peptides | Score | Product | $Log_2$_ratio | Regulation | Functional category | Subcellular localization |
|---|---|---|---|---|---|---|---|
| SaSA53_0162 | 7 | 39.04 | rnpA Ribonuclease P protein component | -2.12 | Down | J | CYT |
| SaSA53_0262 | 4 | 29.12 | Thioredoxin | -1.48 | Down | O | CYT |
| SaSA53_0373 | 5 | 29.93 | oppC Oligopeptide transport system permease protein | -2.03 | Down | EP | MEM |
| SaSA53_0381 | 20 | 111.21 | Reticulocyte binding protein | -4.38 | Down | D | PSE |
| SaSA53_0468 | 4 | 20.23 | RNA-binding protein | -3.26 | Down | R | CYT |
| SaSA53_0494 | 11 | 62.19 | UDP-N-acetylmuramoylpentapeptide-lysine N(6)-alanyltransferase MurM | -1.45 | Down | M | CYT |
| SaSA53_0504 | 27 | 167.67 | ftsX Cell division protein | -1.16 | Down | D | PSE |
| SaSA53_0506 | 7 | 44.70 | Hypothetical protein | -1.56 | Down | R | CYT |
| SaSA53_0535 | 20 | 107.34 | aroA 3-phosphoshikimate 1-carboxyvinyltransferase | -1.36 | Down | E | CYT |
| SaSA53_0591 | 22 | 161.80 | GntR family transcriptional regulator | -1.76 | Down | K | CYT |
| SaSA53_0621 | 31 | 227.07 | N5.N10-methylenetetrahydromethanopterin reductase | -1.06 | Down | HR | CYT |
| SaSA53_0627 | 14 | 95.62 | lgt Prolipoprotein diacylglyceryl transferase | -1.82 | Down | M | MEM |
| SaSA53_0667 | 67 | 570.69 | RNA helicase | -1.06 | Down | L | CYT |
| SaSA53_0689 | 25 | 183.91 | queA S-adenosylmethionine:tRNA ribosyltransferase-isomerase | -1.32 | Down | J | CYT |
| SaSA53_0758 | 60 | 443.06 | atpG ATP synthase gamma chain | -1.08 | Down | C | CYT |
| SaSA53_0767 | 94 | 702.74 | pheT Phenylalanyl--tRNA ligase beta subunit | -1.79 | Down | J | CYT |
| SaSA53_0837 | 11 | 57.66 | GNAT family acetyltransferase | -1.61 | Down | J | CYT |
| SaSA53_0855 | 45 | 342.98 | Phosphate import ATP-binding protein PstB | -1.60 | Down | P | CYT |
| SaSA53_0857 | 6 | 30.89 | Phosphate ABC transporter. permease protein PstA | -1.06 | Down | P | PSE |
| SaSA53_0910 | 5 | 28.06 | Appr-1-p processing protein | -4.60 | Down | J | CYT |
| SaSA53_1178 | 23 | 151.58 | Hypothetical protein | -1.47 | Down | S | CYT |
| SaSA53_1182 | 5 | 26.28 | SAM-dependent methyltransferase | -6.22 | Down | J | CYT |
| SaSA53_1276 | 14 | 92.26 | ybeY Endoribonuclease | -2.07 | Down | J | CYT |
| SaSA53_1291 | 8 | 39.72 | Nickel ABC transporter substrate-binding protein | -1.69 | Down | E | PSE |
| SaSA53_1348 | 10 | 66.78 | tRNA (cytidine(34)-2'-O)-methyltransferase | -1.21 | Down | J | CYT |
| SaSA53_1381 | 30 | 208.69 | Primosomal protein DnaI | -1.34 | Down | L | CYT |
| SaSA53_1392 | 9 | 53.24 | Cobalt ABC transporter permease | -3.71 | Down | H | MEM |
| SaSA53_1476 | 25 | 131.84 | ATP-dependent DNA helicase RecD-like protein | -2.08 | Down | L | CYT |
| SaSA53_1484 | 6 | 46.76 | Glycerol uptake permease | -3.88 | Down | G | MEM |
| SaSA53_1487 | 6 | 33.13 | Crp/Fnr family transcriptional regulator | -2.18 | Down | T | CYT |
| SaSA53_1504 | 22 | 140.11 | Hypothetical protein | -1.28 | Down | R | CYT |
| SaSA53_1522 | 30 | 242.33 | rpsG 30S ribosomal protein S7 | -1.49 | Down | J | CYT |
| SaSA53_1580 | 113 | 845.26 | ATP-dependent Clp protease ATP-binding protein | -1.49 | Down | O | CYT |
| SaSA53_1624 | 10 | 54.84 | dexB Glucan 1.6-alpha-glucosidase | -2.94 | Down | G | CYT |
| SaSA53_1675 | 30 | 187.15 | Ribosomal RNA small subunit methyltransferase E | -1.27 | Down | J | CYT |
| SaSA53_1679 | 7 | 47.08 | GNAT family acetyltransferase | -1.86 | Down | J | CYT |
| SaSA53_1734 | 18 | 190.32 | groS 10 kDa chaperonin | -1.17 | Down | O | CYT |
| SaSA53_0041 | 77 | 778.26 | tkt Transketolase | 1.42 | Up | G | CYT |
| SaSA53_0177 | 14 | 90.81 | purM Phosphoribosylformylglycinamidine cyclo-ligase | 1.88 | Up | F | CYT |
| SaSA53_0180 | 44 | 342.70 | purH Bifunctional purine biosynthesis protein | 1.33 | Up | F | CYT |
| SaSA53_0196 | 16 | 108.09 | purK N5-carboxyaminoimidazole ribonucleotide synthase | 1.23 | Up | F | CYT |
| SaSA53_0197 | 22 | 136.77 | Hypothetical protein | 1.62 | Up | O | CYT |
| SaSA53_0268 | 5 | 24.57 | lytR Sensory transduction protein | 3.73 | Up | KT | CYT |
| SaSA53_0358 | 39 | 322.36 | Amino acid ABC transporter ATP-binding protein | 1.23 | Up | E | CYT |
| SaSA53_0466 | 17 | 142.16 | sepF Cell division protein | 1.54 | Up | D | CYT |
| SaSA53_0478 | 28 | 186.28 | folD Bifunctional protein | 1.14 | Up | H | CYT |
| SaSA53_0481 | 12 | 118.73 | xseB Exodeoxyribonuclease 7 small subunit | 1.69 | Up | L | CYT |
| SaSA53_0522 | 5 | 74.86 | Flavodoxin | 1.79 | Up | C | CYT |
| SaSA53_0601 | 15 | 92.29 | Family 1 glycosyl transferase | 2.04 | Up | M | CYT |
| SaSA53_0730 | 5 | 44.15 | Hypothetical protein | 1.75 | Up | M | SEC |
| SaSA53_0736 | 36 | 274.28 | Phosphomethylpyrimidine kinase | 1.73 | Up | H | CYT |
| SaSA53_0740 | 5 | 32.94 | GNAT family acetyltransferase | 1.05 | Up | JO | CYT |
| SaSA53_0811 | 106 | 1000.67 | Pyruvate kinase | 1.31 | Up | G | CYT |
| SaSA53_0815 | 5 | 26.89 | Amino acid ABC transporter permease | 1.61 | Up | E | MEM |
| SaSA53_0867 | 14 | 71.90 | Hypothetical protein | 1.58 | Up | S | CYT |
| SaSA53_0890 | 7 | 90.07 | ESAT-6-like protein | 1.00 | Up | S | CYT |
| SaSA53_0896 | 6 | 43.51 | pyrC Dihydroorotase | 1.53 | Up | F | CYT |
| SaSA53_0932 | 10 | 63.85 | NADPH-dependent FMN reductase | 1.83 | Up | S | CYT |
| SaSA53_0975 | 19 | 117.47 | folP Dihydropteroate synthase | 1.10 | Up | H | CYT |
| SaSA53_1034 | 32 | 245.18 | cpsB Tyrosine-protein phosphatase | 1.07 | Up | T | CYT |
| SaSA53_1091 | 3 | 23.67 | Amino acid transporter | 1.89 | Up | E | MEM |
| SaSA53_1101 | 39 | 260.34 | 3-hydroxy-3-methylglutaryl coenzyme A reductase | 1.12 | Up | I | CYT |
| SaSA53_1156 | 22 | 219.87 | Hypothetical protein | 1.04 | Up | T | CYT |
| SaSA53_1239 | 7 | 33.05 | Beta-1,6-galactofuranosyltransferase | 1.35 | Up | M | CYT |
| SaSA53_1245 | 47 | 463.68 | Peptide ABC transporter ATP-binding protein | 1.02 | Up | E | CYT |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | Tetracenomycin polyketide synthesis O-methyltransferase | | | | |
| SaSA53_1277 | 6 | 28.64 | TcmP | 1.03 | Up | Q | CYT |
| SaSA53_1325 | 2 | 11.05 | PTS lactose transporter subunit IIC | 1.19 | Up | G | MEM |
| SaSA53_1346 | 30 | 281.46 | upp Uracil phosphoribosyltransferase | 1.21 | Up | F | CYT |
| SaSA53_1383 | 9 | 84.65 | nrdR Transcriptional repressor | 1.05 | Up | K | CYT |
| SaSA53_1416 | 6 | 37.90 | SAM-dependent methyltransferase | 1.36 | Up | H | CYT |
| SaSA53_1440 | 27 | 181.27 | recG ATP-dependent DNA helicase | 1.57 | Up | L | CYT |
| SaSA53_1469 | 23 | 221.64 | trx Thioredoxin | 2.64 | Up | O | CYT |
| SaSA53_1502 | 10 | 77.30 | Hypothetical protein | 1.52 | Up | L | CYT |
| SaSA53_1585 | 19 | 157.05 | Alkyl hydroperoxide reductase subunit C | 1.05 | Up | V | CYT |
| SaSA53_1641 | 11 | 65.99 | dtd D-aminoacyl-tRNA deacylase | 2.00 | Up | J | CYT |
| SaSA53_1682 | 7 | 52.91 | Hypothetical protein | 1.28 | Up | E | CYT |
| SaSA53_1706 | 7 | 40.62 | cAMP factor | 1.46 | Up | R | SEC |
| SaSA53_1735 | 48 | 374.57 | ABC transporter ATP-binding protein | 1.82 | Up | R | CYT |
| SaSA53_1737 | 54 | 484.23 | ABC transporter substrate-binding protein | 2.18 | Up | R | PSE |
| SaSA53_1796 | 9 | 46.73 | Energy-coupling factor transporter ATP-binding protein EcfA1 | 1.26 | Up | PR | CYT |
| SaSA53_1812 | 34 | 354.77 | arcC Carbamate kinase | 1.26 | Up | E | CYT |
| SaSA53_0353 | 2 | 17.02 | Hypothetical protein | | Exc. 32ºC | S | CYT |
| SaSA53_1139 | 5 | 32.18 | Macrolide ABC transporter ATP-binding protein | | Exc. 32ºC | M | CYT |
| SaSA53_1378 | 6 | 35.03 | Hypothetical protein | | Exc. 22ºC | R | CYT |

Figure 1. Heatmap analysis for biological triplicate of GBS strains tested at 22ºC and 32ºC, as evaluated by microarray (A) and LC-HDMS$^{E}$ (B).

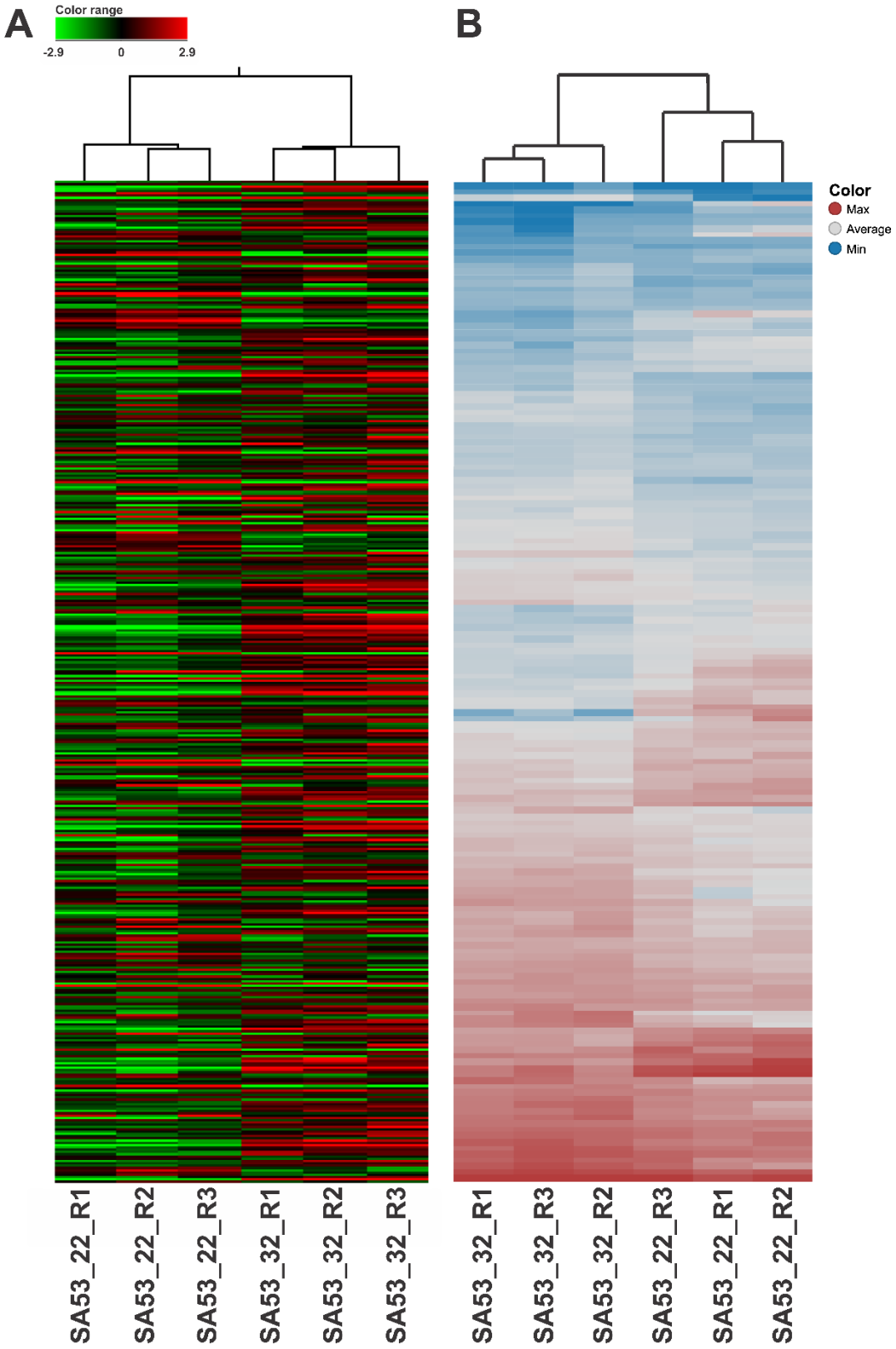


Figure 2. Prediction of COG functional category of the DEGs (A) and DEPs (B) identified in SA53 strain at two temperatures tested. Blue bar represents down-regulation and red bar represents up-regulation.

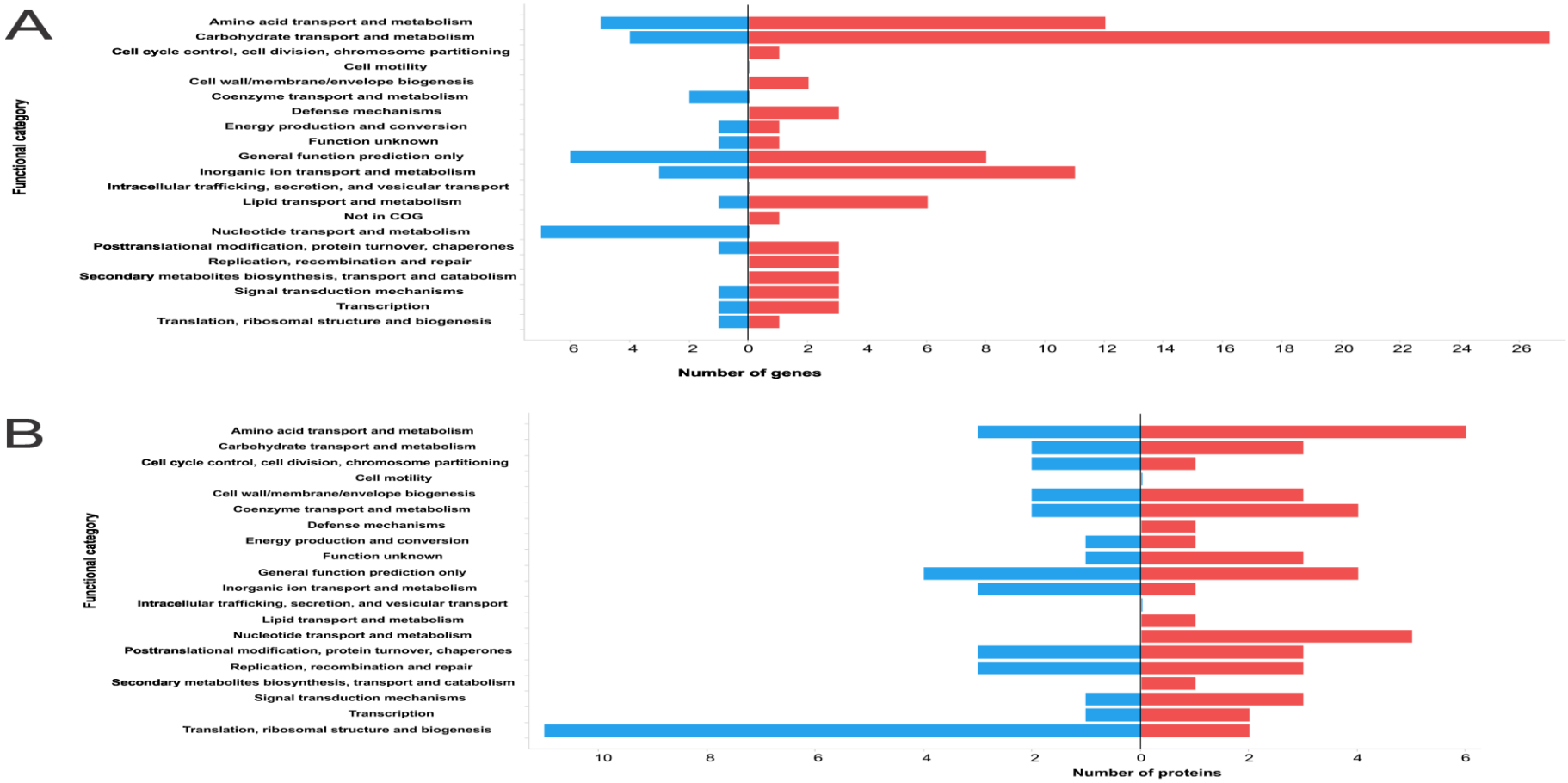


Figure 3. Interaction networks of DEGs (A) and DEPs (B) identified in SA53 strain between the two temperatures. The red circle represents proteins up-regulated at 32ºC, while blue circles represents proteins down-regulated at 32ºC. (1) PTS systems; (2) ABC transport system; (3) Ascorbate and aldarate metabolism; (4) Purine metabolism; (5) metabolic pathways.

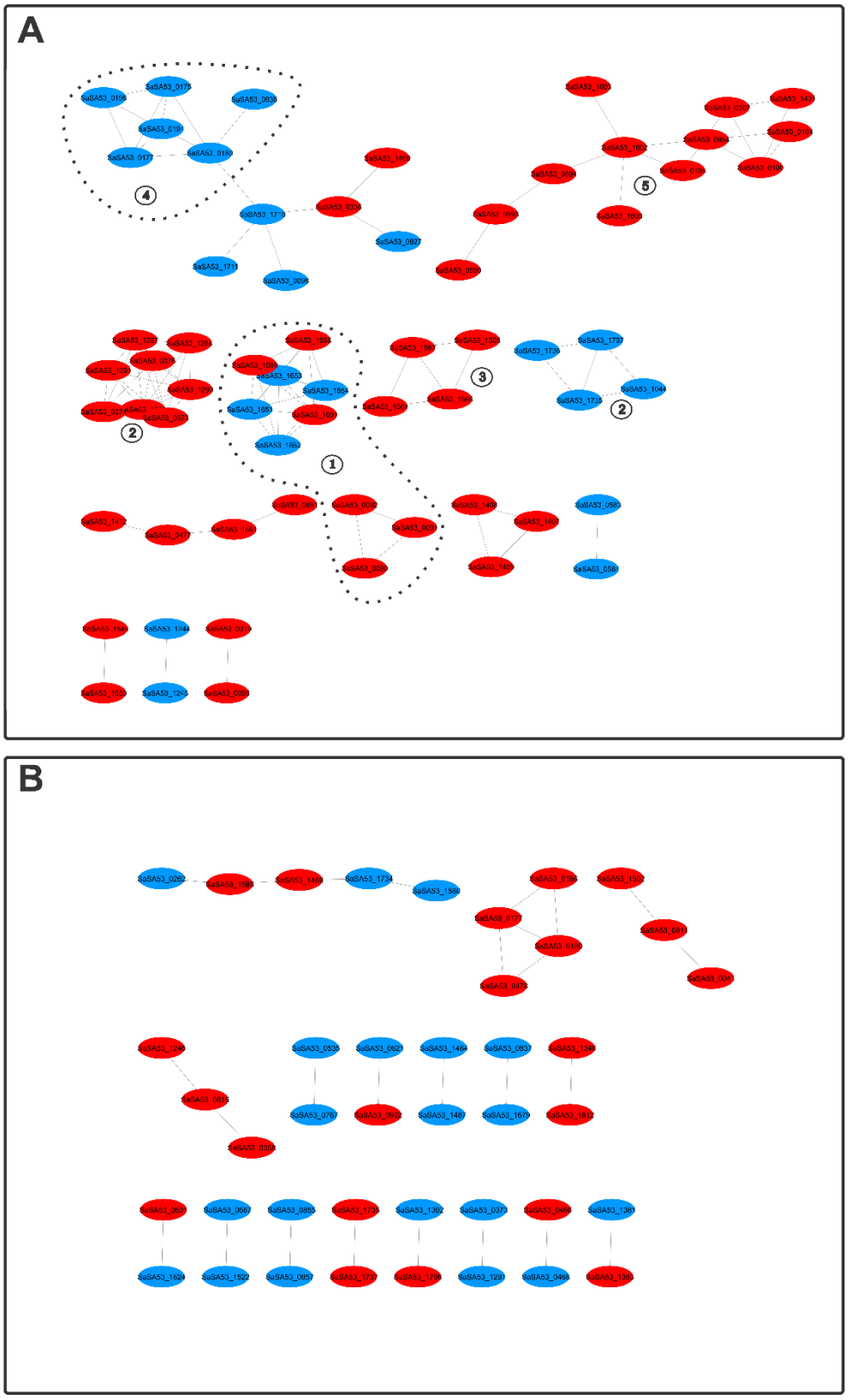


Figure 4. Dynamic range of the protein abundances found in SA53 strain between the two temperatures tested. The data are binned according to the log10 of their normalized abundance. Red circles represent proteins up-regulated at 32ºC. while blue circles represent proteins down-regulated at 32ºC. (A) Thermal proteins resistance; (B) Virulence proteins.

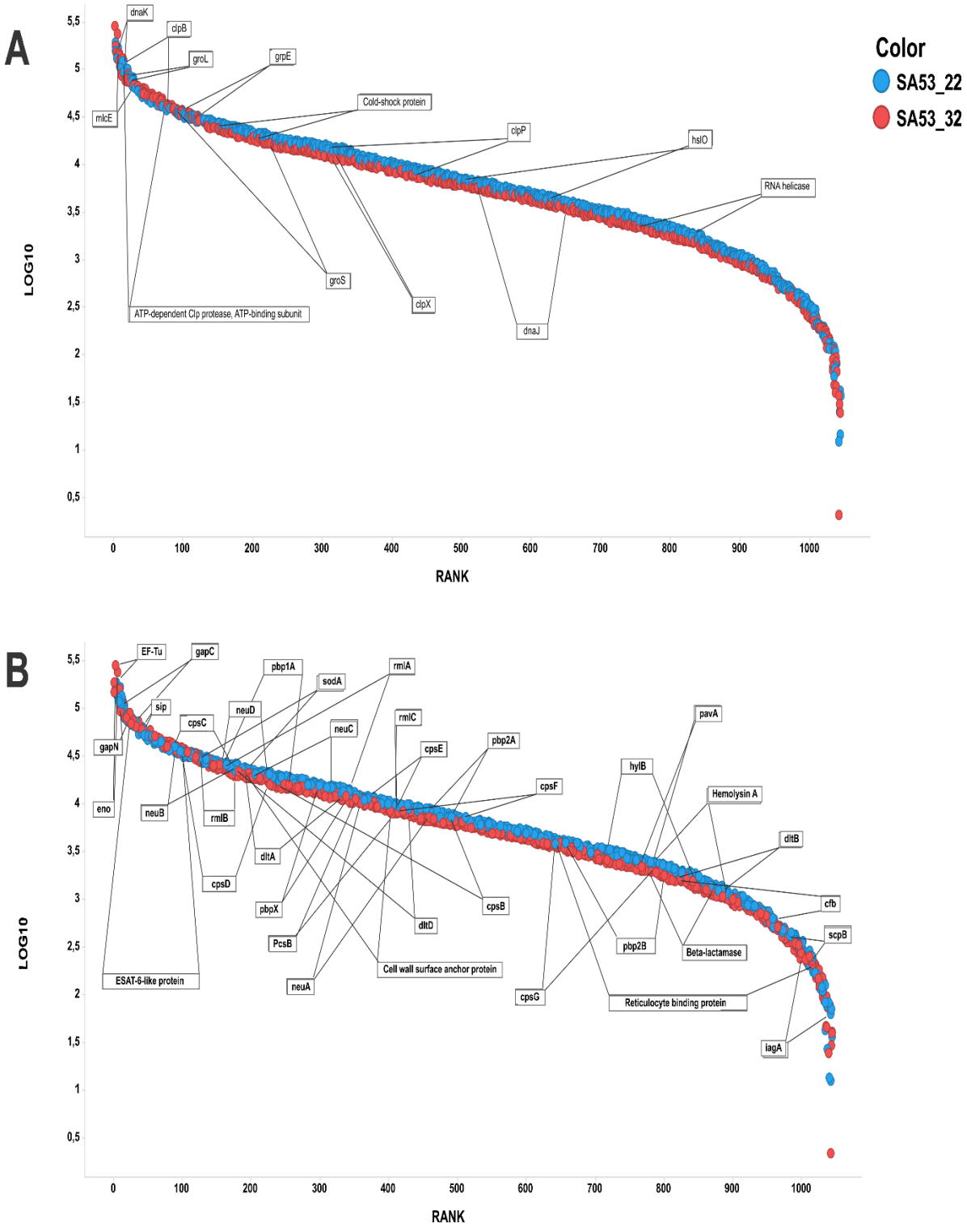
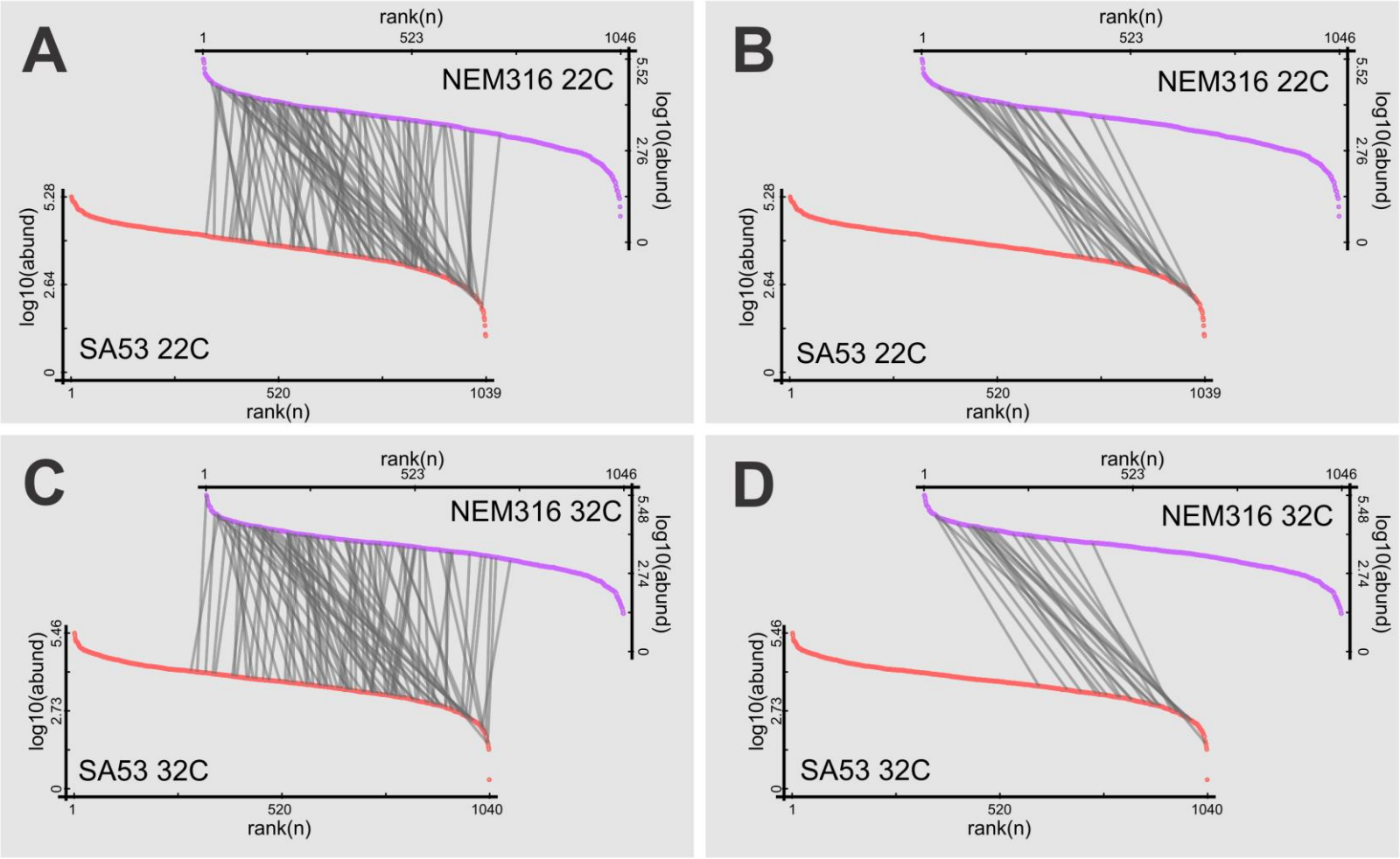
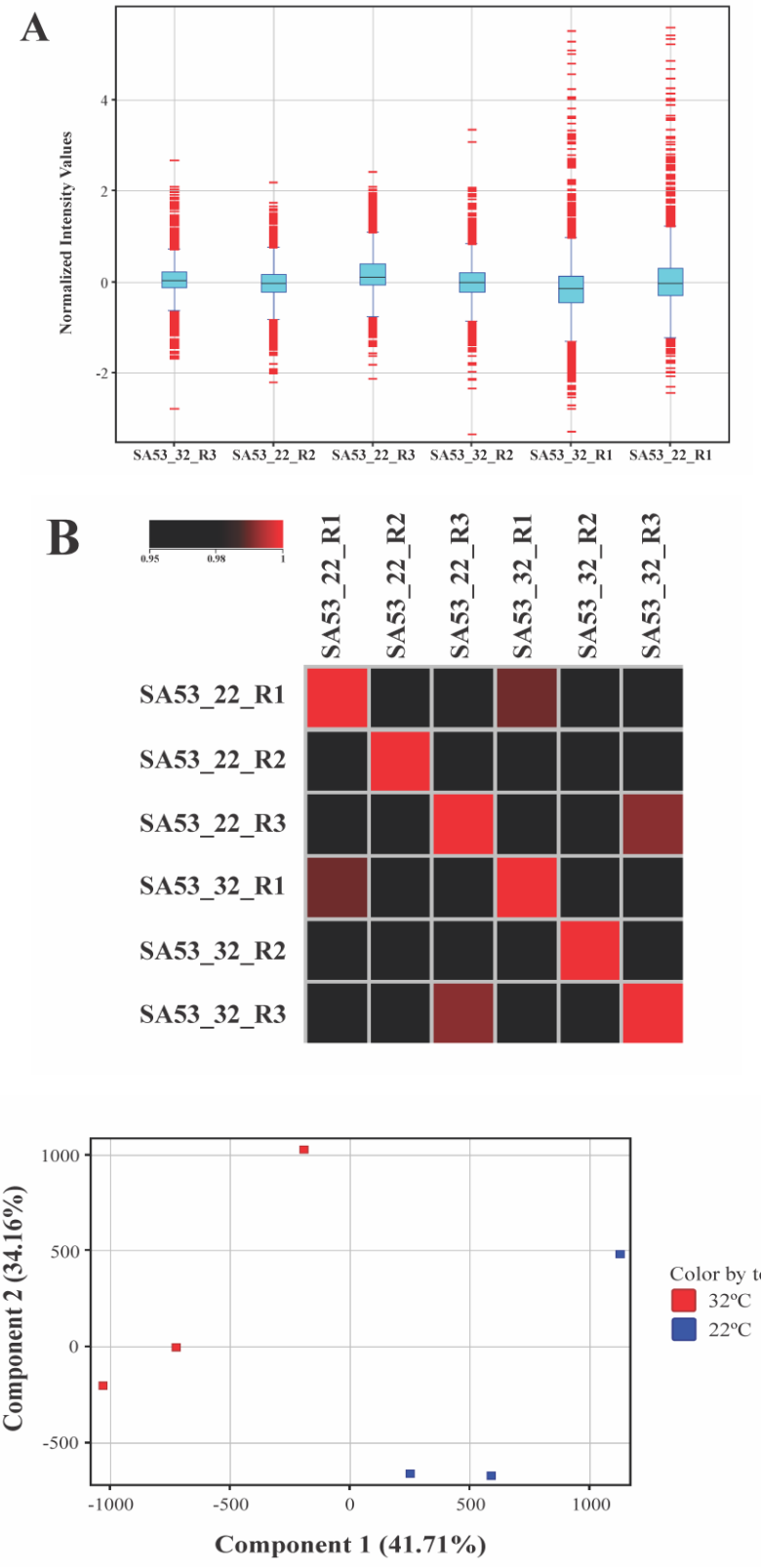


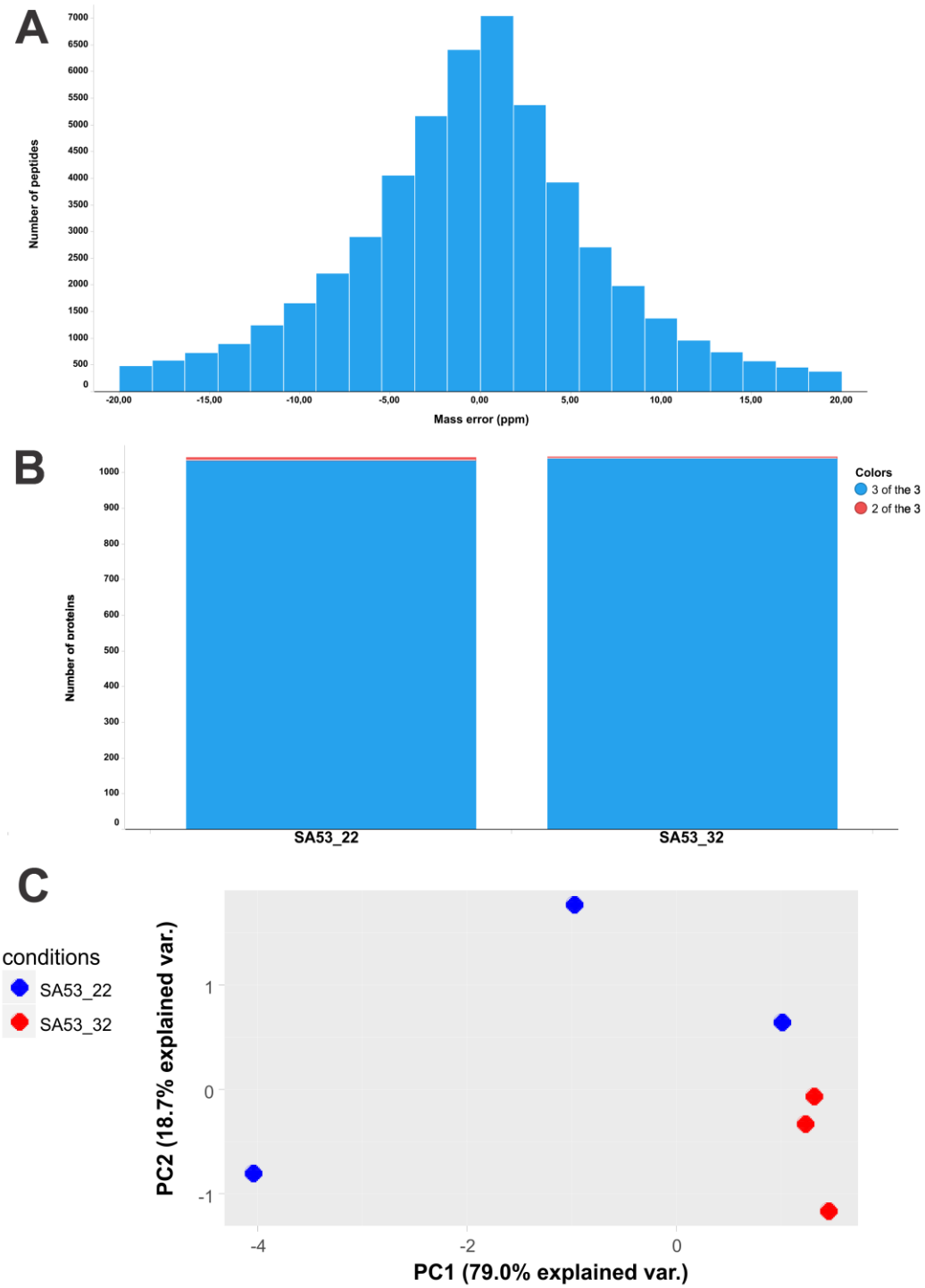
Figure 5. Comparison of protein abundance between SA53 and NEM316 strains at 22 ºC and 32 ºC. (A) at 22ºC with threshold of 25%; (B) at 22ºC with threshold of 50%; (C) at 32ºC with threshold of 25%; (D) at 22ºC with threshold of 50%.



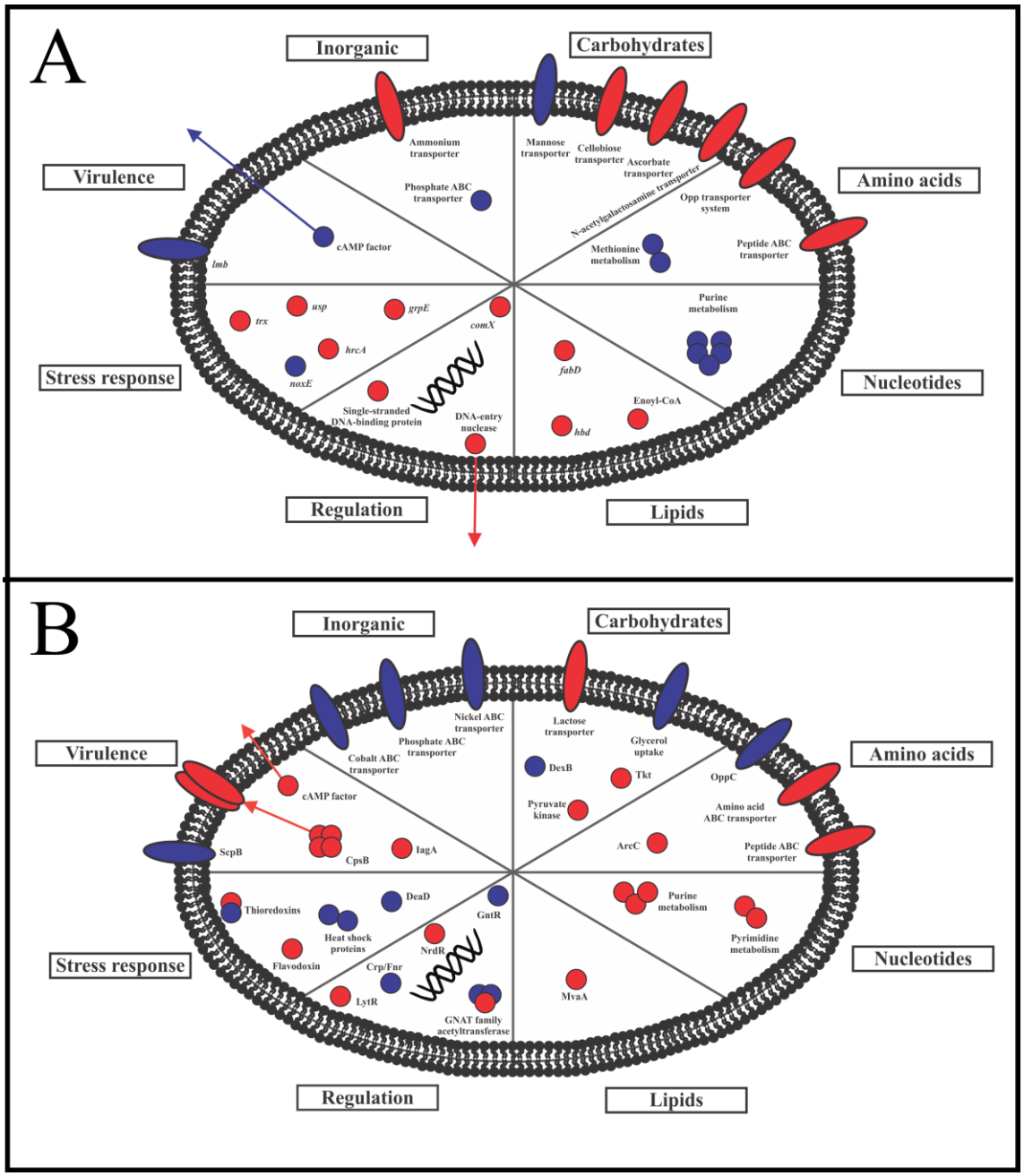
Supplementary Figure 1. Quality assessment of replicates biological used in transcriptome analysis. (A) distributions of the intensities evaluated by box plot; (B) correlation analysis matrix. (C) PCA plot, red circles represent samples growth at 32ºC. while blue circles represent samples at 22ºC.



Supplementary Figure 2. Quality assessment of replicates biological used in proteome analysis. (A) normal distribution of 10 ppm error of the total identified peptides; (B) repeat rate indicating the number of times that an identified protein appears in the replicates: 3 of 3 (blue) and 2 of 3 (red). (C) PCA plot, red circles represent samples growth at 32ºC. while blue circles represent samples at 22ºC.



Supplementary Figure 3. Schematic summarizing the transcriptional (A) and proteomic (B) response of fish-adapted GBS strain to incubation at different temperatures. Red circles indicate up-regulation at 32ºC, while blue circles represent down-regulation at 32ºC.



# 6. FINAL CONSIDERATIONS

The comprehension about the pathogenesis of the infectious process caused by GBS in fish is little understood. Besides that, different GBS genotypes have been associated with disease in fish, being that these genotypes may have specific characteristics which allow the bacterial survival and dissemination in fish. In this context, the study of GBS lineages belonging to clonal complex adapted to aquatic animals, so impacting for Brazilian and world fish farming, is need for understanding the pathogen. However, there are no previous studies that characterize the global expression of genes and proteins of these specific lineages.

Therefore, the present study demonstrated different approaches to characterize the functional genome of fish-adapted GBS strains at transcriptomic and proteomic levels, identifying genes and proteins involved in different biological process, which may favor the pathogenic process de GBS in fish host. In addition, the results provide important information about adaptation of this pathogen in aquatic environment.

In general, the protein content among genotypes of fish-adapted GBS strains is conserved, allowing the identification the targets for the development of vaccines for fish against streptococcosis. And the temperature is able to influence the transcriptome and proteome of a fish-adapted GBS strain.

The results obtained in this study open perspectives for subsequent projects with emphasis on mutant construction and vaccine development, and moreover, enable to use the approaches tested as a basis for evaluating the expression trends of genes and proteins in studies of regulation of signaling pathways, demonstrating the main pathways that enable the bacterial adaptation in aquatic environment and survival and pathogenicity in fish.

# 7. APPENDIX

## Publications during candidature
## Articles in Scientific Journals

LEIBOWITZ, M.P.; TAVARES, G.C.; PEREIRA, F.L.; REZENDE, C.P.; AZEVEDO, V.A.C.; FIGUEIREDO, H.C.P.. Shotgun label-free proteomic analyses of the oyster parasite *Perkinsus marinus*. *J. Proteomics Genomics Res.*, v. 2, p. 13-21, 2017.

ASSIS, G.B.N.; PEREIRA, F.L.; ZEGARRA, A.U.; TAVARES, G.C.; LEAL, C.A.G.; FIGUEIREDO, H.C.P.. Use of MALDI-TOF mass spectrometry for the fast identification of Gram-positive fish pathogens. *Front. Microbiol.*, v. 8, p. 1492, 2017.

BARONY, G.M.; TAVARES, G.C.; PEREIRA, F.L.; CARVALHO, A.F.; DORELLA, F.A.; LEAL, C.A.G.; FIGUEIREDO, H.C.P.. Large-scale genomic analyses reveal the population structure and evolutionary trends of *Streptococcus agalactiae* strains in Brazilian fish farms. *Sci. Rep.*, v. 7, p. 13538, 2017.

ASSIS, G.B.N.; TAVARES, G.C.; PEREIRA, F.L.; FIGUEIREDO, H.C.P.; LEAL, C.A.G.. Natural coinfection by *Streptococcus agalactiae* and *Francisella noatunensis* subsp. *orientalis* in farmed Nile tilapia (*Oreochromis niloticus* L.). *J. Fish Dis.*, v. 40, p. 51-63, 2017.

TAVARES, G.C.; COSTA, F.A.A.; SANTOS, R.R.D.; BARONY, G.M.; LEAL, C.A.G.; FIGUEIREDO, H.C.P.. Nonlethal sampling methods for diagnosis of *Streptococcus agalactiae* infection in Nile tilapia, *Oreochromis niloticus* (L). *Aquaculture*, v. 454, p. 237-242, 2016.

BARONY, G.M.; TAVARES, G.C.; ASSIS, G.B.N.; LUZ, R.K.; FIGUEIREDO, H.C.P.; LEAL, C.A.G.. New hosts and genetic diversity of *Flavobacterium columnare* isolated from Brazilian native species and Nile tilapia. *Dis. Aquat. Org.*, v. 117, p. 1-11, 2015.

LEAL, C.A.G.; TAVARES, G.C.; FIGUEIREDO, H.C.P.. Outbreaks and genetic diversity of *Francisella noatunensis* subsp *orientalis* isolated from farm-raised Nile tilapia (*Oreochromis niloticus*) in Brazil. *Genet. Mol. Res.*, v. 13, p. 5704-5712, 2014.

## Articles in Newspapers/Magazines

OLIVEIRA, T.F.; TAVARES, G.C.; FIGUEIREDO, H.C.P.; LEAL, C.A.G.. Septicemia por *Aeromonas* móveis em peixes. *Cadernos Técnicos de Veterinária e Zootecnia*, Minas Gerais, p. 31 - 42, 02 jun. 2014.

TAVARES, G.C.; FIGUEIREDO, H.C.P.; LEAL, C.A.G.. Infecção por *Streptococcus iniae* em peixes. *Cadernos Técnicos de Veterinária e Zootecnia*, Minas Gerais, p. 43 - 54, 02 jun. 2014.

TAVARES, G.C.; LEAL, C.A.G. ; FIGUEIREDO, H.C.P.. Antibioticoterapia em peixes. *Cadernos Técnicos de Veterinária e Zootecnia*, Minas Gerais, p. 64 - 76, 02 jun. 2014.

TAVARES, G.C.; FIGUEIREDO, H.C.P. ; LEAL, C.A.G.. Coleta e remessa de peixes para diagnóstico de doenças infecciosas. *Cadernos Técnicos de Veterinária e Zootecnia*, Minas Gerais, p. 77 - 86, 02 jun. 2014.

FIGUEIREDO, H.C.P.; TAVARES, G.C.; LEAL, C.A.G.. *Streptococcus agalactiae* e o controle pela vacinação: o que sabemos e que desafios temos pela frente. *Panorama da Aquicultura*, v. 27, n. 164, p. 36-41, 2017.

## Articles submitted for publication

TAVARES, G.C.; PEREIRA, F.L.; BARONY, G.M.; REZENDE, C.P.; SILVA, W.M.; SOUZA, G.H.M.F.; VERANO-BRAGA, T.; AZEVEDO, V.A.C.; LEAL, C.A.G.; FIGUEIREDO, H.C.P.. Delineation of the pan-proteome of fish-pathogenic *Streptococcus agalactiae* strains using a label-free shotgun approach. *BMC Genomics*, 2018.

TAVARES, G.C.; CARVALHO, A.F.; PEREIRA, F.L.; REZENDE, C.P.; LEAL, C.A.G.; FIGUEIREDO, H.C.P.. Transcriptome and proteome of fish-pathogenic *Streptococcus agalactiae* are modulated by temperature change. *Frontiers in Microbiology*, 2018.

PEREIRA, F.L.; GONÇALVES, L.A.; TAVARES, G.C.; SOARES, S.C.; DORELLA, F.A.; CARVALHO, A.F.; LEIBOWITZ, M.P.; LEAL, C.A.G.; AZEVEDO, V.A.C; FIGUEIREDO, H.C.P.. High-resolution molecular epidemiology showed that Brazilian *Francisella noatunensis* subsp. *orientalis* strains belong a clonal population and had a single entry into the country. *Emerging Microbes and Infections*, 2018.

LEAL, C.A.G; QUEIROZ, G.A.; PEREIRA, F.L.; TAVARES, G.C.; FIGUEIREDO, H.C.P. Emergence of *Streptococcus agalactiae* sequence type 283 in farmed fish, Brazil. *Emerging Infectious Diseases*, 2018.

SILVA, W.M.; SOUSA, C.S.; SOARES, S.C.; SOUZA, G.H.M.F.; TAVARES, G.C.; REZENDE, C.P.; FOLADOR, E.L.; OLIVEIRA, L.C.; FIGUEIREDO, H.C.P.; AZEVEDO, V.A.C.. Characterization of the *Lactococcus lactis* core-proteome through label-free quantitative proteomic. *Microbial Biotechnology*, 2018.

BRITO, M.F.; AULER, P.A.; TAVARES, G.C.; REZENDE, C.P.; ALMEIDA, G.M.F.; PEREIRA, F.L.; LEAL, C.A.G.; MOURA, A.A.; FIGUEIREDO, H.C.P.; HENRY, M. Label-free proteome of water buffalo (*Bubalus bubalis*) seminal plasma. *Reproduction in Domestic Animals*, 2018.

MUSSI, J.M.S.; TAVARES, G.C.; SANTOS, E.L.S.; FERREIRA, L.O.; RANGEL, D.A.A.; DORNELES, E.M.S.; SOARES FILHO, P.M.; SOUZA, P.G.; SOUZA, M.R.; LAGE, A.P.. Antagonistic activity of lactic acid bacteria isolated from Minas artisanal cheeses against *Brucella abortus*. *International Journal of Food Microbiology*, 2018.

TAVARES, G.C.; LEIBOWITZ, M.P.; LEAL, C.A.G.; FIGUEIREDO, H.C.P.. Zoonoses emergentes associadas ao consumo e manipulação de pescado. *Cadernos Técnicos de Veterinária e Zootecnia*, Minas Gerais, 2018.

## Articles *in preparation*

TAVARES, G.C.; QUEIROZ, G.A.; ASSIS, G.B.N.; LEIBOWITZ, M.P.; TEIXEIRA, J.P.; FIGUEIREDO, H.C.P.; LEAL; C.A.G.. *Streptococcus* spp. outbreaks in farmed Amazon catfish (*Leiarius marmoratus* x *Pseudoplatystoma corruscans*), *in preparation*, 2018.

LEIBOWITZ, M.P.; SANTOS, N.R.V.; TAVARES, G.C.; FIGUEIREDO, H.C.P.; LEAL, C.A.G. Metacercariae of *Centrocestus formosanus* (Trematoda: Heterophyidae) infecting farmed raised ornamental fish species in Brazil, *in preparation*, 2018.

SANTO, A.H.E.; BRITO, T.S.; BRANDAO, L.L.; TAVARES, G.C.; PRADO, S.A.; FERRAZ, V.P.; HOYOS, D.C.M.; TURRA, E.M.; TEIXEIRA, E.A.; FIGUEIREDO, H.C.P.; LEAL, C.A.G.; RIBEIRO, P.A.P.. Supplementation of dry oregano leaves on Nile tilapia (*Orecochromis niloticus*) diet increases the innate immunity and resistance against *Streptococcus agalactiae*, *in preparation*, 2018.

PEREIRA, F.L.; TAVARES, G.C.; CARVALHO, A.F.; LEAL, C.A.G.; FIGUEIREDO, H.C.P.. The temperature change modulates the pathogenicity of *Francisella noatunensis* subsp. *orientalis* to Nile tilapia but not the expression of its virulence genes, *in preparation,* 2018.

CARMO, F.L.R.; SILVA, W.M.; TAVARES, G.C.; CARVALHO, R.D.O.; RABAH, H.; CAUTY, C.; SILVA, S.H.; CORDEIRO, B.F.; CANARIO, M.V.; FOLADOR, E.L.; FIGUEIREDO, H.C.P.; JAN, G.; AZEVEDO, V.A.C. Comparative proteomic analysis of *Propionibacterium freudenreichii* wild type strain CIRM-BIA 129 and CB129ΔslpB nutant for SlpB surface protein, *in preparation,* 2018.

## Conference abstracts

TAVARES, G.C.; BARONY, G.M.; PEREIRA, F.L.; REZENDE, C.P.; LEAL, C.A.G.; FIGUEIREDO, H.C.P.. Comparative proteomic analysis of *Streptococcus agalactiae* isolates reveals differences between human and fish strains. In: 29º Congresso Brasileiro de Microbiologia, 2017, Foz do Iguaçu. 29º Congresso Brasileiro de Microbiologia, 2017. p. 1511-1.

TAVARES, G.C.; CARVALHO, A.F.; PEREIRA, F.L.; LEAL, C.A.G.; FIGUEIREDO, H.C.P.. Análises de transcriptômica e proteômica revelam as adaptações fisiológicas de *Francisella noatunensis* subsp. *orientalis* ao crescimento sob diferentes temperaturas. In: XIV ENBRAPOA, 2016, Florianópolis. XIV Encontro Brasileiro de Patologistas de Organismos Aquáticos (XIV ENBRAPOA), 2016. p. 202-202.

TAVARES, G.C.; LEAL, C.A.G.; FIGUEIREDO, H.C.P.. Distribution of pilus islands loci and the adhesin gbs2018-6 in *Streptococcus agalactiae* strains isolated from fish. In: XXVIII Congresso Brasileiro Microbiologia, 2015, Florianópolis. ANAIS DO 28º CBM 2015, 2015.

TAVARES, G.C.; CARVALHO, A.F. ; COSTA, F.A.A.; PEREIRA, F.L.; DORELLA, F.A.; SOARES, S.C.; LEAL, C.A.G. ; FIGUEIREDO, H.C.P.. Combined genomics, transcriptomics and proteomics approaches reveals a high number of transcribed and untranslated pseudogenes in *Francisella noatunensis* subsp. *orientalis*. In: XXVIII Congresso Brasileiro Microbiologia, 2015, Florianópolis. ANAIS DO 28º CBM 2015, 2015.

QUEIROZ, G.A.; TAVARES, G.C.; FIGUEIREDO, H.C.P.; LEAL, C.A.G.. Diversidade filogenética de amostras patogênicas e ambientais de *Aeromonas hydrophila*. In: XIII Encontro Brasileiro de Patologistas de Organismos Aquáticos, 2014, Aracaju. XIII ENBRAPOA, 2014.