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Novel Strategies for the Control of *Streptococcus agalactiae* (Group B *Streptococcus*) in Aquaculture

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Abstract

Streptococcus agalactiae (Group B *Streptococcus*, GBS) has a wide host range that includes aquatic animals. GBS are commensal bacteria of intestinal and genitourinary tracts in humans and animals but some lineages are adapted to specific hosts and have become hypervirulent clones, e.g. clonal complex (CC)17 is the main cause of neonatal invasive GBS infection. CC17 is transferred from the maternal vaginal tract to the neonate during birth, and neonatal GBS has been a major focus of scientific study. However, since the end of the 20th century, GBS sequence type (ST)283 infection has caused a quiet epidemic in Southeast Asia. It was confirmed as a zoonotic disease in 2015 and consumption of raw fish was the major route of transmission to nonpregnant humans whom did not have comorbidities. Common clinical manifestations were meningitis, endocarditis and septic arthritis and mortality rate was 3.4%. Control of GBS disease in humans, fishes and cattle currently relies primarily on use of antimicrobials. To reduce the burden of human GBS disease, economic losses resulting from GB infections in aquaculture and the threat of rising antibiotic resistance, this project's main aims were to find bacteriocins active against GBS and to explore host-adaptation through metabolic profiling to develop alternative control strategies against this bacterium.

One hundred and twelve *S. agalactiae* isolates (19 human, 36 bovine and 57 piscine) were tested for bacteriocin production ability in antagonism assays. A class III bacteriocin, produced by ST17 human isolate, was identified and designated "agalacticin A". Its spectrum was determined on solid medium assay. Agalacticin A showed killing activity against human, bovine and piscine GBS strains supporting its potential as GBS-specific antimicrobial agent and it had a narrow spectrum towards other pyogenic *Streptococcus* species, *Streptococcus dysgalactiae* subsp. *dysgalactiae* and *Streptococcus canis.* Vancomycin-resistant *Enterococcus faecalis* could also be inhibited by agalacticin A at high concentration but there was no impact on *Lactobacillus* species, which are important in the human vaginal microbiome. Pre-screening of agalacticin A in a *Galleria mellonella* (the greater wax moth) challenge model showed its bacteriolytic effect and its usefulness for treatment of GBS-challenged wax moth larvae. However, the duration of agalacticin A action is limited to 6 hrs due to elimination by an insect excretory system, which suggests agalacticin A is likely to be removed by the glomerular filtration in vertebrates.

In an attempt to find vaccine candidates or metabolic manipulations to control GBS in aquaculture, investigation was conducted into metabolic pathways associated with GBS from particular hosts and potential mechanisms of competitive advantage or niche restriction. Biolog GEN III was used to identify phenotypic markers of niche adaptation using metabolic profiling. Eighty-eight isolates (29 bovine, 17 human and 42 piscine) were tested for their use of sugars, sugar alcohol, hexose-phosphate, amino acids, hexose acids and carboxylic acids, esters and fatty acids. Fish-specifc phenotypes were not identified across all CCs associated with aquaculture. Instead, CC-associated phenotypes were described. Loss of catabolism of D-salicin and other β-glucosides (β-methyl-D-glucoside) was observed in CC283 and CC17. Further preliminary study on salicin (sal) utilization and hyaluronidase (hyl) production by GBS from different CC showed that the sal^-/hyl^+ phenotype is associated with the only known hypervirulent GBS clades in humans, i.e. CC283 and CC17. Mechanisms underlying the lack of salicin hydrolysis and its association with virulence are unknown. Several potential pathways are described, leading to suggestions for further research.

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Declaration

I declare that, except where explicit reference is made to the contribution of others, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or at any other institution.

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Abbreviations

AMR	Antimicrobial resistance
BBB	Blood brain barrier
BLIS	Bacteriocin-like inhibitory substance
CAT	N-terminal catalytic domain
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming unit
CLSI	Clinical and Laboratory Standard Institute
CovSR	Control of virulence Sensor and Regulator system
CPS	Capsular polysaccharide
DAMPs	Danger-associated molecular patterns
ECM	Extracellular matrix
EOD	Early-onset disease caused by *Streptococcus agalactiae*
FAO	Food and Agriculture Organization of the United Nations
FIC	Fractional inhibitory concentration index
GBS	Group B *Streptococcus* or *Streptococcus agalactiae*
H&E stain	Hematoxylin and eosin stain
HIER	Heat-induced antigen retrieval
HvgA	Surface-anchored hypervirulent GBS adhesin
IAP	Intrapartum antimicrobial prophylaxis
ICEs	Integrative and conjugative elements
IHC	Immunohistochemistry
IPTG	D-isopropyl-β-thiogalactopyranoside
kDa	Kilodalton
LAB	Lactic acid bacteria
LAMP	Loop-mediated isothermal amplification
LD_{50}	Lethal dose 50%, or median lethal dose

LGT	Lateral gene transfer
lmb	Laminin-binding protein
LOD	Late-onset disease caused by *Streptococcus agalactiae*
mg	Milligrams
mg/L	Milligrams per litre
MGE	Mobile genetic element
MIC	Minimal inhibitory concentration
ml	Millilitres
MLST	Multilocus sequence typing
OD_{600}	Optical density at 600 nm
PAMPs	Pathogen-associated molecular patterns
Rib	Resistance to proteases, immunity, group B
RPM	Revolutions per minute
ScpB	C5a peptidase
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sip	Surface immunogenic protein
ST	Sequence type
TLRs	Toll-like receptors
TRD	C-terminal target recognition domain
µl	Microliters
WHO	World Health Organization

Chapter 1 Introduction: *Streptococcus agalactiae* and its role in human health, animal health and food safety

1.1 *Streptococcus agalactiae*

Streptococcus agalactiae is a gram positive coccus also known as Group B *Streptococcus* (GBS) of the Lancefield classification, which is based on the carbohydrate composition of bacterial cell walls. *Streptococcus agalactiae* is the only species within this Lancefield group, so the two names are effectively synonymous, whereby GBS is most commonly used in human medicine whereas *S. agalactiae* is most commonly used in veterinary medicine and aquaculture. GBS is a commensal bacterium of the human gastrointestinal and genitourinary tracts and the oropharynx (Van Der Mee-Marquet et al. 2008). Colonization or carriage in humans is asymptomatic, but GBS can lead to fatal disease in neonates (Martins et al. 2007) and immunocompromised persons (Sunkara et al. 2012). GBS is an important pathogen not only in humans but also in terrestrial and aquatic animals. GBS infection of mammary glands of cattle, camels (Younan et al. 2001) or buffaloes (Farooq et al. 2008) causes clinical and sub-clinical mastitis, affecting milk quality and quantity (Rajala-Schultz, Hogan, and Smith 2005; Åkerstedt et al. 2012). Emergence of GBS disease in poikilothermic hosts has been reported worldwide, especially in farmed fish species (Duremdez et al. 2004; Evans et al. 2002; Ye et al. 2011) but also in reptiles (crocodiles) (Bishop et al. 2007) and amphibians (bullfrogs) (Amborski et al. 1983). GBS has also been reported from aquatic mammals (bottlenose dolphins, grey seals) (Delannoy et al. 2013), companion animals (dogs and cats) (Yildirim et al. 2002), monkeys (Lammler, Abdulmawjood, and Weiss 1998), horses (Yildirim, Lämmler, and Weiß 2002) and lizards (emerald monitors) (Hetzel et al. 2003). The distribution of GBS across a broad range of host species, including food-producing animals, raises questions about the existence of host-adaptation in *S. agalactiae* and the possibility of interspecies transmission. Based on its impact on animals, particularly cattle and fishes, *S. agalactiae* is a threat to food security. In addition, there are concerns about GBS as a One Health issue, i.e. affecting human, animal and environmental health, particularly in relation to potential zoonotic transmission, antimicrobial resistance (AMR) and food safety.

An emerging ST283 clone associated with invasive disease of adult humans in Asia originates from fish, where it can also cause disease (Kalimuddin et al. 2017; Delannoy et al. 2013). Thus, this clone is a good example of GBS being a food security issue, both through its impact on food production and through its effect on food safety. This introduction shall provide a brief overview of the occurrence and impact of GBS in humans, fish and dairy cattle, current treatment and control strategies, and the challenges

GBS poses to public health and food safety. Finally, it will introduce the concept of the novel therapeutics used in an attempt to control infection or disease.

1.1.1 Human *Streptococcus agalactiae*

Human GBS status can be classified as infection, which is accompanied by signs and symptoms of disease, and GBS colonization (called carriage), which is asymptomatic. The most common colonization sites in humans are the rectum, perianal area, vagina, urethra and throat. Prevalence of colonization in healthy humans in the United States has been estimated at 20% of men and 34% of women. GBS in men was found from urine, anal orifice, and throat (Manning et al. 2004). The prevalence of GBS vaginal colonization in Europe has been estimated to range from 6.5 to 36% (Barcaite et al. 2008). In the United Kingdom, GBS prevalence was 20 to 40% (Hughes et al. 2017). Several risk factors for carriage of GBS have been identified, including but not limited to sexual activity and ethnicity (Meyn et al. 2002). Reported differences in prevalence estimates are influenced by selection criteria for inclusion in GBS screening.

The manifestation of clinical infections is different between neonates, elderly people and healthy adults. Key features of the three manifestations are described here. Neonatal invasive GBS infection can be described in terms of early-onset disease (EOD) and late-onset disease (LOD) according to the age of onset (Phares et al. 2008). Maternal colonization is a major risk factor for invasive infection in neonates, particularly for EOD, within 7 days from birth (Sendi and Johansson 2008). Ascension of GBS from vagina to placenta and amniotic fluid can occur (Whidbey et al. 2013), which may explain why elective caesarean section does not prevent transmission. The incidence of EOD in the UK and Ireland in 2015 was 0.57/1000 births (Hughes RG et al. 2017) and of LOD was 0.37/1000 births (O'Sullivan et al. 2019). Clinical symptoms of EOD include sepsis followed by pneumonia and meningitis (Phares et al. 2008). In case of LOD, disease generally occurs between 7 and 89 days of age according to case definition by the Centers for Disease Control and Prevention (CDC). Cases occurring after 90 days and up to 14 years (not pregnant) were classified as pediatric cases (Edwards and Baker 2014; Phares et al. 2008). Bacteraemia remains the most common clinical expression in LOD but meningitis is higher than in EOD (27% and 7%, respectively) (Phares et al. 2008). Capsular serotype III is associated with both EOD and LOD. Importantly, the hypervirulent ST17 clone is the most prevalent among neonatal meningitis GBS strains (Poyart, Réglier-poupet, et al. 2008). The rupture of placental membranes has been reported to be a risk factor for EOD (Håkansson et al. 2008). Breast milk represents

a source of transmission to the neonates with LOD. However, transmission of GBS from human mastitis is rare, unless the bacterial load in mother's milk is high (Filleron et al. 2014).

GBS is an invasive disease not only in infants but also in adults (15 years or older and not pregnant). Edwards and Baker reported GBS infection in elderly adults (≥ 65 years of age) in the United States between 1997 to 2003. GBS colonization among healthy elderly adults was 25%. Invasive GBS infection cases have been reported and fatality rates were high (15%). Pneumonia, bacteraemia and arthritis are the most common clinical manifestation of invasive GBS disease in elderly adults. Soft-tissue and urinary tract infections were also frequently found in the elderly cases. Meningitis, osteomyelitis and endocarditis are rare (Edwards and Baker, 2005). The most prevalent among adults in the United States were serotype V (31%) followed by Ia (24%), II (12%) and III (12%) (Phares et al. 2008). In Japan, serotypes Ib and V were most common among adults (Murayama et al. 2009). *S. agalactiae* has been described as a causative agent of infective endocarditis (Sambola et al. 2002) and pyogenic arthritis (Nolla, Gómez-vaquero, and Corbella 2003) especially in patients undergoing prosthetic valve or articular surgery. Toxic shock syndrome due to GBS has also been reported (Tang et al., 2000). Streptococcal toxic shock syndrome (STSS) is often associated with severe skin infections and can rapidly progress to multiple organ failure. Diabetes mellitus, cancer and compromised immunity are predisposing conditions increasing the risk of GBS disease (Sendi and Johansson 2008).

Recently, GBS has been recognised as a zoonosis and foodborne disease. Piscine CC283 GBS strains can be transmitted to humans by raw fish consumption (Ip et al. 2006). The emergence of zoonotic piscine ST283 GBS, causing meningitis and arthritis in humans, has consequences not only for public health but also for food security and food safety. This will be described in detail in the section on foodborne disease (section 1.1.5).

1.1.2 *Streptococcus agalactiae* in aquaculture

The global fisheries production continuously grows to supply more fish for human consumption. The total sale value of aquaculture production in 2016 was estimated at USD 232 billion (FAO, 2018). The influence of climate change and a progressive catch reduction policy has caused a slowdown in production from capture fisheries (Roessig et al. 2004; FAO 2018). To meet the growing demand for seafood whilst limiting capture fisheries, aquaculture has become an increasingly important source of fish production in the past 50 years. Developing countries especially in Southeast Asia are major aquaculture

producers, e.g. Indonesia (4.9 million tonnes), Vietnam (3.6 million tonnes) and Thailand (0.96 million tonnes) (FAO 2016). Domestic production and export of fish and fish products contribute to economic growth in these countries (FAO, 2018). Aquaculture species have been intensively produced to supply the expansion of domestic consumption and export. Major species produced in world aquaculture are carp, Atlantic salmon, pangasius and tilapia (FAO, 2018). Commercially important tilapia species are Nile tilapia (*Oreochromis niloticus*), Blue tilapia (*O. aureus*), Mozambique tilapia (*O. mossambicus*) and red tilapia hybrids (Mozambique tilapia × Nile tilapia) (Gupta and Acosta 2004). Global tilapia farming is based on two culture systems: water-based and land-based. Water-based systems use cage culture in lakes, reservoirs or shared water sources such as rivers. Tilapia cage culture is practiced in Asia, Africa and the Americas (Gupta and Acosta 2004; Hasan and New 2013; Fitzsimmons 2016). In land-based systems, tilapia is cultivated in ponds, raceways and tanks. In addition, ricefield fisheries (Ruddle 1982) and integrated livestock systems (Wohlfarth and Schroeder 1979) have been common practice in Southeast Asia. Livestock manure and by-products can be used as nutrients for fish production (Wohlfarth and Schroeder 1979).

To increase production, semi-intensive and intensive production systems have been established. Intensive production and related risk factors for disease emergence are environment, population density (Reno, 1998) and introduction of infected stock (Murray and Peeler 2005). Temperature, salinity and water quality evoke corticosteroid stress responses causing immunosuppression and affect fish health (Marzouk 2005; Barton 2002). High stocking density promotes contact between infectious and susceptible animals increasing the probability of pathogen transfer (Reno, 1998). Streptococcal infection caused by *S. agalactiae* is an important disease in tilapia and red hybrid tilapia (Ye et al. 2011; Musa et al. 2009). Moreover, *S. agalactiae* from tilapia can infect bighead carp (*Aristichthys nobilis*) (Zhang, Ke, and Liu 2018).

S. agalactiae impacts on food security because it causes massive fish kills, reducing food availability from fish farming and caught fish, e.g. cultured seabream and wild mullet in Kuwait (Evans et al. 2002) and tilapia in Indonesia and China (Ye et al. 2011). Massive kills of large-sized fish lead to major economic losses because all investment in producing and feeding the fish is lost (The Fish Site 2006). Infected fish show neurological clinical signs such as swirling swimming behaviour and exophthalmia (eye protrusion) (Figure 1-1) because of meningoencephalitis and septicaemia. Hatcheries can act as a source of GBS and carrier fingerlings and contaminated water can transfer *S. agalactiae* into

new tilapia establishments (Amal et al. 2013). Furthermore, the use of antimicrobial agents in treatment or prophylaxis may contribute to the emergence of antimicrobial resistance (AMR) in the environmental bacterial population (Cabello et al. 2016). Further detail on occurrence of AMR in fish pathogens and the environment will be provided in the section on antimicrobial resistance (1.1.7).

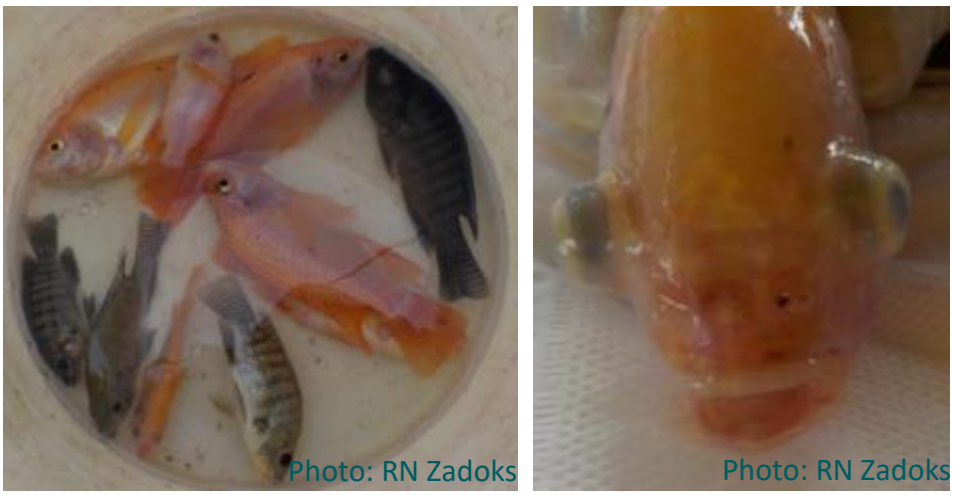


Figure 1-1 Clinical presentation of *Streptococcus agalactiae* in fish.
Central nervous system damage and septicaemia caused abnormal swimming (left) and exophthalmia (popeye) (right). Photos used by permission of Professor Ruth N Zadoks.

Phenotypic characteristics of *S. agalactiae* based on haemolytic activity can distinguish piscine GBS into two subpopulations: non-haemolytic and haemolytic strains. The non-haemolytic subpopulation belongs to clonal complex (CC) 552, serotype Ib which grows at 28°C (Delannoy et al. 2013; Godoy et al. 2013). Outbreaks of fish-specific GBS stains of CC552 have been reported worldwide, e.g. in tilapia in Latin American countries (Honduras, Colombia, Costa Rica, and Brazil), in hybrid striped bass in Israel and in wild fishes in Australia (Bowater et al., 2012). Non-haemolytic GBS infection has also been found in cultured bullfrogs in Brazil (Amborski et al. 1983).

Haemolytic piscine GBS isolates comprise CC7 and CC283, both of which grow at 37°C. CC7 strains have been isolated from a wide range of host species, including fishes, frogs (Delannoy et al. 2013), sea mammals (dolphins) and humans (Evans et al. 2019). CC7 serotype Ia and CC283 serotype III are strains predominantly found in Asian countries (Delannoy et al. 2013; Kayansamruaj et al. 2018; Kalimuddin et al. 2017). Human effluent caused an outbreak of CC7 disease in aquatic animal species (fish and mammal) in Kuwait Bay (Jafar et al. 2008). Experimental challenges of Nile tilapia with ST7 provided further

evidence of the possibility of human-to-fish transmission of GBS ST7 (Evans et al. 2009). However, not all human associated GBS isolates are pathogenic to fish. Delannoy et al. challenged Nile tilapia with ST23 (CC23) which was isolated from a grey seal, *Halichoerus grypus* (Fabricius). CC23 can infect multiple host species such as humans, cattle and aquatic mammals but it does not cause morbidity or mortality in fish (Delannoy et al. 2016). Transmission of GBS from animals to humans is well recognized for serotype III, CC283, which caused invasive GBS infection in humans via raw fish consumption (Ip et al. 2006; Kalimuddin et al. 2017). More detail on host adaptation, virulence factors and their links to interspecies transmission or niche restriction of *S. agalactiae* will be provided in the section 1.3.2.1.

1.1.3 Bovine *Streptococcus agalactiae*

S. agalactiae is an important cause of udder infections in cows (Zadoks et al. 2011). Persistent intramammary infections cause long-term increases in somatic cell counts which affect milk yield, composition and quality, leading to considerable negative economic impacts. Prevalence of *S. agalactiae* bovine mastitis in Colombia is estimated at 11 to 60% at the herd level and 35% at the cow level (Reyes et al. 2017). Europe and Canada have low prevalence of GBS bovine mastitis, with estimated prevalence of 0.1 to 3% at the herd level (Tenhagen et al. 2006; Riekerink et al. 2008). The milking routine can spread bacteria between udder quarters within an animal and between udders from different animals. This is called the contagious transmission cycle (Ramírez 2014). Sources and transmission routes of *S. agalactiae* in dairy cattle herds have been identified to mitigate risk of reinfection by using good animal husbandry practices (Keefe 2012). In addition, the bovine gastrointestinal tract and the dairy cow environment have recently been recognized as potential reservoirs of *S. agalactiae.* Fecal shedding and leakage of milk from infected udders contribute to environmental contamination. Contamination of the environment with GBS (bedding and drinking water) may contribute to an environmental GBS transmission cycle (Jørgensen et al. 2016).

Molecular epidemiology studies of *S. agalactiae* in dairy cattle in South America, North America and European countries have indicated different predominant strains in each region. Based on multilocus sequence typing (MLST), clonal complex (CC) 67 was responsible for the majority of bovine mastitis in the United Kingdom (Bisharat et al. 2004) and the closely related CC61 is responsible for most bovine GBS mastitis in Portugal (Almeida et al, 2016). CC61 and CC67 are exclusive in cattle (Almeida et al. 2016).

In Northern Europe, the most common strains were sequence type (ST)1 (the founder of CC1) in Denmark (Zadoks et al. 2011), Finland and Sweden (Lyhs et al. 2016) followed by ST23 (CC23), ST103 (CC103), ST196 (CC196) and ST8 (CC8). CC103 predominated in Colombia and strains belonging to CC67 were not detected in Northern Europe and Colombia. The fact that ST1, 8, 12, 23 and 196 have been found both in humans and in cattle (Lyhs et al. 2016) raises questions on the possibility of interspecies transmission. Comparative genomics of human and bovine isolates revealed that GBS strains from those host species have distinct characteristics. Lactose utilization is exclusive for bovine GBS strains and facilitates bacterial survival and infection in mammary glands (Finch et al. 1984; Richards et al. 2011). Most bovine strain-specific genes were acquired through lateral gene transfer (LGT) (Richards et al. 2011). LGT has been reported to occur between GBS and other mastitis pathogens such as *Streptococcus uberis* and *Streptococcus dysgalactiae* subsp. *dysgalactiae* (Richards et al. 2011). Likewise, some virulence factors showed different types and distribution between bovine and human strains. For example, pilus island (PI) 1 in combination with one of the PI-2 variants is most frequent in human isolates. In contrast, PI-2b is predominantly found in bovine isolates. It is likely that virulence factors play a role in host specificity with specific mechanisms in those host species. PI-1 confers an advantage to evade the human immune cells but is not necessary for colonization in bovines (Springman et al. 2014).

Genetic factors are an important mechanism of host adaptation. However, based on serotype, ribotype characteristics and tetracycline resistance genes, human-to-cattle transmission of *S. agalactiae* may occasionally occur (Dogan et al. 2005). Cattle-to-human transmission has also been suggested. Manning and colleagues collected stool samples from people engaging in livestock production and their animals. Strain ST1 was found in a couple and their cattle. Cattle exposure may be associated with interspecies transmission (Manning et al. 2010).

Like the infection with human GBS in fishes, the possibility of causing infection with human GBS in cattle has also been demonstrated in challenge studies, although the clinical manifestation of mastitis differed between human and bovine strains (Jensen 1982).

1.1.4 Strain typing of *Streptococcus agalactiae*

Within bacterial species, different strains may exist, i.e. isolates exhibiting phenotypic and/or genotypic traits which are distinctive from those of other isolates of the same species

(see Glossery of Terms). Infection with specific strains can cause particular disease manifestations. In addition, typing information to classify GBS strains is important for studying its epidemiology. There are many ways of identifying strains, including phenotypic methods (e.g. antimicrobial susceptibility testing, serotyping) and genotypic methods (e.g. molecular serotyping, MLST). Strain typing methods can be definitive, meaning that they provide typing results with universal meaning, or comparative, meaning that interpretation of results is limited to comparison of data obtained within individual studies (see Glossary of Terms). The two most widely used typing methods for GBS are serotyping and MLST. Serotyping is in human microbiology because the capsular polysaccharide (CPS), which is formed of structurally unique oligosaccharide units (Michon et al. 1987; Paoletti and Kasper 2019), is an important virulence factor for GBS and antibody to GBS's CPS are type-specific (Baker and Kasper 1976; Smith et al. 1990). Currently, ten CPS serotypes are recognized: Ia, Ib, and II through IX (Mackie et al. 1979; Ferrieri and Flores 1997; Slotved et al. 2007). Serotyping has been widely used in the vaccine-related studies (Harrison et al. 1998) and can be conducted phenotypically using antibodies or genotypically using primers that target specific regions of the capsule operon (Dogan et al., 2005). However, because only 10 serotypes exist, the method has very limited discriminatory power (ability to differentiate between isolates), which limits is usefulness for epidemiological studies (van Belkum et al. 2007). Moreover, a high proportion of GBS isolates from animals are nonserotypeable using classical serotyping, which may be because the typing antisera were initially developed for human isolates (Dogan et al., 2005; Ekin and Gurturk 2006). In addition, pseudogenization of the capsule operon has been described in bovine isolates (Almeida et al. 2016). This phenomenon suggests that the capsule is not important as virulence factor in cattle, and limits the typeability of serotyping, i.e. the ability of the method to classify every isolate as belonging to a particular strain.

To increase discriminatory capacity, DNA based-typing methods, e.g. ribotyping (Blumberg et al. 1992), random amplified polymorphic DNA typing (Limansky et al. 1998) and pulsed field gel electrophoresis (Gordillo et al. 1993) were developed. All methods can detect banding patterns of DNA fragments, either based on digestion of bacterial genomic DNA by restriction enzymes (ribotyping, pulsed field gel electrophoresis) or based on enzymatic

amplification of DNA by polymerase chain reaction (random amplified polymorphic DNA typing; Dogan et al. 2005, Duarte et al. 2004). These methods are reproducible within laboratories and discriminatory but interpretation of banding patterns has not been standardised among laboratories, precluding data exchange or comparison between studies (van Belkum et al. 2007), unless expensive automated ribotyping equipment is used. Multilocus sequence typing (MLST) (Enright and Spratt 1999) was developed to overcome those limitations and to facilitate data sharing, including standardised interpretation using free software. MLST is currently widely used as a definitive typing method providing data that can be compared between laboratories over the internet and that is suitable for epidemiological studies (van Belkum et al. 2007). GBS isolates are characterised using the sequences of internal fragments of seven house-keeping genes (loci) to obtain the allelic profiles, and each allele at each locus is assigned a number that provides unique identification and that is linked to a unifying database (Enright and Spratt 1999; Jones et al. 2003). For ease of use, allelic profiles are converted into sequence types (ST), whereby each ST designated a unique combination of alleles and hence a unique combination of nucleotides at the 7 loci used for MLST. Closely related STs are grouped into CCs using a bespoke methodology called based upon related sequence types or BURST (Enright and Spratt 1999; Feil et al. 2000). The BURST methodology and its electronic successor eBURST make no assumptions with regards to underlying evolutionary mechanisms such as recombination or

Glossary of terms

Allelic profile: The alleles at each of the genes used for MLST that unambiguously define a strain (Enright and Spratt 1999). For example, the hypervirulent neonatal clone of *Streptococcus agalactiae* has the allelic profile 2 - 1 - 1 - 1 - 1 - 1 - 1.

"Clone: Bacterial isolates that, although they may have been cultured independently from different sources in different locations and perhaps at different times, still have so many identical phenotypic and genotypic traits that the most likely explanation for this identity is acommon origin within a relevant time span" (van Belkum et al. 2007).

"Clonal complex: A group of bacterial isolates showing a high degree of similarity, ideally based on near-identity of multilocus enzymeprofiles and multilocus sequence types. Clonal complexes are identical to clonal groups" (van Belkum et al. 2007).

"Comparative typing: A typing strategy aimed at assessing relatedness within a set of isolates without reference to other isolates." (van Belkum et al. 2007).

"Definitive (library) typing: Type allocation of organisms according to an existing typing scheme aimed at the development of (exchangeable) databases for long-term retrospective and prospective multicentre studies as well as epidemiological surveillance studies".(van Belkum et al. 2007).

"**Isolate:** a population of microbial cells in pure culture derived from a single colony on an isolation plate and characterized by identification to the species level" (van Belkum et al. 2007)

"Strain: an isolate or group of isolates exhibiting phenotypic and/or genotypic traits which are distinctive from those of other isolates of the same species" (van Belkum et al. 2007)

"Sequence type (ST): Defined by the allelic profile" (Enright and Spratt 1999). For example, ST17 defines the hypervirulent clone of GBS with the allelic profile 2 - 1 - 1-1 - 1 - 1 - 1" (http://pubmlst.org/sagalactiae/).

point mutation and does not describe deep phylogenetic relationships (Feil et al. 2004). However, STs belonging to the same CC can be assumed to be descended from a recent common ancestor (Enright and Spratt 1999). The original CC definition proposed by Feil and colleagues (Feil et al. 2004) include all allelic profiles that could be linked together based on connecting single locus variants, i.e. STs that differed from each other in a single allele. Over time, a growing number of STs has been detected for GBS, resulting in amalgamation of CCs that were once separate (Springman et al. 2014). The first paper on MLST of GBS included only 29 STs (Jones et al. 2003). The current database includes over 1000 STs for GBS. To avoid loss of discriminatory power, CCs are now commonly identified using a modified rule, such as sharing of 5 of 7 alleles (Springman et al. 2014).

Because the number of STs (over 1000) is much higher than the number of serotypes (10), there cannot be a one-on-one relationship between ST and serotype. Rather, within a serotype, multiple STs must exist. For example, serotype III includes several major STs such as ST17 and ST19 as well as part of ST23 (Lyhs et al. 2016; Sorensen et al. 2010). The remainder of ST23 isolates mostly belongs to serotype Ia, showing that a single ST can include multiple serotypes (Sorensen et al. 2010). This is, however, an exception, and ST23 appears to consist of two sub-clades that are more distantly related than other clades that belong to different STs (Sorensen et al. 2010; Delannoy et al. 2016). Within most ST, a single serotype predominates. For example, ST17 isolates from humans were all serotype III and ST103 isolates from cows were serotype Ia (Lyhs et al. 2016). Horizontal transfer of the capsular biosynthetic genes may occur occassionally (Cieslewicz et al. 2005), and a minority of isolates within any given ST (other than ST23), will have a serotype that differs from the rest of the isolates within that ST, as seen in e.g. ST1 (mostly serotype V, rarely serotype Ia or II) or ST19 (mostly seroypte III, rarely serotype II) (Lyhs et al. 2016).

1.1.5 Foodborne disease

The World Health Organization (WHO) defines foodborne diseases as “those conditions that are commonly transmitted through ingested food” (World Health Organization 2007). GBS bacteraemia was strongly associated with eating raw freshwater fish in Hong Kong in the period from 1993 to 2012 (Ip et al. 2006) and in Singapore in 2015 (Tan, Lin, Foo, Ang, et al. 2016). GBS Serotype III Subtype 4 (III-4) raised public awareness of the foodborne potential of GBS. Serotype III-4 is found in ST283 (Delannoy et al., 2013). Tan et al. (2016) have studied the association of GBS Serotype III, ST283 bacteraemia with raw fish consumption in Singapore. Sashimi, sushi and Cantonese-style raw fish salad

called yusheng were considered risk factors in a case–control study of the GBS outbreak in 2015. Eating yusheng was the highest risk factor among the raw fish dishes because it was prepared with freshwater fish such as Asian bighead carp or snakehead fish (Tan et al. 2016) which should not be eaten raw. Sashimi and sushi are intended for raw fish consumption so they are prepared using strict hygienic practices. However, *S. agalactiae* was reported in marine fish species (Bowater et al. 2012), so eating raw saltwater fish products may still be a risk.

To detect ST283 GBS in fish markets, Kalimuddin and coworkers collected 43 raw fish samples from fish ports, wet markets (Figure 1-2), supermarkets, and eating establishments in Singapore and found 13 ST283-positive samples (30%). GBS isolates were recovered from yusheng-prepared with Asian bighead carp and grass carp, snakehead fish from a wet market. The source of the GBS in the freshwater fish may occur during their farming. Another possibility that the fish may be contaminated before being sold in fish markets or eating establishments is unlikely as food handlers and fishmongers did not carry ST283 (Kalimuddin et al. 2017). The emergence of zoonotic piscine ST283 as a cause of streptococcal meningitis and osteoarthritis not only has consequences for public health but also impacts on fish sales, as sales of raw fish dishes were suspended (Rajendram et al. 2016). Furthermore, control of hypervirulent piscine GBS should take place for sustainable food security and food safety. There is no report on cross contamination of ST283 from food handlers (Chau et al. 2017) but food hygiene measures should be followed strictly for microbial safety.

Food consumption has been associated with GBS colonization in non-outbreak risk factor studies too. Eating fish was associated with higher risk of GBS colonization by serotype Ia and Ib but not other serotypes. Meat (beef, pork and chicken), milk and dairy products (yogurt, ice cream and cheese) and eggs were not associated with GBS incidence. Moreover, cooked and raw vegetables were not related to GBS colonization in human. (Foxman et al. 2007).

Figure 1-2 Wet market in Singapore.
Photo permitted by Professor Ruth N Zadoks.

1.1.6 Zoonosis or anthroponosis

S. agalactiae strains can be transmissible from vertebrate animals to humans (zoonosis) or from humans to animals (anthroponosis). CC283 from fish has been proven to be zoonotic (Ip et al. 2016). Cattle-to-human transmission may also occur for some strains. Raw milk consumption or exposure to livestock may be a possible route of the interspecies transmission (Manning et al. 2010). The identical serotypes between humans and cows were reported (Brglez 1981; Lyhs et al. 2016; Manning et al. 2010). CC103 was previously described as the bovine-adapted strain which is most prevalent in Denmark (Zadoks et al. 2011), Norway (Jørgensen et al. 2016) and China (Yang et al. 2013). Between 2015-2017, ST485 (CC103) was frequently isolated from human patients in China (Wang et al. 2018; Li et al. 2018) and linkage to animals was unknown. To confirm cattle-to-human transmission, epidemiological connections should be investigated to confirm zoonotic GBS clones.

Human-to-animal transmission was reported for GBS in fish. Effluent contaminated with CC7 human GBS was related to the outbreak that occurred in Kuwait Bay in August 2001 (Jafar et al. 2008). *S. agalactiae* may be shed from the human urogenital or gastrointestinal tract to the environment causing disease transmission to fish (Delannoy et al. 2013). In the Mekong Delta, human urine and wastewater were used as fertilizer to produce natural food supplied to fish culture (Trung 2014). Fish pond toilets (Figure 1-3) and use of wastewater as feed in fish production should be practiced with caution to reduce the risk of anthroponotic diseases.

Figure 1-3 Open toilet in Mekong Delta.
Photo permitted by Professor Ruth N Zadoks.

1.1.7 Antimicrobial resistance

Antibiotics must be approved for disease treatment in food animal production including aquaculture (Miller, and Gieseker 2013). High volumes of antibiotic consumption accelerate the selection of antibiotic resistance in pathogenic bacteria of human or animal origin (Schwarz, Kehrenberg, and Walsh 2001; Aarestrup 2005). As a result, food animals are regarded as a reservoir of antibiotic resistance genes and resistant bacteria. Bacteria are intrinsically resistant to antibiotics, gene conferring antibiotic resistance existed before the introduction of antibiotics. However, the incidence and levels of antibiotic resistance in bacteria have changed since the increased application of antibiotics (Houndt and Ochman 2000). Antibiotics provide selective advantage (fitness) to resistant bacteria allowing them to persist in hosts (Austin, Kristinsson, and Anderson 1999). The volume of drug use is a major selection pressure driving the frequency of antibiotic resistance and selection of antibiotic resistance determinants might occur in the environment by chemical pollution with antibiotic residues (Alonso, Sanchez, and Martinez 2001). Acquiring resistance determinants is a mechanism in the evolution of a resistant pathogen to expand niches (Enright 2003). CDC defines both resistance genes and resistance mutations that give a microbe the ability to resist the effects of one or more drugs to be the resistance determinants: available at https://www.cdc.gov/narms/resources/glossary.html. Transfer of resistance determinants among bacteria of food animals and the resident human microflora has become a public health concern (Salyers, Gupta, and Wang 2004). To reduce the risk posed to humans by

antibiotic use in animal production, antibiotic residues in the edible tissues are controlled to lower selective pressure for antimicrobial resistance in the normal flora in the human gastrointestinal tract (Lee, Lee, and Ryu 2001; Mathew, Cissell, and Liamthong 2007). Some cost-effective agricultural technologies such as integrated fish farming, which uses farm animal waste as fertilizer providing natural feed for fish, encourage the shedding of antibiotic-resistant organisms into the environment (Shah et al. 2012; Xiong et al. 2015; Petersen et al. 2002). Using animal waste as fertilizer in integrated fish farming has been conducted in Thailand (Petersen et al. 2002; Andreas Petersen and Dalsgaard 2003), Pakistan, Tanzania (Shah et al. 2012) and China (Su et al. 2011). Studies on integrated fish farming have shown bacteria isolated from manure and pond sediment, such as *Enterococcus* species were resistant to antibiotics. There was no information on bacteria isolated from fish. The distribution of resistance determinants between animal manures, sediment samples and fish commensal bacteria was not addressed. Even though there is no clear evidence, integrated fish farming may need to be practiced with caution.

Similarly, antibiotic-resistance determinants have been detected from inflow, effluent and sludge from municipal sewage water treatment plants (Szczepanowski et al. 2009). Sewage treatment processes are unable to avoid the dissemination of resistant bacteria such as enterococci into the environment (Martins da Costa, Vaz-Pires, and Bernardo 2006). The use of antimicrobial agents in medicine, agriculture and anthropogenic activities can promote a selective pressure and spread of antimicrobial resistance in the bacterial community including the pathogenic bacteria. Thus, antimicrobial use for GBS control is a One Health issue. In addition, if selection for AMR in GBS in one host species is followed by transmission of GBS between host species, AMR in GBS itself can be considered a One Health problem. Antimicrobial resistance in GBS will be discussed in the next section (1.2.1.4).

1.2 Current control strategies and sustainability

1.2.1 Antimicrobial agents

1.2.1.1 Intrapartum antimicrobial prophylaxis (IAP) and antibiotics in human medicine

Intrapartum antimicrobial prophylaxis (IAP) has been developed to prevent vertical transmission of GBS during labour or delivery with the aim to control neonatal GBS infection. Either bacterial culture or risk factors for perinatal GBS disease are taken into

account before administration of IAP, depending on the country and its public health guidelines. Rectovaginal swabs at 35-37 weeks of gestation are used for the culture-based approach. GBS culture-positive mothers should be treated with IAP during labour. In case of unknown GBS culture status, including in countries where national health guidelines do not support routine screening (Hughes et. al 2017), a risk-based approach is used for IAP strategies (Verani, McGee, and Schrag 2010). Delivery at <37 weeks of gestation, having an intrapartum temperature >38.0ºC, or rupture of placental membranes for >18 hours are indications for IAP (Di Renzo et al. 2015). According to CDC guidelines on prevention of perinatal group B streptococcal disease, penicillin is the agent of choice for IAP but ampicillin is an acceptable alternative (Verani, McGee, and Schrag 2010). Penicillin or ampicillin is administered intravenously for the initial dose and then every 4 hours until delivery. If the woman is or claims to be penicillin-allergic, cefazolin is used as an alternative to penicillin. In patients with high risk of anaphylaxis, erythromycin or clindamycin are used. Vancomycin is offered in case of penicillin allergy and GBS resistance to erythromycin or clindamycin (Laiprasert et al. 2007; Verani, McGee, and Schrag 2010).

IAP successfully prevents early-onset disease but this intervention is not effective against late-onset disease (Jordan et al. 2008). Although IAP has benefits, the possibility of adverse effects and unintended consequences has raised concern. Allergic or anaphylactic reactions to penicillin, emergence of AMR in GBS (Chen et al. 2005), and increasing incidence of non-GBS sepsis, e.g. *Escherichia coli* in infants (Joseph, Pyati, and Jacobs 1998; Levine et al. 1999; Bizzarro et al. 2008) may result from routine screening and IAP use. In addition, there is growing concern about the possible impact of perinatal antimicrobial treatment on the infant microbiome (Jauréguy et al. 2004), as antimicrobial treatment in early life has been linked to a variety of disorders, including asthma, eczema and obesity, some of which may be life-threatening or affect quality of life (Kummeling et al. 2007; Cox and Blaser 2015).

Common clinical presentations of GBS disease in nonpregnant adults are skin, soft-tissue (Lee et al. 2005) and osteoarticular infections (García-Lechuz et al. 1999), pneumonia and urosepsis (Tyrrell et al. 2000). Meningitis and endocarditis are less common but associated with serious morbidity and mortality (High, Edwards, and Baker 2005). Penicillins remain the drug of choice. High doses of penicillin are indicated in serious GBS meningitis (Bayer et al. 1976). Similar to IAP, erythromycin and clindamycin are acceptable drugs in the case of penicillin allergy. Vancomycin, chloramphenicol and first-, second- and third-generation cephalosporins are effective alternatives (Farley 2001). The optimal duration of antibiotic

treatment against invasive group B streptococci is a minimum of 2 weeks. Longer courses, e.g. more than 4 weeks, may be necessary for endocarditis and osteomyelitis. In addition, a combination of aminoglycosides such as gentamicin with ampicillin or penicillin G has also been used in endocarditis (Farley 2001).

1.2.1.2 Antibiotics used and treatment of *S. agalactiae* in aquaculture

In contrast to the situation in dairy cattle, chemical and antimicrobial administration is intended to treat whole fish stocks rather than individual animals. The antimicrobial concentration in medicated feed must be precisely calculated to avoid antibiotic residue from uneaten feed. It is necessary to determine pond areas, water volume, feeding rate and total fish weight. Water solubility of antibiotics, duration of feed remaining in the water and size of pellets are important factors in leaching of drug into the water (Rigos, Alexis, and Nengas 1999). Palatability also plays a role in the successful administration of a medicated feed (Duis et al. 1995). Coating medicated feed with a special binder has been conducted to allow antimicrobials to stay on the pellets and increase palatability (Rigos, Alexis, and Nengas 1999; Duis et al. 1995).

Other factors should be considered before using antibiotics in aquaculture. Firstly, active fish will consume the feed while sick and inactive fish will be off feed. Thus in-feed medication tends to be a prophylactic rather than a therapeutic application. Secondly, antibiotic incorporation into feed must be homogenous and stable under the pelleting process. Third, antibiotic residues may be released to the environment from uneaten medicated pellets and fish metabolic waste, e.g. as a result of urine and gill elimination (Yu et al. 2009; Ranjan, Sahu, and Gupta 2017). Drug residue excreted by fish and drugs from pellets that have not been eaten are found in the sediment and the environment. Finally, bioavailability of antibiotics may be reduced due to interaction of the drug with water components. For example, divalent cations such as Mg^{2+} and Ca^{2+} in seawater bind to tetracycline. Consequently, the antibiotic is less effective and the dose may become subtherapeutic (Ranjan, Sahu, and Gupta 2017).

Chemicals used in aquaculture in Southeast Asia are oxolinic acid, oxytetracyclines, chlortetracycline, sulphonamides, and sulfamerazine. Some antibiotics may be allowed depending on country. Those include fluoroquinolones (sarafloxacin), ormethoprim and sulfadimethoxin & trimethoprim (ASEAN 2013). In contrast, nitrofurans and chloramphenicol are strictly prohibited in food-producing animals and aquaculture because

they present a carcinogenic risk to humans. Common antimicrobial treatments of *S. agalactiae* in fish farming are oxytetracyclin and erythromycin. Incorporation of these compounds into feed at 75 -100 mg/kg of oxytetracycline and 25-50 mg/kg of erythromycin for 4 to 7 days is effective against streptococcal infections (Saad 2011). Withdrawal periods are estimated from dose, route of administration and water temperature (Shao 2001; Concordet and Toutain 1997; Okocha, Olatoye, and Adedeji 2018). Accumulation and elimination of drug vary with fish species and temperatures (Chen et al. 2004). Oxytetracylcine use in finfish is usually associated with a withdrawal time of 17-18 days for oral administration and 12.5 -16 days for medicated bath (Choo 1995). Importantly, the public health hazards related to antimicrobial use in terrestrial animals and aquaculture are a global concern (WHO 2015). Development and spread of antimicrobial resistant bacteria and resistance genes and the occurrence of antimicrobial residues in products are critical to both human and non-human health. Therefore, alternative control strategies are needed, including e.g. vaccines (section 1.2.2) and novel treatment compounds (section 1.3).

1.2.1.3 Antibiotics used in GBS bovine mastitis

Use of antibiotics in bovine mastitis consists of treatment in lactating and nonlactating ("dry") cows. Antibiotics must have a low degree of binding to milk and udder tissue protein for fast drug distribution. *S. agalactiae* remains in the milk ducts rather than in the udder parenchyma. In general terms, inntramammary treatment (IMM) is the preferable route of administration with intramuscular treatment (IM) as second choice, in part because of the amount of drug used to treat an individual animal (much higher for IM treatment than IMM treatment) (Hillerton and Kliem 2002). In addition, after IM administration, excretion of drugs into the lipophilic environment of the mammary gland can be limited whereas IMM drugs results in higher local concentration in the mammary gland where *S. agalactiae* is present (Pyörälä 2006). Some penicillin derivates have been especially modified to make them lipophilic, resulting in good excretion into the udder and high cure rates for streptococcal mastitis (St Rose et al. 2003). IMM shows a better efficacy in treatment and lower amount of antibiotic used than IM (Reyes et al. 2015). Penicillin G was and still is the drug of choice for treatment of bovine mastitis (Wilkinson 1965) in many countries because of their narrow spectrum and high efficacy against GBS. In some emerging economies, e.g. in China and Colombia, reports of treatment failure and penicillin resistance are emerging (Reyes et al. 2015; Hu et al. 2018). In humans, macrolides have been used as drugs of 2nd choice and macrolide antibiotics have also been used for mastitist treatment

(Barkema et al. 2006). WHO has classified macrolides as Highest Priority Critically Important Antimicrobials for humans (https://www.who.int/foodsafety/cia/en/), which means that they should not be used in animal agriculture anymore. Using antibiotics during the lactating period may increase economic cost because the withdrawal period for milk is 4-5 days. However, this cost is offset by improvements in milk quantity and quality when mastitis is treated successfully (Edmondson 1989; Erskine and Eberhart 1990). In nonlactating cows, there is less concern about withdrawal period and antibiotic residue in milk. Antibiotic intramammary formulations are commercially available (Gruet et al. 2001).

1.2.1.4 Occurrence of antimicrobial resistance and consequences

Among GBS human clinical isolates, the prevalence of tetracycline resistance was 84.6% in the United States (Dogan et al. 2005). Erythromycin and clindamycin resistant strains were at 32% to 15%, respectively (Phares et al. 2008). The tetracycline resistance gene *tet*(M) was predominantly found in all tetracycline-resistant human isolates (Dogan et al. 2005; Da Cunha et al. 2014). *Erm*(B), *erm*(TR), and *mef*(A) were found among macrolide resistant isolates (Dogan et al. 2005). Some resistance genes encode resistance against multiple antimicrobial compounds in GBS and other bacterial species. Macrolide, lincosamide and streptogramin B (MLS_B) antibiotics have a different structure but similarly inhibit bacterial protein synthesis by binding to 23s rRNA in 50S ribosomal subunits. The presence of erythromycin resistance methylase (*erm*) genes can modify ribosomal methylation and lead to reduced binding of any member of the group of MLS_B antibiotics to the target site. Increased rates of constitutive and inducible resistance to MLS_B antibiotics may limit treatment options or lead to clinical failure of treatment (Heelan, Hasenbein, and McAdam 2004).

Tetracycline and erythromycin resistance among GBS bovine isolates was less common than among human GBS isolates. In the United States, tetracycline and erythromycin resistant strains were at 14.5% and 3.6%, respectively, for temporally and geographically matched human and bovine isolates (Dogan et al. 2005). Brazilian GBS bovine isolates were resistant to tetracycline and erythromycin at 44.7% and 10.5%, respectively (Duarte et al. 2004). Unlike human isolates, the most common tetracycline resistance gene was *tet*(O). Erythromycin resistance was predominantly due to *erm*(B) (Duarte et al. 2004). Emergence of tetracycline and erythromycin resistance appears to largely occur independently among human and animal isolates (Dogan et al. 2005; Duarte et al. 2004; Da Cunha et al. 2014). Dogan et. al. reported a bovine GBS isolate that carried *tet*(M), which is usually found in

human GBS (Dogan et al. 2005). This suggested a human GBS subtype harbouring a *tet*(M) gene can transfer to a cow.

In aquaculture, a subtype of serotype III (III-4) GBS isolated from tilapia in Thailand was resistant to tetracycline. The tetracycline resistance gene was *tet*(M), which was carried by a conjugative transposon and its integrase (*int*-Tn) (Suanyuk et al. 2008). Interestingly, the *tet*(M) gene was frequently detected in the human GBS genomes (95%) but only in 25% of bovine isolates and 11.8% of the piscine GBS strains (Dangwetngam et al. 2016). Distribution of *tet(M)* among GBS is not limited to serotype III. Tetracycline resistance genes were identified in GBS isolates representing various serotypes (Dogan et al. 2005). Da Cunha and colleagues provided evidence that the use of antibiotics has resulted in evolutionary bottlenecks and the emergence of human-pathogenic clones (Da Cunha et al. 2014). CC17 was rare before the use of tetracycline. After tetracycline was first used in 1948 and subsequently used widely, the incidence of neonatal GBS infections has increased. Hypervirulent CC17 strains in neonates carry integrative and conjugative elements (ICEs) containing the tetracycline resistance gene *tet*(M) (Da Cunha et al. 2014). ICE conferring tetracycline resistance (TcR) are mostly Tn*916* and Tn*5801* (Da Cunha et al. 2014). The transposition and the transfer functions of Tn916 proceed by the transcription of an operon containing *tet*(M) and the regulatory genes. Tetracycline induces and increases the frequency of conjugative transposition of Tn916 (Celli and Trieu-Cuot 1998). Even though the use of tetracycline has been reduced, the prevalence of tetracycline resistance remains remarkably stable. After TcR is inserted in the GBS genome, Tn*916* and Tn*5801* genes are maintained (Violette Da Cunha et al. 2014). The *tet*(M)-associated TcR is very common in human GBS strains but less frequent in bovine GBS strains (Da Cunha et al. 2014). This conjugative element leads to recombinant events causing the diversification of GBS lineages. Da Cunha et al. found all CC17 strains have acquired Tn916 or Tn5801 and a low rate of recombination. In summary, the use of antibiotics can create selective pressure for clonal expansion of pathogenic clones in humans.

1.2.2 Vaccination

1.2.2.1 Human GBS vaccination

Vertical transmission of GBS was estimated at 29-85% (Baker 1997). Neonates that acquire GBS from colonized mothers can be asymptomatic carriers and 1-2% of infants develop an invasive disease (Baker and Edwards 1988). GBS polysaccharide capsule is a major virulence factor in human infection (Rubens et al. 1987). Antibody to GBS was found to be

serotype-specific (Smith et al. 1990). To eliminate GBS infection, antibody binds to GBS and subsequently activates complement pathway mediated phagocytosis (Edwards et al. 1980). Baker and Kasper investigated the difference in level of specific antibodies in maternal sera at delivery between GBS carriers whose infants were healthy or had invasive GBS disease. A low level of antibodies to the capsular polysaccharide (CPS) of type III GBS in maternal serum was strongly associated with invasive neonatal GBS infection (Baker and Kasper 1976). IgG transferred from mother to neonates through umbilical cord (Baker and Kasper 1976) can reduce risk of EOD (Troendle et al. 2001) and LOD (Rinaudo et al. 2016). The active immunisation of pregnant women could prevent GBS invasive disease in neonates and infants (Baker and Edwards 2003). Capsular polysaccharide has been a first vaccine candidate because the CPS-specific antibody can be transferred from mother to newborns (Baker and Kasper 1976). However, CPS-based vaccines do not provide cross protection between serotypes (Paoletti et al. 1994).

In order to develop sufficient coverage against the prevalent global serotypes, multivalent vaccines are needed. Conjugate polysaccharide vaccine has been established to increase the CPS immunogenicity (Baker et al. 1999). For protein-based vaccines, alpha and beta components of the C protein complex, protein Rib (resistance to proteases, immunity, group B) (Stalhammar-Carlemalm, Stenberg, and Lindahl 1993), Sip (surface immunogenic protein) and C5a peptidase are highly conserved in human GBS strains. These proteins were investigated as potential vaccine candidates (Larsson, Stålhammar-Carlemalm, and Lindahl 1996; Xue et al. 2010). Recently, new approaches to GBS vaccinology have been developed, including reverse vaccinology. Genome sequences of GBS from different serotypes were analysed to identify genes encoding putative surface-associated and secreted proteins (Johri et al. 2006). The newly identified antigens have been characterised into three putative pilus-like structures (Galeotti et al. 2009). GBS pili have shown to be promising multivalent live vaccine candidates (Buccato et al. 2006).

However, challenges in GBS vaccine development are 1) The distribution pattern of the prevalent serotypes and sequence types in all global regions should be monitored. 2) Vaccine given to pregnant women must be safe with low risks of birth defects and the potential for subsequent liability. 3) Placental antibody should reach a seroprotection threshold in neonates (Doare et al. 2019). 4) Alteration of the main vaccine target can occur by capsular switching which should be considered for future vaccination strategies (Bellais et al. 2018; Martins, Melo-Cristino, and Ramirez 2010).

In summary, vaccine development can offer a promising strategy in GBS control and eradication. However, maternal GBS vaccination is awaiting public acceptance and licensure pathways (Kobayashi et al. 2016). Considering that CPS-based vaccines may not cover all relevant serotypes, additional control strategies to specifically kill GBS regardless of their serotypes are of interest. Biologically active peptides of bacterial origin and the control of GBS metabolic pathways are selected to be a novel GBS treatment in this study (section 1.3).

1.2.2.2 GBS vaccination in aquatic animals

Administration routes for vaccine in finfish production comprise immersion, oral route and injection. Formalin-killed cells or concentrated extracellular products have been used to develop GBS vaccines for fish farms. The experimental vaccinations offer significant protection with a relative percent of survival (RPS) rate of 80% at 30 days post vaccination but coverage strikingly dropped in 180 days (Evans, Klesius, and Shoemaker 2004). Commercial GBS vaccines manufactured by MSD Animal Health (Madison, USA) are AQUAVAC® Strep *Sa* to protect against piscine *S. agalactiae* biotype 2 (serotype Ib, non-haemolytic strains) and AQUAVAC® Strep *Sa*1 to protect against and biotype 1 (serotype Ia and III, haemolytic strains). AQUAVAC® Strep *Sa* contains inactivated *S. agalactiae* with a water-in-oil emulsion and metabolizable non-mineral oil adjuvant. This vaccine provides protection against non-haemolytic strains which have global prevalence in the major tilapia-producing countries in Asia (Indonesia, China, Vietnam and the Philippines) and Latin America (Ecuador, Honduras, Mexico and Brazil). Injection of 0.05 ml intraperitoneally in fish of 15 gram or more results in at least 30 weeks of protection. AQUAVAC® Strep *Sa*1 is an oil adjuvanted vaccine. Administration of vaccine is the same as for AQUAVAC® Strep *Sa*, except that fish must weigh >10g. Protection lasts 12 weeks post vaccination. Information on AQUAVAC® vaccines is available at https://www.aquavac-vaccines.com/products/.

However, these commercial vaccines have no cross protection between non-haemolytic and haemolytic strains. Injectable vaccines are costly and time-consuming. Future trends in vaccines in aquaculture are development of feed-based, DNA and live attenuated vaccines as well as immunomodulatory compounds (Brudeseth et al. 2013). Oral vaccines in feed must ensure that antigen is resistant against gastric degradation and available for uptake in the gut. Consequently, encapsulation technology has been introduced in oral vaccination (Embregts and Forlenza 2016). Use of DNA and live attenuated vaccines should be

practiced with caution in terms of Genetically Modified Organism (GMO) regulations and the possible reversion to virulence (Brudeseth *et al*, 2013).

Importantly, specific humoral immunity in fish is different to mammals. The predominant immunoglobulin in fish is IgM. IgM has low affinity to multiple antigens and no isotype switching from IgM to IgG resulting in lack of immune recognition (Watts, Munday, and Burke 2001). Given that, an enhanced secondary exposure to antigen is necessary in fish immunoprophylaxis. Vaccination is a promising GBS control method but it may be costly and its efficacy is dependent on the development of immune competence in the vaccinated fish and is serotype specific. By contrast, bacteriocin may potentially be applied to all serotypes, it could work in fish that are immunocompromised, e.g. due to heat, pollution or low oxygen levels, and it would only be needed when there is an imminent threat of disease. This makes the connection between the investment and the effect more directly visible to farmers and could enhance uptake. Potential novel antistreptococcal strategies will be described in a following section (1.3.1).

1.2.2.3 Bovine mastitis vaccination

The immune response of the bovine mammary gland consists of innate and adaptive immune systems (Schukken et al. 2011). The innate defence mechanisms are physical barriers (teat sphincter), chemical barriers (keratin and lactoferrin), and immune cells (macrophages and leukocytes) (Riollet, Rainard, and Poutrel 2000). Specific receptors on host immune cells (Toll-like receptors: TLRs) play a role in recognizing specific conserved pathogen-associated molecular patterns (PAMPs) (Farhat et al. 2008). PAMPs of bacterial species trigger different host immune response and cytokine products (Gunther et al. 2011; Schukken et al. 2011). In the adaptive immune response, antigen-specific B cells with memory functions are responsible for antibody production (Kehrli and Harp 2001). Immunoglobulin is important in opsonisation, aiding phagocytosis of bacteria. The cell-mediated immunity and immunoglobulin classes of antibodies have been researched in bovine mastitis-vaccine development. In non-vaccinated and vaccinated heifers challenged with GBS in the udder, at least two different classes of antibodies were involved in humoral immune response. In early infection, IgA antibody is predominant in infected quarters but is of short duration. IgA antibody is the frontline response to limit bacterial multiplication in the udder quarter. In case of reinfection, IgG_1 class was elevated in serum (Logan, Mackie, and Meneely 1986; Trigo et al. 2008). Similarly, a second dose of GBS vaccine was required to produce IgG antibody (Logan, Mackie, and Meneely 1986;

Rainard et al. 1991). A surface protein, e.g. protein X (Rainard et al. 1991), the B cell epitopes of the surface immunogenic protein (Sip) (Xu et al. 2011) and the recombinant CAMP factor (Liu et al. 2017) were evaluated as potential vaccine candidates against GBS bovine mastitis.

Current control of GBS mastitis is largely based on prevention of transmission and antibacterial treatment. This approach has been very successful in developed countries (Jorgensen et al., 2016) but is difficult to implement in low and middle-income countries (Cobo-Angel et al., 2018). In addition, there is concern about poor response to treatment of bovine GBS in some countries, e.g. Colombia (Reyes-Valdez et al., 2017). Therefore, alternative control strategies for bovine mastitis are still of interest. Direct killing of bacteria at the site of infection might offer more effective treatment of bovine mastitis. Antimicrobial peptides of bacteria origin are candidates to inhibit bacterial infection (section 1.3.1).

1.3 Alternative control strategies

1.3.1 Bacteriocins

Administration of antibiotics can lead to several problems as mentioned in section 1.1 and 1.2. In order to reduce antibiotic consumption and its consequences, antimicrobial peptides of bacterial origin are promising alternatives to antibiotics (Cotter, Ross, and Hill 2012). Bacteriocins are distinctly different from conventional antibiotics in synthesis and mode of action (Kolter and Moreno 1992). These antimicrobial peptides are synthesized on ribosomes by the translation process. Physical and chemical characteristics of the target cell membrane allow bacteriocins to have highly specific activity against targeted bacterial species (Baba and Schneewind 1996; Oscáriz and Pisabarro 2001) while causing no harm to the host cells (Baba and Schneewind 1996). Common mechanisms of action of bacteriocins are inhibiting cell wall synthesis or RNase or DNase activity, or permeabilizing the target cell membrane (Jack, Tagg, and Ray 1995). Changes in cell surface properties can affect the susceptibility of bacteria to bacteriocin (Crandall and Montville 1998). Bacteriocins can be produced by gram-positive and gram-negative bacteria. Classification of bacteriocins from gram-positive bacteria relies on structural features and bacteriocins of gram-negative bacteria are classified based on functions such as uptake and killing mechanisms. The first classification of bacteriocins produced by lactic acid bacteria (LAB) defined four classes (I, II, III and IV). It was proposed by Klaenhammer (1993) and revised in 1996 and 2000 (Nes et al. 1996; Nes and Holo 2000).

However, new bacteriocins have been discovered and they needed new classification. Cotter and colleagues proposed the revised bacteriocin classification in 2005 (Cotter et al. 2005). Details of classification of bacteriocins from gram-positive bacteria will be described in Chapter 3.

Bacteriocins have been applied in food preservation and they can potentially be applied in clinical settings (Cotter, Ross, and Hill 2012; Bastos, Coutinho, and Coelho 2010). Colicins produced by many *Escherichia coli* strains and colicin-like bacteriocins produced by other gram-negative bacteria showed potential in treatment of chronic bacterial infection (Brown et al. 2012). Colicins (E1 and E9) showed potent activity against Crohn's disease (CD) caused by adherent-invasive *E. coli* (AIEC) (Brown et al. 2015). Pyocin S2 produced by *Pseudomonas aeruginosa* (Denayer, Matthijs, and Cornelis 2007) displayed potent activity against growth of clinical isolates of *P. aeruginosa* in biofilms, suggesting Pyocin S2 may be useful for the treatment of chronic lung infections associated with cystic fibrosis (CF).

Nisin is a lantiobiotic (Class I) bacteriocin produced by *Lactococcus lactis* that is applied in food production. The mode of action is pore formation resulting in bacterial cell leakage. Nisin has been used to control *Listeria monocytogenes* in cheese (Ferreira and Lund 1996), *Bacillus cereus* spores in skimmed milk (Wandling, Sheldon, and Foegeding 1999) and lactic acid bacteria (LAB) that cause spoilage of Bologna-type sausage (Davies et al. 1999). However, activity of nisin is influenced by environmental conditions, e.g. pH (Liu and Hansen 1990). The effectiveness of nisin increased in acidic conditions and it lacked activity in cured meat due to high pH (Rayman, Malik, and Hurst 1983). Another bacteriocin, pediocin (Class II) was used in meat as alternative to nisin (Nielsen, Dickson, and Crouse 1990). Application of nisin in animal production and aquaculture is limited. There were reports on attempts to use nisin in treatment of subclinical mastitis (Wu, Hu, and Cao 2007) and to inhibit the fish pathogen *Lactococcus garvieae* (Sequeiros et al. 2015).

Bacteriocins which are highly selective for *S. agalactiae* have not been identified. GBS control strategies should have high specificity to avoid the disruption of microbiota or development of antimicrobial resistance. For use in humans, the potential to target GBS without disrupting the maternal or neonatal microbiota is of interest. Administration of bacteriocin in fish production may lower the risk of antimicrobial resistant bacteria or AMR genes being released into the environment because of its specific mode of action.

Thus, bacteriocins active against *S. agalactiae* might be a promising GBS control tool with low toxicity and a low likelihood of the development of antimicrobial resistance.

1.3.2 Metabolic pathways as potential targets for dietary manipulation and vaccine development

1.3.2.1 Host adaptation

Bacteria show diversity in genome content and metabolic capabilities for fitness and lifestyle in the host environment. Functional diversity of bacteria evolves to exploit substrates in the environment (Gravel et al. 2010). Generalist bacterial lineages have high ability to exploit a large number of substrates (Gravel et al. 2010). They are more likely to infect multiple host species (Woolhouse, Taylor, and Haydon 2001). In contrast, specialist bacterial lineages grow in selective environments and have more restricted niches (Buckling, Wills, and Colegrave 2003). Niche restriction enables bacteria to have a pathogenic lifestyle and strict host specificity (Pascopella et al. 1995; Sørensen et al. 2010).

Genetic diversity affecting genes involved in the host-pathogen interaction results in the evolutionary advantages of niche adaptation (Woolhouse, Taylor, and Haydon 2001). Genomic rearrangements due to acquisition and loss of genes can either promote virulence or disrupt function (Champion et al. 2009). Mobile genetic elements (MGE) such as genomic islands (GIs), insertion sequences and transposons may enhance the fitness and pathogenicity of bacterial strains. Lactose metabolism (Lac.2) is an important niche adaptation to the bovine mammary glands (Richards et al. 2013). The lactose operon appears to have been acquired via lateral gene transfer (LGT) (Richards et al. 2013). Pseudogenisation and recombination in the genomes contribute to genome degradation or gene inactivation (Rohmer et al. 2007). Deletion of genes could lead bacteria to cooperate with their hosts or evade host defense mechanisms (Ochman and Moran 2001). Pseudogenes were identified in sortase and serine protease cspA genes of a CC552 piscine GBS. Moreover, human GBS strains possess an inactive sialidase (NonA) retaining the sialylated capsule to inhibit activation of the complement and anti-opsonophagocytosis, (Yamaguchi et al. 2016; Wessels et al. 1989). The *NonA* in human *S. agalactiae* may evolve to a pseudogene (Yamaguchi 2018).

Delannoy et al. (2016) provided comparative genomic analysis of piscine, human and bovine *S. agalactiae* isolates and showed that CC552 piscine GBS has reduced genome content. The *cyl* operon was incomplete in ST260 which explained the non-haemolytic phenotype by

the disruption of hemolysin production (Delannoy et al. 2016). Genes encoding the virulence determinant Pilus island (PI-2b) in ST260 isolated from fish were truncated due to a pseudogene and introduction of stop codons. While pseudogenisation or gene loss occured, there was also gene gain in fish isolates, similar to the gain of the lactose operon in bovine isolates. Locus 3, which is thought to be involved in the transport and degradation of galactose via the Leloir pathway, was found in all *S. agalactiae* from fish, including in non-haemolytic (CC552) and haemolytic strains (CC7) (Delannoy et al. 2016). Galactose metabolism may relate to fitness of piscine GBS in fish brain, where galactose is a component of glycolipids and glycoproteins. However, the functionality of locus 3 and metabolic markers are not known. ST283 piscine *S. agalactiae* and its single-locus variant ST491 isolated from fish shared the presence of the C-alpha encoding gene as well as identical mobile genetic element (MGE) profiles to GBS isolates from invasive disease in human adults in Southeast Asia (Delannoy et al. 2013). Further study on host adaptation and specific metabolic pathways may contribute to the finding of new targets for highly selective antimicrobial compounds and to the development of a GBS vaccine which provides coverage for the zoonotic strain ST283.

1.3.2.2 Determination of the metabolic diversity of GBS isolates using Biolog phenotype microarray

Phenotype microarrays offer several carbon sources which can be used to identify both the organism and its underlying metabolic pathways. Biolog manufactures a broad range of products to support research involving phenotype microarray for microbial cells. Shea *et al.* described information of Biolog phenotype microarrays and their application: "Biolog's third-generation microbial ID system (GEN III) contains 94 biochemical tests. The biochemical tests comprise 71 carbon sources, including sugars, hexose phosphates, amino acids, hexose acids, carboxylic acids, esters and fatty acids as well as 23 chemical sensitivity assays. Tetrazolium redox dyes are used to indicate utilization of the carbon sources or resistance to inhibitory chemicals. Tetrazolium is colourless water soluble molecule and changes colour into purple when it is reduced. Reduction of tetrazolium irreversibly forms a formazan which is insoluble crystal." Metabolic activities can be tracked by cell viability during cultivation in the different carbon sources. Succinate dehydrogenase (SDH) enzyme complex of viable cells oxidizes succinate to fumarate during the citric acid cycle coupling with the electron transport system. Tetrazolium salts accept electrons when SDH is oxidized in the electron transport system. Reduction of tetrazolium salts forms a water-insoluble violet formazan (Stubberfield and Shaw 1990). The formazan product is used as the indicator for the cell growth. If a specific carbon source is utilized by

bacteria and supports their growth, this can be seen as a purple colour. Colorimetric response to metabolites can be measured using a standard microplate reader or the Biolog-supplied Omnilog to support identification and characterization of bacterial pathogens (Shea et al. 2012).

In this study, Biolog GEN III microplate is applied for metabolic profiling to determine metabolic diversity of human, bovine and piscine *S. agalactiae*. Metabolic profiles provide insight into metabolic specificity of piscine isolates which may potentially link their metabolic pathway to specific genes. Findings shall be of benefit to gain understanding of host adaptation in order to develop novel GBS control strategies for aquaculture.

1.4 Aims of the PhD project

1.4.1 Explore novel GBS control strategy

This project's main aim was to find an improved *S. agalactiae* treatment to reduce the need for antimicrobial treatment leading to selection for antimicrobial resistance and its consequences. *S. agalactiae* isolates from three host categories (human, dairy cattle and fish) were examined for production of bacteriocin. CC552 fish-specific GBS was excluded from this work because it is not recently described as a potential threat to humans. A bacteriocin from *S. agalactiae* was identified, characterized and tested for efficacy *in vitro* and *in vivo*. The project findings shall demonstrate potential of GBS bacteriocins to be pathogen-specific antibiotics as follows: 1) providing coverage against GBS of multiple sequence types across human, bovine and piscine host species, 2) having activity towards antimicrobial resistant strains and 3) having the potential to reduce the use of conventional antibiotics.

General materials and methods used for the project are described in Chapter 2. Detection of bacteriocin-producing strains, identification of bacteriocin genes, plasmid construction, protein expression and purification and killing spectrum are described in Chapter 3. Evaluation of the efficacy of the bacteriocin *in vivo* using a *Galleria mellonella* model is described in Chapter 4 and is compared with conventional antibiotics. Moreover, this project is the first to study GBS multiplication and distribution as well as the degradation of an antimicrobial peptide in the *G. mellonella* larval model.

1.4.2 Explore host-adaptation through metabolic profiling

To gain better understanding of the fitness of the zoonotic piscine GBS in humans and fish, metabolic pathways based on carbon utilization were examined. This study did not cover CC552 because we focused on zoonotic strains. *S. agalactiae* isolates from three host species, representing several major CCs, were biochemically tested on the Biolog GEN III microplate assay. After identification of significant differences in utilization of carbon sources, a selected beta-glucoside (salicin) was further tested in an attempt to find an association with virulence factors in GBS pathogenesis which focused on the structural analogue of a host tissue component (hyaluronic acid) and hyaluronidase (related virulence factor). Metabolic profiling provided novel phenotypes of piscine GBS with potential to further investigate for vaccine development or metabolic manipulation to control GBS in aquaculture. Identification of phenotypic markers of niche adaptation using metabolic profiling is described in Chapter 5. Further exploration of metabolic pathways in major meningitis-associated strains of GBS (ST283 and ST17) and hypotheses on the role of specific virulence factors are described in Chapter 6. A brief synthesis of results and suggestions for further research are given in Chapter 7.

Chapter 2 Materials and methods

2.1 Reagents

Chemicals and reagents used in this work were purchased from Sigma-Aldrich or Thermo-Fisher Scientific unless stated otherwise. Biolog microplates and inoculating fluids were purchased from Biolog (Hayward, California, USA).

2.2 Bacterial strains and growth media

2.2.1 Bacterial strains

Streptococcus agalactiae strains used in this work are shown in Appendix i (Table A-1). The one hundred and eleven strains of *S. agalactiae* were characterised by multilocus sequence typing (MLST) profiling by Professor Ruth N. Zadoks (the Institute of Biodiversity Animal Health and Comparative Medicine, University of Glasgow). Piscine GBS isolates (STIR CD isolates) were provided by Dr Margaret Crumlish (Institute of Aquaculture, University of Stirling). *S. agalactiae* were grown aerobically in brain heart infusion (BHI) broth at 37°C for 24 h with orbital shaking at 170 RPM, except ST260 strains which were grown at 28°C for 48 h. To obtain isolated colonies, *S. agalactiae* were grown on 5% sheep blood agar plates (E&O Laboratories, Bonnybridge, United Kingdom) under the same temperature and time.

Other bacterial species used in evaluation of the spectrum of GBS bacteriocin are shown in Appendix i (Table A-2). *Streptococcus dysgalactiae* subsp. *dysgalactiae*, *Streptococcus canis and Streptococcus uberis* were provided by Professor Ruth N. Zadoks. *Enterococcus faecalis* and *Enterococcus faecium* were provided by Professor Daniel Walker (the Institute of Infection, Immunity and Inflammation, University of Glasgow). *Lactobacillus jensenii*, *Lactobacillus crispatus* and *Lactobacillus gasseri* were purchased from the Belgian Coordinated Collections of Microorganisms (BCCM) (Gent, Belgium). Closely related streptococci and enterococci were aerobically grown in BHI broth at 37°C for 24 h with orbital shaking at 170 RPM. Three *Lactobacillus* type strains were grown under conditions following the instruction of the Belgian Coordinated Collections of Microorganisms (BCCM). *L. jensenii*, and *L. crispatus* were aerobically grown in De Man, Rogosa, Sharpe (MRS) broth at 37°C for 24 h. *L. gasseri* was anaerobically grown in MRS broth at 30°C for 48 h without shaking. Bacteria colonies were grown on agar plates and stored in 20% glycerol at -80°C.

2.2.2 Antibiotic susceptibility profile of *S. agalactiae*

Streptococcus agalactiae isolates were tested for susceptibility to antibiotics using the disc diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI) Antimicrobial susceptibility discs were purchased from Oxoid (Thermo Scientific™ Oxoid™, UK). Concentrations of antibiotics in discs and zone diameter interpretative criteria are shown in Table 2-1. *Streptococcus pneumoniae* ATCC® 49619 was used as a quality control strain. *Streptococcus agalactiae* were streaked on 5% sheep blood agar. Plates were incubated under the growth conditions in section 2.2. Colonies were suspended in 0.85% saline. Inoculum density was standardized to OD_{625} = 0.08 to 0.13 (equivalent to a 0.5 Mcfarland standard or approximately 1 to 2 x 10^8 CFU/ml). Three hundred microliters of inoculum were dispended and spread on Mueller – Hinton agar (MHA) with 5% sheep blood. Once the surface was dry, discs were placed on plates. Plates were incubated under the same growth conditions as colony culture. Zone of complete inhibition including the diameter of disc was measured in millimetres. Zone diameters from triplicate tests are shown in Appendix ii. Findings from antibiotic susceptibility were used as characteristic profile of test stains in Chapter 3 and 4.

Table 2-1 Antibiotic concentrations and CLSI interpretative criteria for disc diffusion method.

Antibiotics	disc content	zone diameter interpretative criteria (mm)		
		Susceptible (S)	Intermediate (I)	Resistance (R)
penicillin G	10 units	≥ 24	-	-
ampicillin	10 µg	≥ 24	-	-
erythromycin	15 µg	≥ 21	16-20	≤ 15
clindamycin	2 µg	≥ 19	16-18	≤ 15
tetracycline	30 µg	≥ 23	19-22	≤ 18
ceftriaxone	30 µg	≥ 24	-	-

Note: a dash mark (-) indicates the interpretative criteria are not applicable. In the case where only susceptible criteria exist, means intermediate or resistance criteria cannot be defined because the absence or rare occurrence of resistant strains to those antibiotic agents.

2.3 Detection of bacteriocin-producing strain

The bacteriocin-producing strain was detected by antagonism assay as previously described by Fyfe (Fyfe, Harris, and Govan 1984). The method for identifying GBS bacteriocin was modified by eliminating the use of chloroform evaporation. Briefly, a single colony of each isolate was inoculated on BHI agar. Plates were incubated overnight at 37°C. GBS from various clonal complexes and sequence types isolated from humans, cows and fishes were selected and used as the indicator strains (Appendix i, Table A-3). The indicator strains were grown in BHI broth at 37°C. Fifty millilitres of mid-log phase culture were added to 5 ml of 0.7% soft agar and subsequently overlaid on plates. After overnight incubation, the bacteriocin-producing strain was detected by a zone of inhibition. To identify putative bacteriocin genes, the genomic sequence of a producing strain was analysed using the Bacteriocin mining tool: Bagel 3, accessible at http://bagel2.molgenrug.nl/index.php/bagel3. This gene or its protein sequence was used for plasmid synthesis in section 2.4.1.

2.4 Plasmid construction, expression and purification of agalacticin A

2.4.1 pZoo1 plasmid

The gene of agalacticin A (Appendix iii) was used for plasmid construction. pZoo1 which contains the open reading frame of agalacticin A in the pj404 vector with unique NdeI and XhoI sites at the start codon and in place of the stop codon, respectively, was synthesized by DNA 2.0 (ATUM, California, USA) (Figure 2-1). This plasmid encodes a C-terminal His_6 tag and ampicillin resistance.

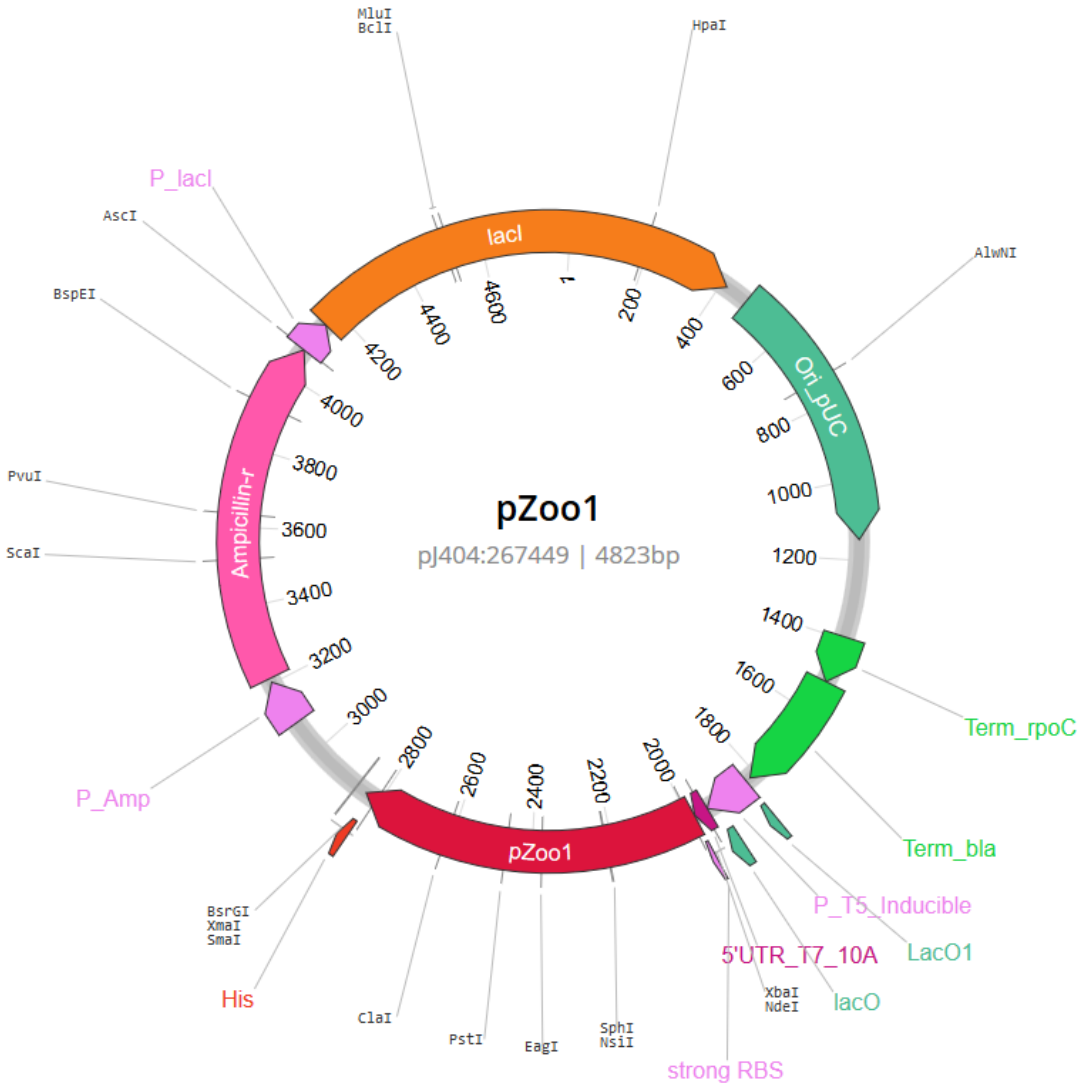


Figure 2-1 pZoo1 plasmid map.
pZoo1 encodes agalacticin A, with expression under the control of a T5 promoter.

2.4.2 Cloning expression and purification of agalacticin A

Agalacticin A was expressed in *E. coli* BL21 (DE3) carrying the plasmid pZoo1. Cells were grown in LB at 37°C. At an OD_{600} of approximately 0.6, protein expression was induced by adding 0.3mM D-isopropyl-β-thiogalactopyranoside (IPTG). Cells were grown for a further three hours. The cell pellet was collected by centrifugation at 5000 RPM at 15°C for 15 min. Cells were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl). Cells were lysed by ultrasonication with 2 mg/ml lysosome and a complete EDTA free protease inhibitor cocktail tablet (Roche, Mannheim, Germany). The cell extract was clarified by centrifugation at 18000 RPM at 4°C for 20 min. Supernatant was applied to a HisTrap™ Nickel column (GE Healthcare, Uppsala, Sweden) equilibrated in 5 mM imidazole, 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl. Bound protein was eluted with a linear gradient of 20–600 mM imidazole in lysis buffer. Fractions containing agalacticin A were analysed by SDS-PAGE and pooled then dialysed overnight into 50 mM Tris-HCl, pH 7.5,

20 mM NaCl at 4°C. Agalacticin A was further purified by size exclusion chromatography. Dialysed sample was loaded onto a Superdex S75 26/60 column, (GE Healthcare). Fractions were analysed by SDS-PAGE. Purified fractions were combined and concentrated using 5 kDa molecular weight cut-off centrifugal concentrator.

For the wrok involving *Galleria mellonella*'s, agalacticin A fractions from size exclusion chromatography were combined and dialysed in phosphate buffered saline (PBS) to remove Tris which is toxic to *G. mellonella* larvae. Dialysed agalacticin A was aliquoted and frozen at -80°C.

2.4.3 SDS-PAGE

Protein samples were mixed with SDS sample buffer (Invitrogen) at a 4:1 ratio. Samples were heated at 95°C for 5 minutes and centrifuged at 12000 g for 1 min. Samples were loaded onto the 12% Tris–glycine gel (Novex™ WedgeWell, Thermo Fisher Scientific). Blue prestained protein standard, broad range of 11-190 kDa (NEB, UK) was used for molecular weight determination. SDS-PAGE was performed using NuPAGE™ MOPS SDS Running Buffer (50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.7) (Thermo Fisher Scientific) at 30 mA. Gel was stained in Coomassie blue at room temperature for 20 min. Coomassie blue staining solution contains 0.1% (w/v) Coomassie blue R350, 20% (v/v) methanol, and 10% (v/v) acetic acid. Gel was detained in detaining solution 30% (v/v) methanol in water with 10% (v/v) acetic acid.

2.4.4 Agalacticin A concentration

The protein sequence (see Appendix iii) of agalacticin A was used to calculate its physical and chemical parameters by the ExPASY online program ProtParam, accessible at https://web.expasy.org/protparam/. The molar extinction coefficient of agalacticin A is at 1.4 (mg/ml) cm^{-1}. Measurement of protein samples at OD_{280} can be converted to concentration using the following equation (Promega, technical note):

$$c = \frac{A}{\varepsilon l}$$

C = protein concentration
A = Absorbance of protein samples at 280 nm
ε = Molar extinction coefficient
l = light pathlength (1 cm)

2.4.5 Determination of killing activity of agalacticin A by spot test

Agalacticin A was tested for its killing activity by spot test (Grinter et al. 2012; Fyfe et al. 1984). Fifty millilitres of mid-log phase culture of a test bacterial strain were added to 5 ml of 0.7% soft agar and subsequently overlaid on BHI agar plates. Two microliters of agalacticin A were spotted directly onto the surface of the overlay. Plates were incubated in the same growth conditions in section 2.2.1 for a further 12 hours. Zone of inhibition was examined to detect killing activity of agalacticin A.

2.5 Evaluation of the killing spectrum of agalacticin A

Bacterial species and strains (Appendix I, Table A-1 and A-2) were tested on the spectrum of agalacticin A. Initial agalacticin A concentration of 2 mg/ml (equal to 62.5 µM or 2000 mg/L) was three fold diluted in PBS. The different concentrations of agalacticin A ranging from 2000 to 0.3 mg/L were spotted on BHI agar plate overlaid with a test strain as method previously described in section 2.4.5. The proportion of strains of each bacterial species or GBS isolates was plotted against the minimal inhibitory concentration (MIC) and evaluated by the log-rank test using Graghpad Prism 8 software. Graphs were plotted by Kaplan –Meier estimator, growth inhibition was equivalent to the death event (1). No inhibition was censored (0).

2.6 Evaluation of the efficacy of agalacticin A in *in vivo*

2.6.1 Galleria mellonella larvae

Galleria mellonella larvae (the greater wax moth or honeycomb moth) were purchased from Livefood UK Ltd (Rooks Bridge, UK). Larvae were stored in the dark at room temperature and used within 5 days of arrival. No feed was given during storage. Larvae from the same batch were used in a single experiment. Ten *G. mellonella* larvae were used in the survival

test for each treatment. For evaluation of bacterial load, three *G. mellonella* larvae were collected at each time point. There were 21 *G. mellonella* larvae in total for each treatment. All experiments were performed in triplicate.

2.6.2 Antibiotic solution

Ampicillin sodium salt (Melford Biolaboratories Ltd, Suffolk, UK) and erythromycin (Duchefa Biochemie, Haarlem, Netherlands) were prepared in 2 mg/ml solution. Ampicillin was resuspended in sterile deionized water. Erythromycin was resuspended in 70% ethanol. Antibiotics were tested against test strains on plate assay using spot test (section 2.4.5) to ascertain their efficacy when administered in *G. mellonella* larvae. For combined agents, the combination of agalacticin A with ampicillin or erythromycin at a ratio of 1:1 at 2 mg/ml concentration was used.

2.6.3 GBS inoculum

Three ST283 piscine and three ST17 human isolates were chosen for *G. mellonella* challenge experiments. The inoculum of test strains used is shown in Table 2-2 Preparation of inoculum and *G. mellonella* challenge were performed by the method developed by Six et al. (Six et al. 2019) with minor modification. Briefly, test strains were cultured in BHI broth at 37°C to an OD_{600} = 0.6. Cells were collected by centrifugation at 7000 RPM at 4°C for 5 min. The pellet was washed twice in PBS and cells were resuspended and adjusted in PBS to an OD_{600} = 0.7. Bacterial suspension of each strain was plated on BHI with 5% Sheep blood agar plate to determine colony-forming units (CFU).

For inoculum preparation, test strain was grown and prepared to OD_{600} = 0.7. Bacterial suspension was serially diluted. Bacterial suspension of each dilution was plated on blood agar to check number of cells in inoculum. Larvae (n=10) were injected with 10 µL of inoculum in the last right proleg using a 10 µl Hamilton syringe (Sigma-Aldrich Ltd, Poole, UK). Infected larvae were incubated at 37 °C for 48 h. Death was defined where no movement even after stimulation was apparent. Number of deaths over 48h and CFU were calculated to define LD_{50} using the Probit method (XLSTAT software). In this study, LD_{80} was used to challenge larvae.

Table 2-2 GBS strains used in *G. mellonella* challenge.

Strains	Host species	Serotype	CC	ST	CFU per larva	Reference
STIR CD 25	Tilapia	III	283	283	3×10^6	Six et al. 2019
MRI Z2-366	Tilapia	III	283	283	1×10^6	Six et al. 2019
MRI Z2-399	Tilapia	III	283	283	2×10^6	This study
MRI Z2-093	human	III	17	17	1.5×10^7	Six et al. 2019
MRI Z2-121	human	III	17	17	1.5×10^7	This study
MRI Z2-132	human	III	17	17	1.5×10^7	This study

2.6.4 Immunohistochemistry (IHC) and histological analysis

To confirm that GBS is not a commensal bacteria of *G. mellonella* and that it can multiply in larvae, tissue sections of healthy larvae were examined by IHC with anti-*Streptococcus* Group B antibody ab53584 (Abcam, Cambridge, UK). Histological sections stained with hematoxylin and eosin (H& E) were used to study the microanatomy of *G. mellonella*. Larvae were euthanized by cooling to -20°C for up to 10 min to prevent over freezing. Whole larvae were placed in 10X volume of 10% neutral buffer formalin and kept at room temperature for 48h. Formalin fixed larvae were washed with PBS and transferred into 70% ethanol and paraffin embedded. For histological examination, sections were de-waxed in xylene and rehydrated through graded alcohols then sections were stained with H&E.

For determination of the presence of *S. agalactiae* using IHC, both longitudinal and cross sections were prepared to detect GBS multiplication in an early, middle and late phase of challenge. For antigen retrieval, heat-induced epitope retrieval (HIER) was carried out using a Menarini Access Retrieval Unit (Biocare LLC, California, USA), in 10 mM Sodium Citrate buffer, pH 6.0 for 1 minute 40 seconds at 125°C full pressure. Slides were loaded onto a Dako Autostainer (Dako Colorado, INC., Colorado, USA) and rinsed 5 min with a Tris-buffered saline solution (TBS), pH 7.6 containing 0.05% Tween 20. Endogenous peroxidase was blocked with Dako REAL™ Peroxidase-Blocking Solution (code S2023) for 5 min and then buffer rinsed. Slides were incubated 30 min at room temperature with anti-Streptococcus Group B antibody ab53584 diluted 1: 200 in Dako universal diluent (code S2022). Unbound primary antibody was removed followed by incubation with anti-rabbit horseradish-peroxidase labelled polymer (Dako, K5007ENV) for 30 min at room temperature. After buffer rinsed, slides were incubated 5 min at room temperature with 3,3'-diaminobenzidine (Dako, K5007 DAB). The slides were rinsed three times

with hydrogen peroxide and counterstained with Gills Haematoxylin for 27 seconds before dehydration in alcohols and mounted. *S. agalactiae* was staining in brown.

2.6.5 Larval Survival model

Inoculum of test strain was prepared to obtain the desired CFU as described in section 2.6.3. Survival test was performed in a group of 10 larvae in each treatment. Larvae were injected with 10 µL of inoculum in the last right proleg using a 10 µl Hamilton syringe. After incubation at 37 °C for 2 h, ten microliters of 2 mg/ml of agalacticin A or antibiotic were injected to the last left proleg. Ten microliters of PSB were injected to a control. Larvae were incubated at 37 °C and the number of death observed, post challenge, over 72 h. Survival analysis was performed by GraphPad Prism 8. Observation of deaths from three experiments of each test strain (n=30) was plotted on a survival curve using the Kaplan-Meier method. Comparison between survival curves was analysed by the log-rank test.

2.6.6 Bacterial counts from larval samples

Brilliance GBS Agar (Oxoid Limited, Hampshire, UK) or CHROMID® Strepto B (bioMérieux UK Limited, Hampshire, UK) was used as the selective GBS agar in this study. These chromogenic agar plates contain enzyme substrates linked to indoxyl chromogens. Specific bacterial enzymes, β-glucuronidase and esterase can cleave these substrates and release indoxyl molecules (Orenga et al. 2009). Indoxyl molecules are oxidized in the presence of oxygen forming indigoid dye precipitated within bacterial colonies (Orenga et al. 2009; Rosa-Fraile and Spellerberg 2017). GBS colonies grow in pink colonies on *Brilliance* GBS Agar and pale pink to red on CHROMID® Strepto B. However, non-GBS organisms may grow but as blue colonies.

Larvae were challenged with GBS strains using the method described in section 2.6.5. Evaluation of bacterial load was performed at 15 min before treatment, at the onset of treatment, and every 2 h for 12 h. For each time point, three larvae of each group were collected and anesthetized by temperature shock at -20°C for up to 7 minutes. They were placed in a 2 ml microtube containing 400 µl of PBS and Lysing Matrix M (MP Biomedicals, Leicester, UK). Samples were homogenised using FastPrep (MP Biomedicals) at a speed of 4m/sec for 20 s in two cycles. Homogenised samples were tenfold serially diluted in PBS and plated on selective chromogenic agar. Plates were incubated at 37°C for 48 h. *S. agalactiae* growing in pink colonies were counted and

the average CFU from the experiment performed in triplicate was used to evaluate bacterial load.

2.6.7 Statistical analysis

GraphPad Prism 8 (GraphPad Software, California, USA) was used for survival analysis, and for statistical significance by Kaplan survival curve and the log-rank test. *S. agalactiae* CFU between groups was determined by nonparametric One-way ANOVA (Kruskal Wallis).

2.6.8 Detection of agalacticin A distribution and degradation in *G. mellonella* larvae

After agalacticin A injection in the absence of infection, *G. mellonella* larvae from different time points were collected for Western-Blot and IHC.

2.6.8.1 Immunohistochemistry

Uninfected larvae were injected with 2 mg/ml of agalacticin A and incubated at 37°C. Three larvae were collected at each time point, at onset of injection (0h), 30 min, 1h, 2h, 4h and 6h. Two larvae were for longitudinal section and the other was for cross section. Larvae without agalacticin A injected were used as a negative control. Method of IHC detecting the presence of agalacticin A in larval tissue was the same as section 2.6.4, except primary antibody was the anti-agalacticin A antibody (Eurogentec, Belgium) diluted 1: 200. To obtain anti-agalacticin A antibody, agalacticin A was sent to Eurogentec to immunize rabbits. Production of polyclonal antibody and purification were performed by Eurogentec.

2.6.8.2 Western Blot Analysis

Uninfected larvae were injected with 2 mg/ml of agalacticin A and incubated at 37°C. Three larvae were collected at each time point, at onset of injection and every two hours. Homogenised samples were prepared by method in section 2.6.6 without dilution. Homogenised samples were weighed to 160 mg tissue for protein extraction. Agalacticin A was extracted from larval tissue using ReadyPrep Protein Extraction Kit (Bio-Rad Laboratories, California, USA) following the manufacturer's instructions. Briefly, samples were incubated in reagent on ice for 2 h. Supernatant was collected by centrifugation at maximum speed for 20 min at 20°C. Agalacticin A was separated from cell extraction by SDS-PAGE and transferred to a nitrocellulose membrane. Membrane was incubated

with PBS containing 5% (w/v) dried skimmed milk at room temperature for 1 h. Membrane was washed three times with PBST and incubated overnight with a polyclonal antiserum (anti-His piece) in PBST at 1:3,000 dilution. Membrane was washed with PBST twice and incubated with horseradish peroxidase-conjugated anti-mouse antibody diluted 1:1,000 in PBST for 30 min. Blot was developed by enhanced chemiluminesence using Clarity Western ECL substrate (Bio-Rad).

2.7 Biolog system for metabolic profiling

Biolog GEN III microplates, inoculating fluid and its consumables were purchased from Biolog (Hayward, California, USA). Carbon source utilization assays and biochemical tests are shown in plate layout (Figure 2-2). *S. agalactiae* strains used in this work are shown in Appendix i (Table A-2). *S. agalactiae* were freshly grown on 5% sheep blood agar at 37°C for 24 h. Colonies were picked by Inoculatorz™ swab (catalog No.3321) and gently mixed with inoculating Fluid: IF-A (catalog No.72401), avoiding formations of bubbles. Bacterial suspension was measured at OD_{600} of 0.02 - 0.05. Plates were inoculated by 100µl of bacterial suspension and incubated at 37°C for 24 h. Cell growth can be seen in purple colour from the reduction of the tetrazolium redox dye. Plates were measured for absorbance at OD_{600}. Absorbance was normalised by the following formulation:

$$\text{Absorbance} = (OD._{\text{sample}} - OD_{\text{negative control}}) / (OD._{\text{positive control}} - OD_{\text{negative control}}).$$

GEN III MicroPlate

A1 Negative Control	A2 Dextrin	A3 D-Maltose	A4 D-Trehalose	A5 D-Cellobiose	A6 Gentiobiose	A7 Sucrose	A8 D-Turanose	A9 Stachyose	A10 Positive Control	A11 pH 6	A12 pH 5
B1 D-Raffinose	B2 α-D-Lactose	B3 D-Melibiose	B4 β-Methyl-D-Glucoside	B5 D-Salicin	B6 N-Acetyl-D-Glucosamine	B7 N-Acetyl-β-D-Mannosamine	B8 N-Acetyl-D-Galactosamine	B9 N-Acetyl Neuraminic Acid	B10 1% NaCl	B11 4% NaCl	B12 8% NaCl
C1 α-D-Glucose	C2 D-Mannose	C3 D-Fructose	C4 D-Galactose	C5 3-Methyl Glucose	C6 D-Fucose	C7 L-Fucose	C8 L-Rhamnose	C9 Inosine	C10 1% Sodium Lactate	C11 Fusidic Acid	C12 D-Serine
D1 D-Sorbitol	D2 D-Mannitol	D3 D-Arabitol	D4 myo-Inositol	D5 Glycerol	D6 D-Glucose-6-PO4	D7 D-Fructose-6-PO4	D8 D-Aspartic Acid	D9 D-Serine	D10 Troleandomycin	D11 Rifamycin SV	D12 Minocycline
E1 Gelatin	E2 Glycyl-L-Proline	E3 L-Alanine	E4 L-Arginine	E5 L-Aspartic Acid	E6 L-Glutamic Acid	E7 L-Histidine	E8 L-Pyroglutamic Acid	E9 L-Serine	E10 Lincomycin	E11 Guanidine HCl	E12 Niaproof 4
F1 Pectin	F2 D-Galacturonic Acid	F3 L-Galactonic Acid Lactone	F4 D-Gluconic Acid	F5 D-Glucuronic Acid	F6 Glucuronamide	F7 Mucic Acid	F8 Quinic Acid	F9 D-Saccharic Acid	F10 Vancomycin	F11 Tetrazolium Violet	F12 Tetrazolium Blue
G1 p-Hydroxy-Phenylacetic Acid	G2 Methyl Pyruvate	G3 D-Lactic Acid Methyl Ester	G4 L-Lactic Acid	G5 Citric Acid	G6 α-Keto-Glutaric Acid	G7 D-Malic Acid	G8 L-Malic Acid	G9 Bromo-Succinic Acid	G10 Nalidixic Acid	G11 Lithium Chloride	G12 Potassium Tellurite
H1 Tween 40	H2 γ-Amino-Butyric Acid	H3 α-Hydroxy-Butyric Acid	H4 β-Hydroxy-D,L-Butyric Acid	H5 α-Keto-Butyric Acid	H6 Acetoacetic Acid	H7 Propionic Acid	H8 Acetic Acid	H9 Formic Acid	H10 Aztreonam	H11 Sodium Butyrate	H12 Sodium Bromate

Figure 2-2 Carbon sources and biochemical tests in Biolog GEN III.

2.7.1 Statistical analysis

Statistical analysis was performed by GraphPad Prism 8 (GraphPad Software, California, USA). Normalised absorbance values of samples were plotted and compared by parametric One-way ANOVA for normal distribution and Kruskal Wallis test for bimodal distribution. Metabolic profiles were stratified in host species and clonal complex.

2.8 Investigation of the linkage of specific metabolic pathway to the virulence factor

2.8.1 Hyaluronidase activity assay

Hyaluronic acid (HA) sodium salt from rooster comb (CAS number 9067-32-7) and bovine Serum Albumin Fraction V were purchased from Sigma-Aldrich Ltd (Poole, UK). Hyaluronidase activity was determined by using an agar activity assay (Smith and Willett 1968; King et al. 2004). Briefly, 1% BHI agar was cooled to 42°C. HA aqueous was prepared at concentration of 4 mg/ml and filter-sterilized. Filter sterilization was performed by using 0.2 µm Minisart® syringe filter (Sartorius, Goettingen, Germany). Sterilized HA was added to 1% BHI agar to a final concentration of 400 µg/ml. A 10% (wt/vol) filter-sterilized solution of bovine serum albumin fraction V was added to a final concentration of 1% (wt/vol) in the medium. Agar containing HA and bovine serum albumin was gently stirred to avoid bubbles and then poured on the plates. *S. agalactiae* was grown in BHI broth at 37°C to mid-exponential phase. Two microliters of liquid culture were spotted on HA agar. Plates were incubated at 37°C, overnight. To visualize hyaluronidase activity, each plate was flooded with 2 M acetic acid for 10 min. Nondegraded HA was conjugated with albumin and precipitated under acidic condition which can be seen as cloudy. Hyaluronidase can break down HA into smaller products. These HA products were not precipitated showing as a clear zone. Zone diameter was measured to categorize hyaluronidase positive or negative phenotypes.

2.8.2 Detection of Hyaluronidase gene and insertion elements by PCR

2.8.2.1 DNA extraction

DNA extraction was performed by using the GenElute bacterial Genomic DNA Kits (Sigma-Aldrich Ltd, Poole, UK). *S. agalactiae* was grown in BHI broth at 37°C, overnight. Cells were collected by centrifugation of 1.5 ml culture at 14000 RPM for 2 min. Lysozyme

from chicken egg white was prepared at 45 mg/ml of concentration. The 200 µl of lysozyme solution were added to the cell pellet and incubated at 37°C for 1 h. Cells were lysed by adding 20 µl of 20 mg/ml proteinase K, followed by 200 µl of lysis solution C (B8803). Samples were vortexed and incubated at 55 °C for 10 min. 200 µl of absolute ethanol were added and mixed by vortex. Columns were washed before loading samples to maximize DNA binding. 500 µl of each column preparation solution were added to column and centrifuged at 14000 RPM for 1 min. 500 µl of Lysate were loaded to the binding column and centrifuged at 7200 RPM for 1 min. Column was first washed by adding 500 µl of Wash Solution 1 (W0263) and centrifuged at 7200 RPM for 1 min. A second wash was performed by adding 500 µl of wash solution concentrate and centrifuged at 12000 RPM for 3 min with an additional centrifugation step to dry the column. DNA was eluted by adding 100 µl of the elution solution (B6803) and centrifuging at 7200 RPM for 1 min. DNA concentration was measured by Nanodrop.

2.8.2.2 PCR amplification

Hyaluronidase (also called hyaluronate lyase) is encoded by *hylB* gene (Lin et al. 1994). Loss of hyaluronidase activity frequently occurs due to the presence of insertion sequence *1548* (IS*1548*) (Sukhnanand et al. 2005). This study was to confirm hyaluronidase phenotypes by *hylB* gene. The published primers Sa-hylF (5-CAT ACC TTA ACA AAG ATA TAT AAC CCA AA-3, GenBank accession numbers Y15903) and Sa-hylR (5-AGA TTT TTT AGA GAA TGA GAA GTT TTT T-3, accession numbers U15050) are used to amplify a 950-bp fragment of hylB (Sukhnanand et al. 2005).

Amplification of *hyl*B gene was carried out with 1 U Taq polymerase from Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Herts, UK). The primers were purchased from Sigma-Aldrich Ltd (Poole, UK). PCR conditions consisted of an initial 30 s denaturation step at 98°C, 35 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 30 s, and a final 5-min extension cycle at 72°C. PCR products were electrophoresed in 1% agarose gel at 65 V. Gel was visualized by ChemiDoc Imaging System (Bio-Rad Laboratories Ltd., Watford, UK). PCR products from *hyl*B gene were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and were sent to Source Bioscience (Nottingham, UK) for nucleotide sequencing.

Chapter 3 Identification, recombinant production and preliminary analysis of the killing activity of agalacticin A

3.1 Introduction

3.1.1 Bacteriocins from gram-positive bacteria

Bacteria produce ribosomal synthesized-antimicrobial peptides, namely bacteriocins to occupy a niche or inhibit competing strains (Dobson et al. 2012). In this respect, lactic acid bacteria (LAB) have been a major focus of bacteriocin research in gram-positive bacteria due to their importance in commercial development in the food industry and being approved as "generally recognized as safe" (GRAS) by the US Food and Drug Agency (FDA) (Cotter et al. 2005). Bacteriocins produced by LAB were defined into four classes as follows:

1) Class I are lantibiotics, small peptides (<5 kDa) containing lanthionine and β-methyl lanthionine, and dehydrated residues, e.g. nisin.
2) Class II are small heat-stable, non-lanthionine peptides (<10 kDa). They are split into three groups, IIa (Listeria–active peptides), IIb (poration complexes consisting of two peptides) and IIc (thiol-activated peptides).
3) Class III are large heat-labile proteins (>30 kDa).
4) Class IV are complex bacteriocins, composed of protein plus one or more chemical moieties (lipid, carbohydrate) required for activity.

In 1996 and 2000, the classification was revised by Nes and Holo into Class I (lantibiotics), II (nonmodified heat stable bacteriocins), IIa (pediocin-like bacteriocins), IIb (two-peptide bacteriocins), IIc (other class II bacteriocins) and III (large heat-labile proteins) (Nes et al. 1996; Nes and Holo 2000). However, subsequently new bacteriocins have been discovered that did not fit into these existing classifications. Cotter and colleagues proposed a revised bacteriocin classification in 2005. This classification scheme divides the bacteriocins into two distinct categories: the lanthionine-containing (class I, lantibiotic) and the non-lanthionine-containing bacteriocins (class II). Formerly class III bacteriocins, the large (>30 kDa), heat-labile proteins have been separately designated to be bacteriolysins (Cotter et al. 2005). Cotter suggested a reclassification of Class III bacteriocins because they are lytic enzymes rather than peptides.

Bacteriolysins are large, heat-labile antimicrobial proteins and are also called non-bacteriocin lytic proteins because their domain structure and mechanism of action is distinct from other bacteriocins (Figure 3-1). Examples of bacteriolysins are zoocin A produced by *Streptococcus equi* subsp. *zooepidemicus* 4881 (Naidoo, Jones, and Tagg 1995), lysostaphin produced by *Staphylococcus simulans* biovar *staphylolyticus* (Schindler and Schuhardt 1964) and enterolysin A produced by *Enterococcus faecalis* LMG 2333

(Nilsen, Nes, and Holo 2003). The mechanism of action is an endopeptidase activity that hydrolyses the peptide links of susceptible cell wall peptidoglycan. Producer cells have an immunity protein to protect themselves from the lethal action of their own bacteriocin. Modification of the amino acid composition within peptidoglycan cross-links is a mechanism of bacteriolysin producer strains to be resistant to their endopeptidase. For example, the integration of serine in place of glycine residues in the peptidoglycan pentaglycine is the mechanism that makes a lysostaphin producer resistant to its own endopeptidase (DeHart et al. 1995; Thumm and Gotz 1997; Gargis et al. 2010) and addition of leucine to the pentapeptide peptidoglycan precursor has been reported in millericin B producer strains of *Streptococcus milleri* (Beukes and Hastings 2001).

With the rise of AMR, bacteriocins provide a potential alternative to the broad spectrum, small molecule antibiotics that are widely used in clinical practice and animal health. A particularly attractive property of the bacteriocins is their relatively narrow spectrum of activity which could enable specific bacterial pathogens to be targeted, leaving the wider microbial community intact. However, bacteriocins have not been widely used in a clinical setting and more work is required to assess the potential for the development of resistance to bacteriocins which could occur through the spread of bacteriocin immunity genes, mutation to bacteriocin receptors, or the acquisition of other resistance determinants (Draper et al. 2009). For example, the proteolytic cleavage of nisin was reported to remove the C-terminal tail of this bacteriocin (Sun et al. 2009) leading to nisin resistance (Draper et al. 2009).

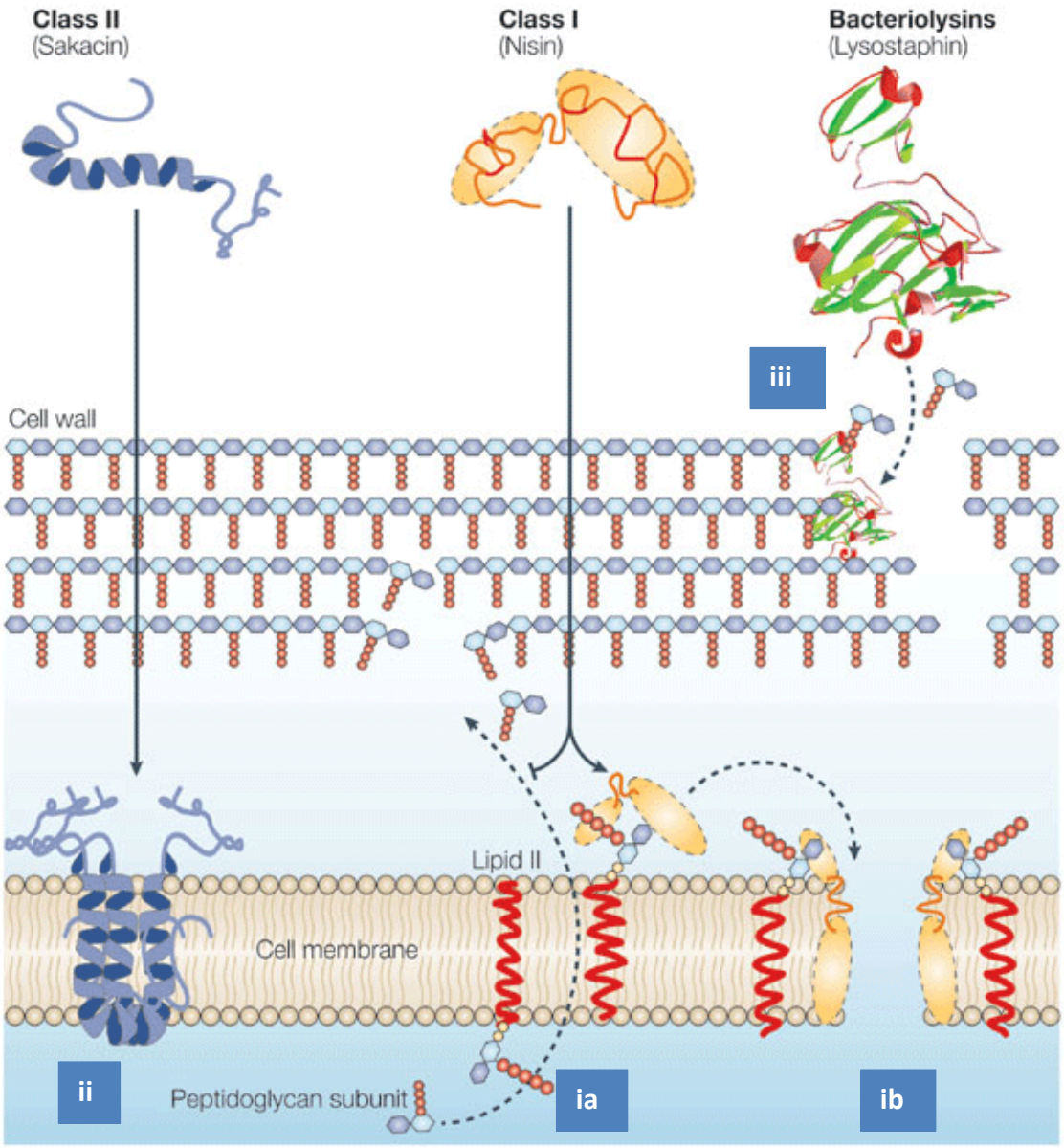


Figure 3-1 Mode of action of lactic acid bacteria bacteriocins.
Mode of action of lactic acid bacteria (LAB) bacteriocins can be classified on the basis of structure. The class I bacteriocins have a dual mode of action. 1) Preventing correct cell wall synthesis by binding to lipid II, the main transporter of peptidoglycan subunits (ia). 2) Pore formation by using lipid II as a docking molecule for membrane insertion (ib). The class II peptides, an amphiphilic helical structure allows membrane insertion of the target cell causing cell depolarisation and death (ii). Bacteriolysins (large bacteriolytic proteins) have a function directly on the cell wall of gram-positive target cells leading to cell lysis (iii). Reprinted by permission from Springer Nature: Nature Reviews Microbiology. Food microbiology: Bacteriocins: developing innate immunity for food, (Paul D. Cotter, Colin Hill, R. Paul Ross), [copyright] 2005.

3.1.2 Bacteriocins active against *Streptococcus* species

Zoocin A is a streptococcolytic enzyme produced by *Streptococcus equi* subsp. *zooepidemicus* 4881, with a mode of action similar to lysostaphin (Simmonds et al. 1996). It hydrolyses the junction between the D-alanine of the stem peptide and the first L-alanine of the cross bridge (Heath et al. 2004; Gargis, Heath, et al. 2009) as shown in Figure 3-2. Zoocin A is composed of two domains, the N-terminal catalytic domain (CAT) and the C-terminal target recognition domain (TRD) (Simmonds et al. 1996). The CAT of zoocin A contains the peptidase activity for cell wall hydrolysis and the TRD contains functional groups determining target recognition (Lai, Tran, and Simmonds 2002). CAT of zoocin A has homology to the M23 family of peptidases, and is 41% identical to the catalytic domain of lysostaphin and 35% identical to LytM, a latent autolysin produced by *Staphylococcus aureus* (Xing, Simmonds, and Timkovich 2017).

To protect zoocin A producers from their own bacteriocin, a producer strain carries the *zif* gene (zoocin immunity factor) (Beatson, Simmonds, and Sloan 1998). Zif provides protection by lengthening the peptidoglycan cross bridge from two L-alanine residues to three. Increasing the length of cross bridges inhibits both binding of the zoocin A TRD and the ability of the zoocin A CAT to hydrolyse the cross bridge (Gargis, Gargis, et al. 2009). Zoocin A has a narrow spectrum which can effectively inhibit growth of pathogenic streptococci such as *Streptococcus mutans* (Naidoo, Jones, and Tagg 1995), *Streptococcus pyogenes*, and *Streptococcus gordonii* (Akesson et al. 2007). Testing of zoocin A activity against *S. agalactiae* has not been documented.

There are a few studies identifying bacteriocins active against GBS. The first bacteriocin-like inhibitory substance (BLIS), designated streptocin B1 produced by human *S. agalactiae* strain 746284 was discovered in 1975 (Tagg, Dajani, and Wannamaker 1975) and it was confirmed as a natural variant of nisin U, Class I lantibiotic bacteriocin in 2006 (Wirawan et al. 2006). There was a synthetic biology approach using the publicly available gene encoding bacteriocin of *S. agalactiae* from Bagel3, combined with the biosynthesis machinery of the model lantibiotic nisin (van Heel et al. 2016). Van Heel and colleagues did not specify the gene(s) encoding bacteriocin of *S. agalactiae*, nor the killing activity of synthetic bacteriocin against *S. agalactiae* strains and the class of bacteriocin is therefore unknown. In this thesis, the focus is on phenotypical discovery of bacteriocin of *S. agalactiae*. Detection of bacteriocin-producing strains and bacteriocin characteristics and killing spectrum are described.

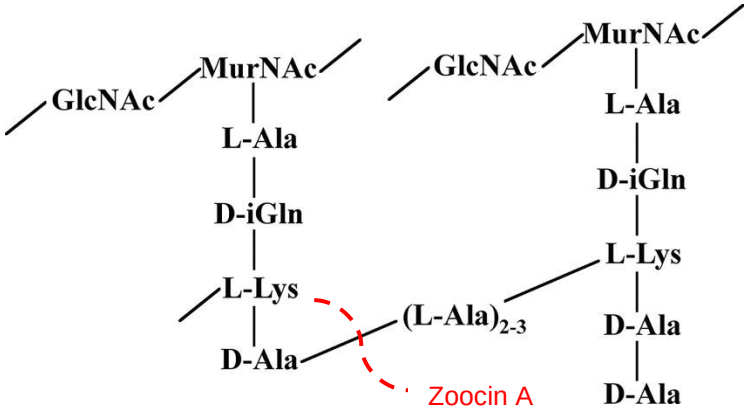


Figure 3-2 Streptococcal peptidoglycan highlighting the peptide link cleaved by zoocin A. Typically, streptococcal peptidoglycan has two or three alanine residues in peptide cross bridges. Zoocin A is a D-alanyl-L-alanine endopeptidase of peptidoglycan cross bridges (dashed line). Reprinted by permission from American Society for Microbiology: Applied and Environmental Microbiology. Use of 4-Sulfophenyl Isothiocyanate Labeling and Mass Spectrometry to Determine the Site of Action of the Streptococcolytic Peptidoglycan Hydrolase zoocin A, (Shaw R. Gargis. et. al.) [copyright] 2008.

3.2 Aims and objectives

The aim of this study was to find bacterioicins active against GBS that may form the basis for an alternative control strategy against this bacterium. Specifically, the objectives of the work described in this chapter were to 1) detect bacteriocin production from human, bovine and piscine *S. agalactiae* isolates, 2) purify and characterize identified bacteriocins, and 3) assess their killing activity and spectrum.

3.3 Results

3.3.1 Detection of bacteriocin production in GBS isolates

Bacteriocin-producing strains were detected by antagonism assay as described in section 2.3. One hundred and twelve *S. agalactiae* isolates (19 human, 36 bovine and 57 piscine) were tested for bacteriocin production ability in antagonism assays. Twenty-four *S. agalactiae* human, bovine and piscine isolates were selected as indicator strains (Appendix i, Table A-3) based on clonal complex and host species. MRI Z2-093, ST17 human *S. agalactiae* isolated from urinary tract infection inhibited growth of the indicator strains from three host species (Figure 3-3a). The antagonism assay using direct colony inoculation showed clear zones of inhibition. However, the spot test using supernatant collected from the mid-log phase culture of the producer strain showed a narrow zone indicating low levels of growth inhibition (Figure 3-3b).

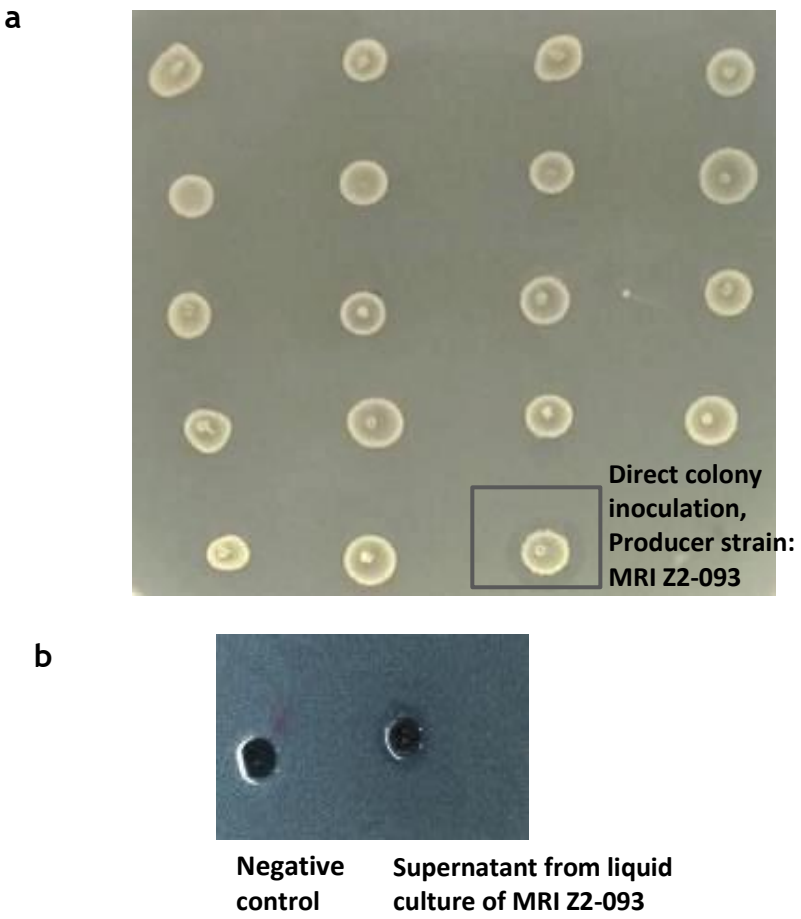
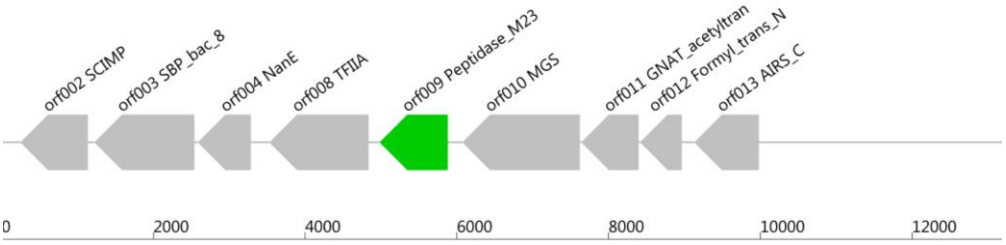


Figure 3-3 Identification of group B *Streptococcus* bacteriocin producer strain.
(a) Bacteriocin-producing strain (MRI Z2-093) was detected by antagonism assay. Direct colony or 2 µl of liquid culture of each GBS isolate was inoculated on BHI agar. Plates were incubated overnight at 37°C. Selected indicator strains were grown in BHI broth at 37°C. Plates were overlaid with 5 ml of 0.7% soft agar containing 50 µl of indicator strain. Zone of inhibition indicated a bacteriocin producer strain can be observed after overnight incubation. (b) Evaluation of bacteriocin production in supernatant collected from the mid-log phase showed low activity. BHI agar plate was overlaid by 0.7% soft agar containing an indicator strain. Agar was cut to make a well for loading 50 µl of cell-free supernatant from the mid-log phase growth of MRI Z2-093. Growth inhibition can be seen as a thin layer at the edge of well (right).

3.3.2 Identification of bacteriocin of *Streptococcus agalactiae*

The genomic sequence of MRI Z2-093, a bacteriocin producer, was analysed for putative bacteriocin encoding genes using the bacteriocin mining tool Bagel 3, accessible at http://bagel.molgenrug.nl/. FASTA files of contig sequences were provided by Professor RN Zadoks. A single putative bacteriocin gene encoding a predicted M23 peptidase of 299 amino acids (32 kDa) was identified (Figure 3-4). Both zoocin A and the well-characterised bacteriocin lysostaphin possess an M23 peptidase domain. The protein sequence of the putative M23 peptidase was further analysed (section 2.4.4). The molar extinction coefficient was calculated as 1.4 (mg/ml) cm^{-1} and the isoelectric point (pI) as 9.24. We named this putative bacteriocin agalacticin A. Agalacticin A has a high degree of amino acid identity to zoocin A (accession number: WP_043039484.1) at 55% (Figure 3-5).



Protein ID AOI_1; orf009
Protein sequence:
LNKWLVKASSLVVLGGMVLSAGSRVLADTYVRPIDNGRITTGFNGYPGHCGVDYAVPTGT
IIRAVADGTVKFAGAGANFSWMTDLAGNCVMIQHADGMHSGYAHMSRVVARTGEKVKQ
GDIIGYVGATGMATGPHLHFEFLPANPNFQNGFHGRINPTSLIANVATFSGKTQASAPSIKPL
QSAPVQNQSSKLKVYRVDELQKVNGVWLVKNNTLTPTGFDWNDNGIPASEIDEVDANGN
LTADQVLQKGGYFIFNPKTLKTVEKPIQGTAGLTWAKTRFANGSSVWLRVDNSQELLYK

Figure 3-4 Bacteriocin gene of *Streptococcus agalactiae*.
To identify putative bacteriocin genes, genomic sequence of MRI Z2-093, a producing strain, was analysed by Bagel 3, which identified only the bacteriocin-encoding region. Group B streptococcus bacteriocin was classified as a Class III bacteriocin or bacteriolysin belonging to the M23 peptidase family. It has 299 amino acids, as in the grey box.

```
Zoocin_A           1 -----MKRIFFAFLSLCLFIFGTQTVSAATYTRPLDTGNITTGFNGYPGH     45
                           :|......|...:...|:: |.|.||.||:|.|.||||||||||
Agalacticin_A      1 LNKWLVKASSLVVLGGMVLSAGSR-VLADTYVRPIDNGRITTGFNGYPGH     49

Zoocin_A          46 VGVDYAVPVGTPVRAVANGTVKFAGNGANHPWMLWMAGNCVLIQHADGMH     95
                     .|||||||.||.:||||:|||||||.|||..||..:|||||:||||||||
Agalacticin_A     50 CGVDYAVPTGTIIRAVADGTVKFAGAGANFSWMTDLAGNCVMIQHADGMH     99

Zoocin_A          96 TGYAHLSKISVSTDSTVKQGQIIGYTGATGQVTGPHLHFEMLPANPNWQN    145
                     :||||:|::...|...||||.||||.||||..||||||||.||||||:||
Agalacticin_A    100 SGYAHMSRVVARTGEKVKQGDIIGYVGATGMATGPHLHFEFLPANPNFQN    149

Zoocin_A         146 GFSGRIDPTGYIANAPVFNGTTPTE-------PTTPTTN----LKIYKVD    184
                     ||.|||:||..|||...|:|.|...       .:.|..|    ||:|:||
Agalacticin_A    150 GFHGRINPTSLIANVATFSGKTQASAPSIKPLQSAPVQNQSSKLKVYRVD    199

Zoocin_A         185 DLQKINGIWQVRNNILVPTDFTWVDNGIAADDVIEVTSNGTRTSDQVLQK    234
                     :|||:||:|.|:||.|.||.|.|.||||.|.::.||.:||..|:||||||
Agalacticin_A    200 ELQKVNGVWLVKNNTLTPTGFDWNDNGIPASEIDEVDANGNLTADQVLQK    249

Zoocin_A         235 GGYFVINPNNVKSVGTPMKGSGGLSWAQVNFTTGGNVWLNTTSKDNLLYG    284
                     ||||:.||..:|:|..|::|:.||:||:..|..|.:|||...:...|||.
Agalacticin_A    250 GGYFIFNPKTLKTVEKPIQGTAGLTWAKTRFANGSSVWLRVDNSQELLYK    299

Zoocin_A         285 K    285

Agalacticin_A    300 -    299
```

Figure 3-5 Alignment of protein sequences of agalacticin A and zoocin A.
Sequence alignment of zoocin A produced by *Streptococcus equi* subsp. *zooepidemicus* (accession number: WP_043039484.1) and agalacticin A was performed by ClustalW multiple sequence alignment tool. Positions with conserved residue are indicated by a solid line (|). Residues conserved between groups which have similar properties are indicated by a colon (:) and residues with weakly similar properties are shown as a period (.). Agalacticin A has 55% identity and 67.4% similarity to zoocin A.

3.3.3 Expression and purification of agalacticin A

In order to test the killing activity of the putative bacteriocin agalactacin A, recombinant protein was produced in *E. coli* BL21 (DE3) harbouring the plasmid pZoo1 which encodes agalacticin A. The agalacticin A gene is under the control of a T5 promoter with an embedded lac operator for IPTG inducible protein expression. Cells were grown in LB at 37°C to an OD_{600} of approximately 0.6 and agalacticin A expression was induced by the addition of 0.3 mM IPTG. Cells were grown for a further three hours. Agalacticin A has a C-terminal histidine tag. Purification of agalacticin A from cell extract was achieved by nickel affinity chromatography using a HisTrap™ Nickel column (GE Healthcare, Uppsala, Sweden) equilibrated in 5 mM imidazole, 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl (Figure 3-6a). Fractions containing agalacticin A (Figure 3-6b peak 2) were pooled and dialysed in 50 mM Tris-HCl, pH 7.5, 20 mM NaCl at 4°C overnight. To obtain highly purified agalacticin A, dialysed fractions of agalacticin A were further purified by size exclusion chromatography using a Superdex S75 26/60 column (Figure 3-6c). Fractions of purified agalacticin A were run on 12% SDS-PAGE. In addition to the major band corresponding to monomeric agalacticin A at 32 kDa, a band at approximately 70 kDa was observed indicating dimerization (Figure 3-6c). Fractions from the main peak were pooled and stored at -80°C.

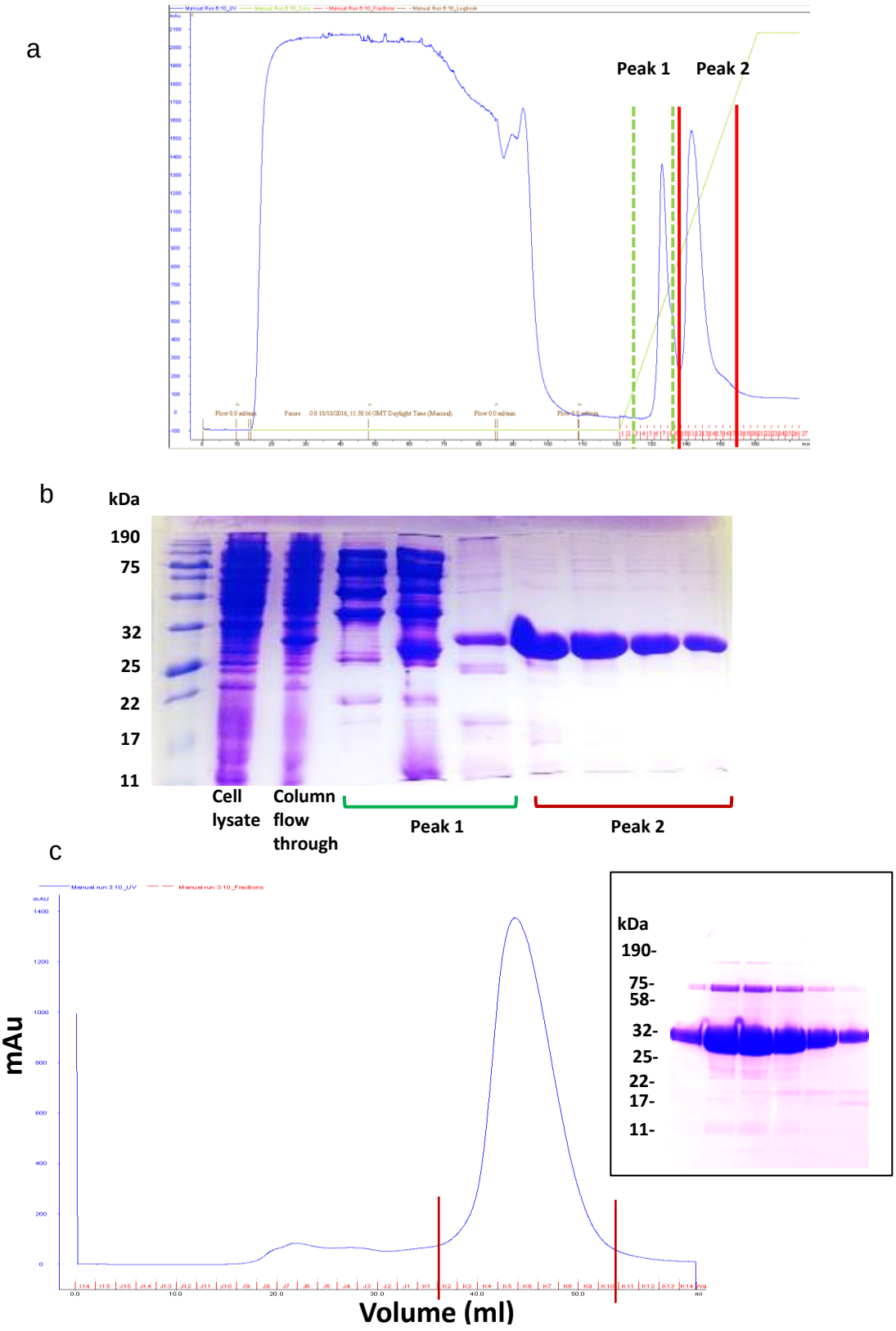


Figure 3-6 Agalacticin A purification by affinity chromatography and size exclusion chromatography.

(a) Nickel affinity chromatography and fractions being examined on SDS-PAGE. (b) Examination of fractions from the nickel affinity chromatography on SDS-PAGE, fractions from peak 2 were collected for further size exclusion chromatography. (c) Size exclusion chromatography and fractions containing agalacticin A were run in SD-PAGE. Bands at 32 kDa are agalacticin A and bands at 70 kDa may be caused by dimerization. Fractions from a main peak were pooled and used in this study.

3.3.4 Agalacticin A concentration used for *in vitro* testing

To test the killing spectrum of agalacticin A, different concentrations of agalacticin A were evaluated for their killing activity by spot test using the method described in section 2.4.5. Twenty eight (12 piscine, 8 human and 8 bovine) *S. agalactiae*, one *Staphylococcus aureus* and two *Pseudomonas aeruginosa* isolates were tested for preliminary optimisation of the range of concentrations. Agalacticin A at 2 mg/ml (for preparation see section 2.4.4) and further three-fold dilutions at a range of 0.67 to 0.0003 mg/ml were spotted on a BHI plate overlaid with test *S. agalactiae* isolate. All test strains were inhibited at concentrations of 2, 0.67 and 0.22 mg/ml. Killing activity was variable at concentrations of 0.07 to 0.025 mg/ml and no killing activity was observed at concentrations less than 0.008 mg/ml (Table 3-1 and Figure 3-7). Selectivity of the killing spectrum was initially evaluated on gram positive and gram negative bacteria. *S. aureus* and *P. aeruginosa* were not inhibited by agalacticin A. Therefore, concentrations from 2 mg/ml to 0.003 mg/ml were selected to test the spectrum of agalacticin A in *in vitro*.

Table 3-1 Preliminary test to optimize the range of concentrations for evaluation of agalacticin efficacy against group B *Streptococcus* (GBS) *in vitro*.

Test isolates	Number of GBS isolates inhibited by agalacticin A's concentrations (mg/ml)					
	2	**0.67**	**0.22**	**0.07**	**0.025**	**0.008**
Piscine GBS (n=12)	12/12	12/12	12/12	9/12	3/12	0/12
Human GBS (n=8)	8/8	8/8	8/8	6/8	2/8	0/8
Bovine GBS (n=8)	8/8	8/8	8/8	8/8	4/8	4/8
% inhibition	**100%**	**100%**	**100%**	**82%**	**32%**	**14%**

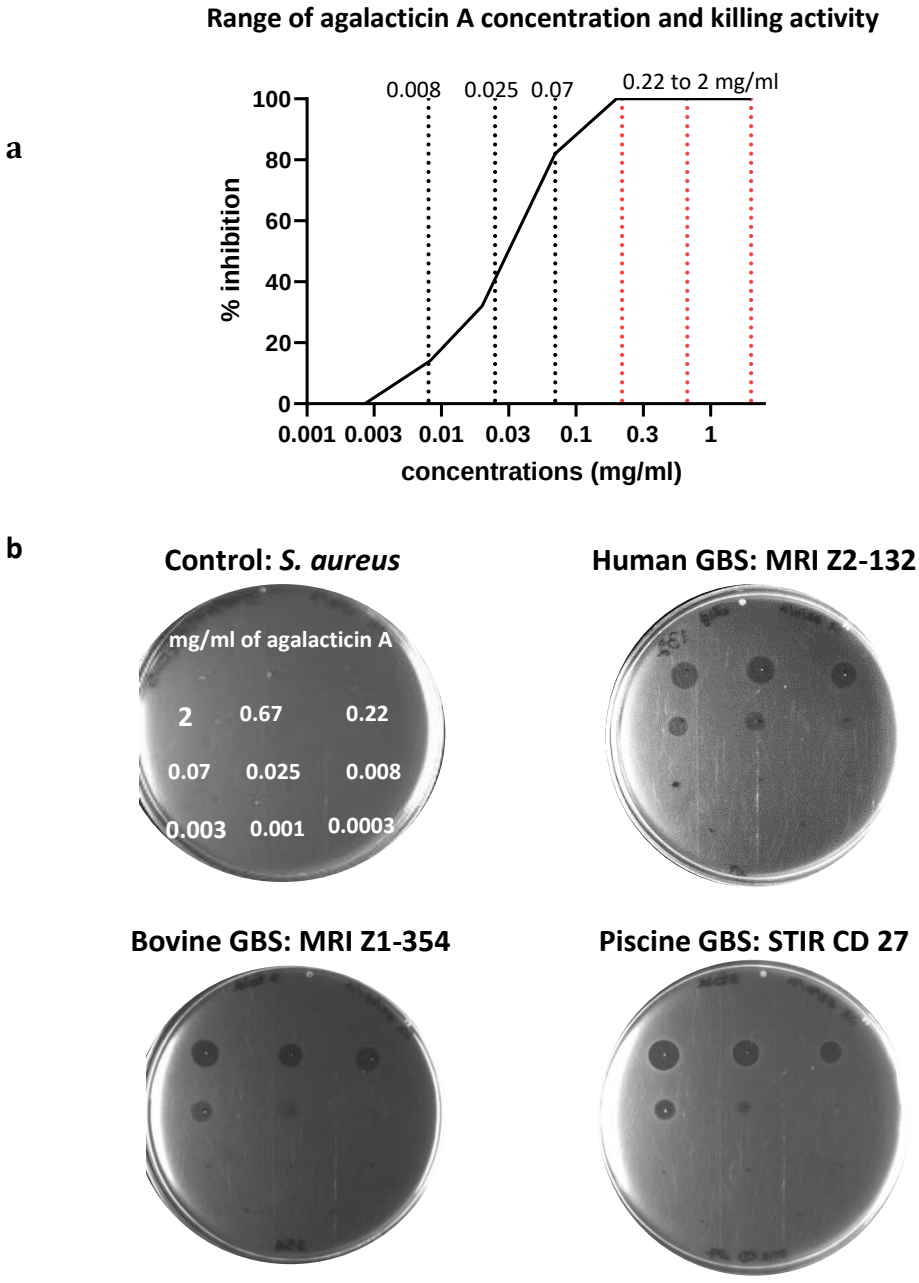


Figure 3-7 Spot test of different agalacticin A concentrations on group B *Streptococcus* (GBS) isolates to optimize range of concentrations.
(a) Inhibition of growth of GBS isolates by agalacticin. Percent inhibition was calculated from 28 GBS isolates (detail in Table 3-1). Inhibition was first observed at a concentration of 0.008 mg/ml and all GBS isolates were inhibited at 0.22 mg/ml. The range of concentrations of 0.003 to 2 mg/ml was chosen for *in vitro* testing. (b) Spot test of agalacticin A on human, bovine and piscine GBS isolates, showing growth inhibition by each concentration. *Staphylococcus aureus* was used as control to determine the selective killing activity of agalacticin A. Numbers on control plate indicate concentrations of agalacticin A (mg/ml) used on all plates.

3.3.5 Killing spectrum of agalacticin A

To explore the killing spectrum of agalacticin A against GBS from different host species, the MICs against *S. agalactiae* isolates from humans, dairy cows and fishes were determined. CC552 piscine GBS isolates, which do not grow at 37°C, were not tested to ensure consistency of growth conditions between GBS groups. The narrow-spectrum property of agalacticin A was explored by testing its killing activity on closely related *Streptococcus* species and relevant representatives of the human vaginal microbiome, i.e. *Lactobacillus* species.

Test isolates and determination of killing activity are described in section 2.5. Because preliminary data showed that bacterial isolates were inhibited at isolate-specific concentrations, a survival analysis function was used to present the susceptibility of bacteria to agalacticin A. MIC of individual isolates from experiments in triplicate was plotted by Kaplan-Meier estimator. Response of bacteria to agalacticin A, at the range of 2000 mg/L (2 mg/ml) to 0.3 mg/L (0.003 mg/ml) was defined as the event of death, seen as a clear zone on the test plate. A log-rank test was used to compare the survival proportion among GBS strains from different host species and to other bacterial species and to compute an LD_{50} concentration of agalacticin A. Kaplan-Meier estimator and a log-rank test were performed on GraphPad Prism 8.

The survival proportion of 102 GBS isolates (18 human, 36 bovine and 48 piscine) exposed to agalacticin A showed no difference between human, bovine and piscine isolates ($P = 0.15$) (Figure 3-8a). At 25 mg/L agalacticin A (0.025 mg/ml), 50% of test isolates were killed (LD_{50}). Increasing concentration to ≥74 mg/L (0.074 mg/ml) is sufficient to kill all GBS strains. *Streptococcus dysgalactiae* subsp. *dysgalactiae* is most agalacticin A sensitive, with LD_{50} at 8.23 mg/L (0.008 mg/ml). Higher concentrations of agalacticin A at 74 mg/L can inhibit *Streptococcus canis* whereas agalacticin A has no activity against the more distantly related streptococcal species, *Streptococcus uberis*. Agalacticin A had no activity against members of the genus *Lactobacillus* (*Lactobacillus jensenii*, *L. gasseri* and *L. crispatus*) or against *Enterococcus faecium* (Figure 3-8b). Thus, agalacticin A appeared to have narrow spectrum ($P < 0.0001$), although it was effective against *Enterococcus faeacalis*.

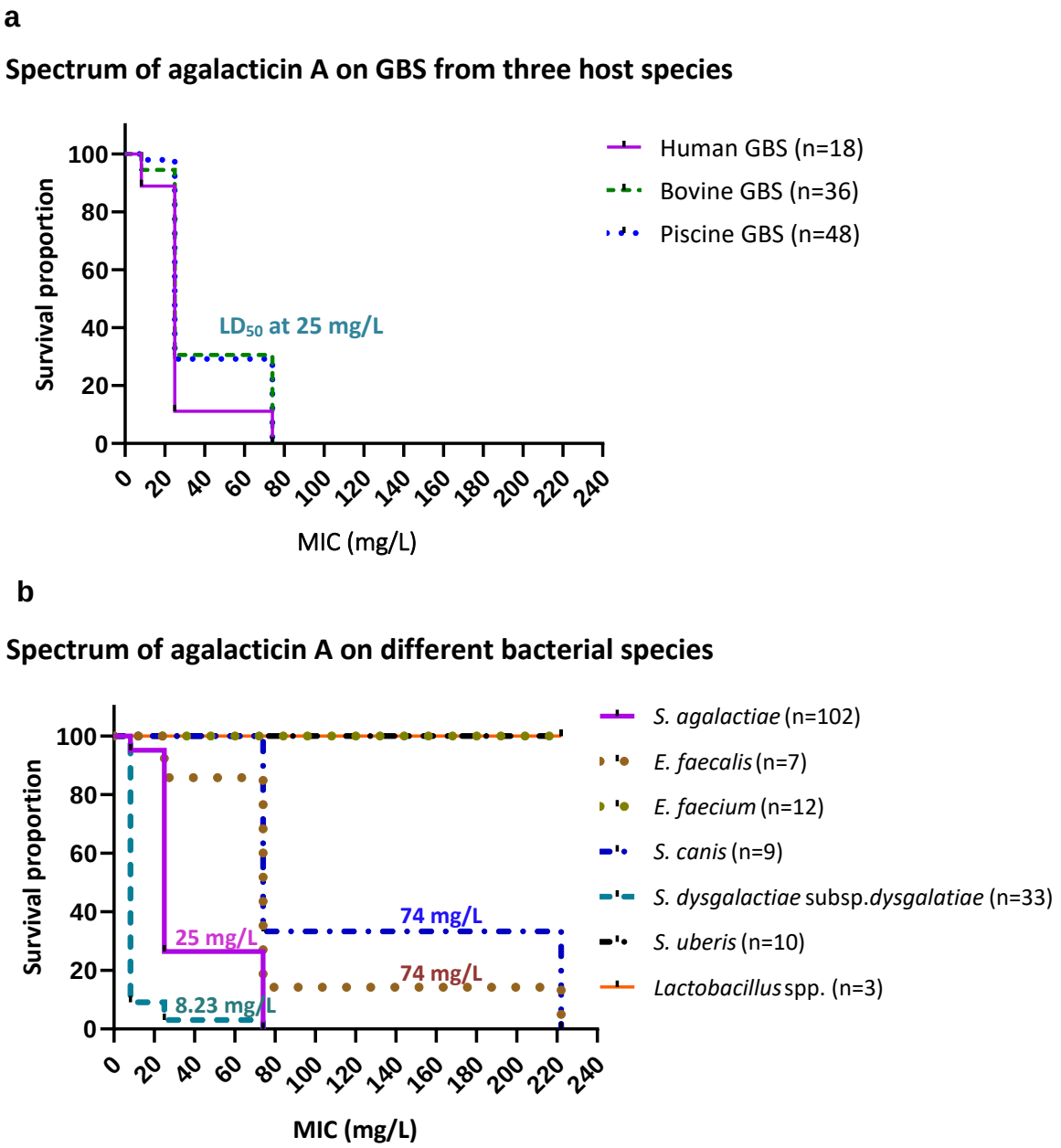


Figure 3-8 Spectrum of agalacticin A against group B *Streptococcus* (GBS) and related bacterial species and genera.
Proportion of bacteria surviving from a range of agalacticin A's concentrations was plotted by the Kaplan-Meier estimator using Graphpad Prism 8. (a) Agalacticin A can similarly inhibit GBS from three host species (P = 0.15). (b) Killing activity of agalacticin A against GBS and closely related bacterial species or genera, including *Lactobacillus* species commonly present in the human vaginal microbiome (P < 0.0001). Numbers indicate LD_{50} of agalacticin A for each species (mg/L).

3.4 Conclusions

3.4.1 Spectrum of agalacticin A

Agalacticin A, a novel GBS bacteriocin discovered in this study, is an endopeptidase in the M23 peptidase family, which are peptidoglycan hydrolases (Heath et al. 2004; Gargis, Heath, et al. 2009). The N-terminal catalytic domain (CAT) of zoocin A produced by *Streptococcus equi* subsp. *zooepidemicus* contains functional groups for cell wall hydrolysis (Simmonds et al. 1996). The C-terminal target recognition domain (TRD) contains functional groups determining target recognition binding to the cell surface (Lai, Tran, and Simmonds 2002). Agalacticin A has a moderate degree of identity to zoocin A (55%) and a moderately high degree of similarity (67%) at amino acid level.

Agalacticin A is produced by MRI Z2-093, ST17 human GBS isolate. The molecular weight is 32 kDa which agrees with the size of large antimicrobial proteins in Class III bacteriocins. Agalacticin A showed high killing efficacy at low concentration to GBS isolated from three host species. Killing activity of agalacticin A is non-serotype dependent and includes antibiotic-resistant *S. agalactiae* isolates (see appendix ii). Some GBS isolates in this study are resistant to tetracycline, erythromycin and clindamycin but all are susceptible to penicillin and ampicillin.

Among non-GBS streptococci, *S. dysgalactiae* subsp. *dysgalactiae* (group C streptococcus) was the most sensitive to agalacticin A. *S. canis* (group G streptococcus) was partially inhibited at the concentration that killed all GBS strains and no inhibition was seen in *S. uberis* at any concentration. The observed susceptibility of *Streptococcus* species parallels phylogenetic relationships among streptococcal species (Täpp, Thollesson, and Herrmann 2003) (Figure 3-9). These findings are consistent with work from Akesson et. al, who tested zoocin A against other streptococci and closely related species (Akesson et al. 2007).

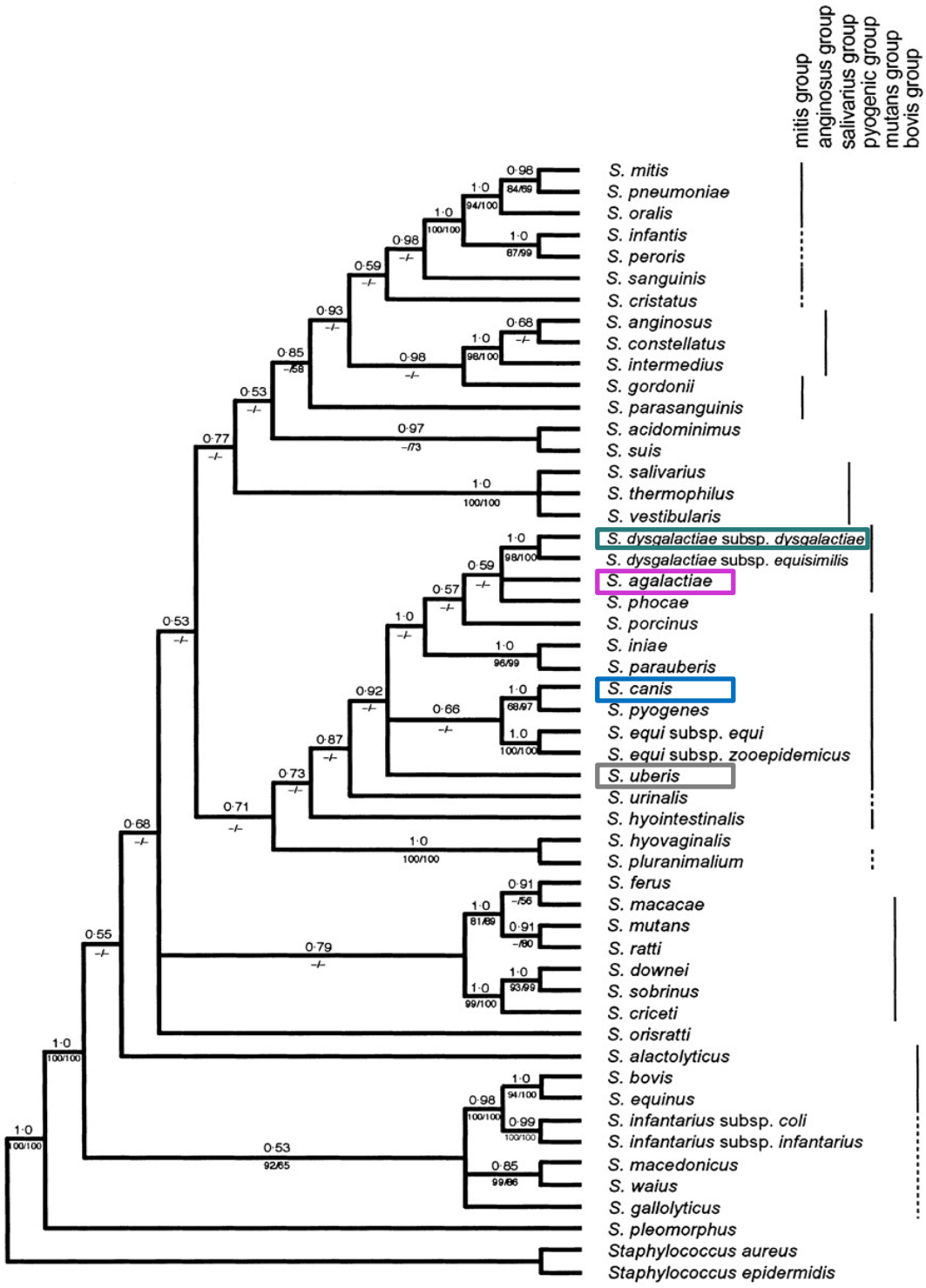


Figure 3-9 Phylogenetic relationships among streptococcal species.
Bacterial species with more distant phylogenetic relationships to *Streptococcus agalactiae* (magenta box) were found to be less sensitive to agalacticin A. Agalacticin A can inhibit *S. dysgalactiae* subsp. *dysgalactiae* (green box) followed by *S. canis* (blue box) but there is no effect on *S. uberis (*grey box). Reprinted and modified by permission from Microbiology Society, International journal of systematic and evolutionary microbiology. Phylogenetic relationships and genotyping of the genus *Streptococcus* by sequence determination of the RNase P RNA gene, rnpB, (Jenny Täpp1, Mikael Thollesson2, Björn Herrmann), [copyright] 2003.

3.4.2 Spectrum of agalacticin A against clinically-relevant bacterial species

Resistance of Enterococci to agalacticin A depended on species but resistance to vancomycin had no influence. *Enterococcus faecium* and *E. faecalis* have similar levels of genetic similarity to GBS (64.9% and 66.4% respectively) (Drancourt, Fournier, and Raoult 2004) but *E. faecalis* and *E. faecium* extracellular polysaccharide and capsule compositions are different based on genes encoding the capsule production or the extracellular polymer biosynthesis (Palmer et al. 2012). Agalacticin A had no activity against *E. faecium* strains. A high concentration of agalacticin A is required to inhibit growth of all *E. faecalis* strains. Two vancomycin–resistant *E. faecalis* (specimen ID: 17M701947B and 17M659576E) were inhibited by agalacticin A at a concentration of 25 and 74 mg/L, respectively, while two vancomycin-sensitive *E. faecalis* were inhibited at higher concentration, 222 mg/L. Vancomycin binds to the D-ala-D-ala termini of peptidoglycan cross bridges and resistance is frequently due to modification of peptidoglycan precursors from D-ala-D-ala to D-ala-D-lactate (Cetinkaya, Falk, and Mayhall 2000). Based on the data, this change in peptidoglycan composition affects the efficacy of vancomycin but not the efficacy of agalacticin A. The primary focus of this thesis is to identify alternative antimicrobials for GBS but the efficacy of agalacticin A against vancomycin resistant enterococci, which are of great clinical concern, is an interesting beneficial finding.

Regarding standard policy for the prevention of perinatal GBS disease, implementation of IAP has been routinely practiced in some countries such as in the United States of America (Verani, McGee, and Schrag 2010). In contrast, IAP is not recommended in the United Kingdom, in part due to concerns about potential adverse effects of IAP in the child (Hughes, Brocklehurst P, Steer PJ, Heath P 2017). IAP changes the neonatal gut microbiota by reducing early colonization of the gut in newborns with lactobacilli (Keski-Nisula et al. 2013) or *Bifidobacterium* (Corvaglia et al. 2016). Early colonization of the gut in newborns plays a role in the development of infants microbiome and immune system (Tamburini et al. 2016). Imbalance or maladaptation of gut microbial community (dysbiosis) has been linked to allergy (Rachid and Chatila 2016), obesity (Mueller et al. 2015) and diabetes in later life (Knip and Honkanen 2017).

The human vaginal microbiome in pregnancy is unique and less diverse than nonpregnant communities (Aagaard et al. 2012). *Lactobacillus jensenii* anaerobically metabolizes vaginal glycogen creating an acidic vaginal environment (Prince et al. 2014). Moreover,

antimicrobial compounds, e.g. hydrogen peroxide and bacteriocin-like substances are produced by Lactobacilli as a means of controlling vaginal pathogens (Prince et al. 2014; Boris and Barbés 2000). Agalacticin A has selective killing activity against GBS but no effect on *Lactobacillus* species. The narrow spectrum of agalacticin A may offer a benefit in prevention of perinatal GBS disease by removing maternal GBS colonization with no adverse effect on the maternal microbiota or the neonatal microbiota, which is largely derived from the mother. Currently, we cannot make solid conclusions that agalacticin A has no impact on the vaginal microbiome because bacterial species other than Lactobacilli not tested in this study, e.g. Clostridiales, Bacteroidales, and Actinomycetales are also predominant in late-pregnancy (Aagaard et al. 2012). Impact on additional vaginal taxa should be tested to ensure that agalacticin A has no negative effect on microbial diversity. Stability of agalacticin A under environmental conditions, e.g. pH, temperature, proteolytic activity, and interaction of agalacticin A with vaginal-antimicrobial compounds should be evaluated. Modification of agalacticin A's structure may offer means to expand its biological activity in the clinical setting.

For application of agalacticin A in aquaculture to control *S. agalactiae* disease, there are several challenges such as the use of agalacticin A at a large scale and environmental degradation of the compound. Because of its selective impact on GBS, however, the use of agalacticin A could be a major improvement in aquaculture because it would allow for treatment of fish without negative impacts on water quality or the environment. *In vivo* testing should be conducted especially in the fish challenge model, which will be useful to obtain more information on agalacticin A's efficacy, impact on fish microbiota, route of administration and the interaction of fish, as a bacterial environment, on chemical and biological properties of agalacticin A.

3.4.3 Conclusion and further study

In summary, this study has shown killing activity of agalacticin A against human, bovine and piscine GBS strains supporting its potential as GBS-specific antimicrobial agent. Agalacticin A has a narrow spectrum towards closely related *Streptococcus* species which are causative pathogens in bovine mastitis, but which are not (*S. dysgalactiae* subsp. *dysgalactiae*) or very rarely (*S. canis*) found in humans (Vandamme et al. 1996; Pinho et al. 2013). Vancomycin-resistant *E. faecalis* can be inhibited by agalacticin A at high concentration. *In vitro*, there is no impact on several key representatives of vaginal microbiome. However, the application of agalacticin A needs more information on its

chemical and biological properties as well as a mode of action. The efficacy should be tested *in vivo* to evaluate the interaction of host factors with agalacticin A, e.g. enzymatic degradation and duration of action. *Galleria mellonella*, murine and fish models can be used for *in vivo* testing (Six et al. 2019; Tazi et al. 2010; C M J Delannoy et al. 2016). Moreover, impact on the diversity of the vaginal and gastrointestinal microbiome in mothers and offspring should be studied. The murine model of vaginal tract colonization with a synchronized estrous cycle (Patras et al. 2013; Patras and Doran 2016) can be used for comparison of agalacticin A and antibiotics in GBS removal and the changing in vaginal microbiome using culture (for GBS) and small-subunit (16S) ribosomal RNA gene-sequence-based characterisation of the microbiome (Rosen et al. 2017; Bernardini et al. 2017; Ding et al. 2018).

For application of agalacticin A in aquaculture, the use of agalacticin A in mass production and route of administration should be taken into account. Physiology related to bacteriocin's distribution and elimination may vary between fish species. Because of the importance of GBS for tilapia aquaculture, further research should initially focus on tilapia spp. The environmental factors in degradation of agalacticin A should be examined, such as water temperature, pond sediment and metal ions in water. Fish challenge models may be a powerful tool to explore the potential of agalacticin A as novel treatment in aquatic animals.

Chapter 4 Determination of the efficacy of agalacticin A *in vivo* using a *Galleria mellonella* larval challenge model

The *Galleria mellonella* challenge method and inoculum preparation protocol were developed by Dr. Anne Six, Institute of Infection, Immunity & Inflammation, University of Glasgow. The site-directed mutagenesis and mutant agalacticin A production were conducted by Catriona Thompson, Institute of Infection, Immunity & Inflammation, University of Glasgow. Larval samples were sent to the Veterinary Diagnostic Services, School of Veterinary Medicine, University of Glasgow, for preparation of histology and immunohistochemistry slides. Anatomy and physiology of *G. mellonella* were examined and discussed with Prof. Julian Dow, Institute of Molecular Cell & Systems Biology, University of Glasgow.

4.1 Introduction

In vivo models are valuable for study of the host-pathogen interactions and drug testing. GBS infection models have been developed in mammals, including mice, rats and cows (Tazi et al. 2010; Noel, Santos, and Vitale 1985; Jensen 1982) and fishes, including zebra fish larvae and Nile tilapia (Kim et al. 2015; Delannoy et al. 2016). Animal welfare concerns have given rise to acts and laws to control the unethical use of animals and minimize the pain caused to animals during experimentation. Alternatives to animal models, including use of invertebrates, are applied to minimise involvement of laboratory animals in scientific procedures (Doke and Dhawale 2015).

Insect models are now widely used to study host-pathogen interactions during bacterial infection and, for some studies, offer a viable alternative to vertebrate models. The costs of establishment and maintenance of insect models are generally very low and they are not subjected to the same ethical scrutiny as vertebrate models (Ramarao, Nielsen-Leroux, and Lereclus 2012). Importantly, the innate immune systems and signalling pathways are conserved between insects and mammals (Kang et al. 1998). For example, superoxide production and microbial killing by insect hemocytes is similar to the mechanism used by human neutrophils (Bergin et al. 2005). The density of hemocytes and hemolymph protein can be elevated on repeated infection (Fallon, Troy, and Kavanagh 2011), but insects do not have an antibody-producing adaptive immune system. *Galleria mellonella* larvae have been widely used in bacterial and fungal infection models and to test the efficacy of antimicrobial compounds (Tsai,Loh and Proft 2016; McArthur 2013; Ba et al. 2015). A range of bacterial pathogens have been tested in the *G. mellonella* model including *Bacillus cereus* (Kamar et al. 2013), *Listeria monocytogenes* (Mukherjee et al. 2010), *Pseudomonas aeruginosa* (Miyata et al. 2003), *Staphylococcus aureus* (Peleg et al. 2009), *Streptococcus pyogenes* (Loh et al. 2013) and *Enterococcus faecium* (Lebreton et al. 2012).

Galleria mellonella or the greater wax moth is of the order Lepidoptera (butterflies or moths). Larvae (also called caterpillars) can be reared at temperatures from 15°C to above 37°C (Réjasse et al. 2012; R. T. Jones et al. 2010). This is useful to study gene expression in response to temperature changes (Smoot et al. 2001) or to study bacterial infection at relevant host-specific (e.g. poikilothermic versus homeothermic) body temperatures (Six et al. 2019). Larvae can be maintained without feed or with feed, such as beeswax and pollen or an artificial diet (Mukherjee et al. 2010). Challenging *G. mellonella* larvae by injection has been widely practiced but oral inoculation has been reported for *B. cereus*

(Fedhila et al. 2006). Six and colleagues have reported that *G. mellonella* is an effective model for studying GBS isolated from multiple host species (humans, cattle and fish) (Six et al. 2019).

Unlike *in vitro* testing, the testing of antimicrobial agents *in vivo* offers predictors of host factors that may influence antibiotic efficacy (Zak and O'Reilly 1991). The *G. mellonella* model can therefore serve as an additional pre-screening test for antimicrobial agents, including antimicrobial peptides and bacteriocins, before proceeding to tests in mammalian models (Tsai, Loh, and Proft 2016; Gibreel and Upton 2013; Smith et al. 2012).

4.2 Aims and objectives

Based on results from Chapter 3, it is hypothesized that agalacticin A can have efficacy against GBS *in vivo*. The objectives of this chapter are to 1) Test the efficacy of agalactacin A in the *G. mellonella* infection model, 2) Compare the activity of agalacticin A to activity of antibiotics, 3) Determine if agalacticin A can be used in combination with small molecule antibiotics, 4) Determine host factors in reduction of the activity of agalacticin A. The workflow for this chapter is shown in Figure 4-1. Details of methodology are given in Chapter 2.

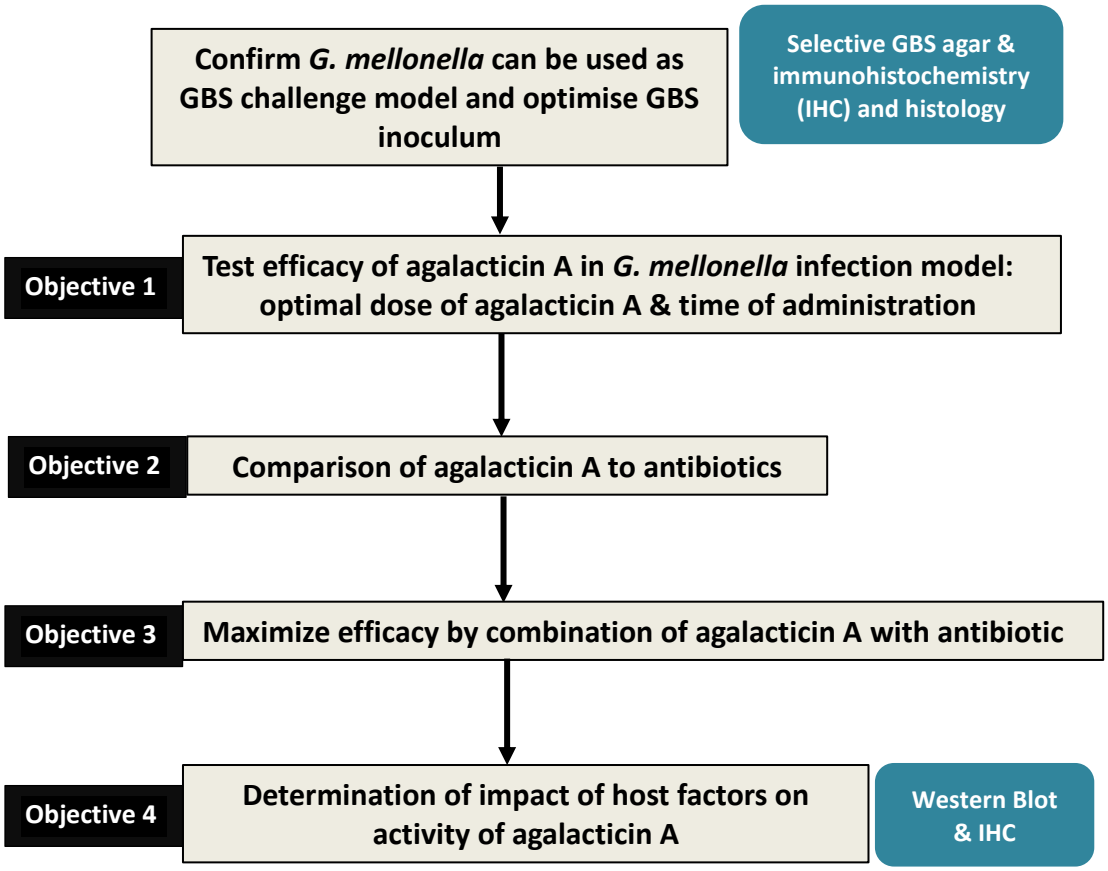


Figure 4-1 Workflow for testing of efficacy of agalacticin A against group B *Streptococcus* (GBS) in *Galleria mellonella* model
Overall steps and procedures used in evaluating the efficacy of agalacticin A against GBS *in vivo*. Firstly, it was confirmed that GBS can multiply in *G. mellonella*. Optimization of bacterial inoculum and administration of agalacticin A were performed before determination of agalacticin A's efficacy by GBS challenge and bacterial count (Objective 1). To explore whether agalacticin A can be used as an alternative to antibiotics, survival and bacterial count were compared between bacteriocin and conventional treatments (Objective 2). Feasibility of using a combination of agalacticin A with antibiotic was also tested by GBS challenge and treatment (Objective 3). Finally, results from comparison of agalacticin A to antibiotics suggested a role of host factors in the distribution and elimination of agalacticin A in *G. mellonella* larvae and this was explored (Objective 4). Methods used in this work consisted of larval survival test and/or bacterial counting on GBS selective agar. Western Blot, histology and immunohistochemistry (IHC) were used for detection of GBS or agalacticin A in larval tissue (green boxes).

4.3 Results

4.3.1 *Galleria mellonella* can be used as a GBS infection model

To begin to explore the use of *G. melonella* as a host for the study of GBS infection it was first determined wheter GBS is a commensal microorganism in *G. mellonella*. To attempt to isolate GBS from *G. melonella* we plated homogenised samples of unchallenged larvae on selective chromogenic GBS agar (Brilliance GBS Agar). No GBS colonies were isolated by this method. In addition, immunohistochemistry with an anti-GBS antibody (see section 2.6.4) was performed on larval tissue sections. GBS could not be detected in larval tissue by this method. The method is able to detect GBS in larvae (see below), so the results were considered to be true negatives rather than a flaw of the methodology. After confirming that larvae are GBS-free, GBS infection of *G. melonella* larvae was assessed by growth and pathogenicity.

Initially, MRI Z2-366, an ST283 piscine GBS strain, was injected into larvae using the method described in section 2.6.3 (1×10^6 CFU per larva). Larvae were incubated at 37°C and colony counts were performed at time points from 30 min to 24 h post injection by homogenizing larvae (n=3 at each time point) and plating dilution series on Brilliance GBS Agar (section 2.6.6). Colony counts suggest rapid growth in vivo from 2 to 4 h post inoculation and a stationary phase from 6 to 24 h after inoculation (Figure 4-2a). IHC of larval sections (Figure 4-3) confirmed *in vivo* GBS multiplication with bacterial growth concentrated around the fat body and serosa of the gastrointestinal tract and respiratory tract. GBS challenge induced a cellular immune response resulting in melanisation (Figure 4-2b) and hemocyte infiltration (Figure 4-3, blue circles).

Challenge with GBS can be lethal to *G. mellonella*, as most larvae were moribund or died within 24 h. To demonstrate that agalacticin A can provide a therapeutic effect on larvae with GBS infection, the number of cells in the GBS inoculum must be sufficient to kill most larvae without treatment but not so high that it kills all larvae before the treatment can be administered. Therefore, LD_{80} within 24 h was chosen for use in the survival test. The hypervirulent or meningitis-associated strains (ST17 and ST283) are important in human medicine and were selected as the focus of this experiment. ST17 was represented by human isolates MRI Z2-093, MRI Z2-121 and MRI Z2-132. ST283 was represented by piscine isolates MRI Z2-366, STIR-CD-25 and MRI Z2-399.

Known inocula for LD_{80} of MRI Z2-093, MRI Z2-366 and STIR-CD-25 were provided by Dr. Anne Six. For the remaining three isolates, inocula for LD_{80} determination were prepared as described in section 2.6.3. For ST17, test doses were 1×10^9, 1.5×10^9 and 2×10^9 CFU/ml. These inocula were administered to a group of 10 larvae each and 2 mg/ml agalacticin A and antibiotics were initially assessed as treatments. A dose less than 1×10^9 CFU/ml was sublethal, causing no differences between control, challenge and treated groups. At 2×10^9 CFU/ml, infection was heavy, leading to indistinguishable results for challenge and treatment groups. The optimal dose is 1.5×10^9 CFU/ml, providing LD_{80} as well as response to treatments. The LD_{80} of ST283 isolate MRI Z2-399 is 2×10^8 CFU/ml (Table 4-1). Melanisation, causing larvae to turn dark, was observed within 2 h post inoculation with ST17 but delayed or weak after inoculation with ST283. Melanisation in *G. mellonella* after challenge with ST17 is not correlated with death.

Table 4-1 Group B *Streptococcus* inoculum used in *Galleria mellonella* challenge model.

Strains	Host species	CC	ST	CFU per ml	CFU per larva	Reference
STIR CD 25	Tilapia	283	283	3×10^8	3×10^6	Six et al. 2019
MRI Z2-366	Tilapia	283	283	1×10^8	1×10^6	Six et al. 2019
MRI Z2-399	Tilapia	283	283	2×10^8	2×10^6	This study
MRI Z2-093	human	17	17	1.5×10^9	1.5×10^7	Six et al. 2019
MRI Z2-121	human	17	17	1.5×10^9	1.5×10^7	This study
MRI Z2-132	human	17	17	1.5×10^9	1.5×10^7	This study

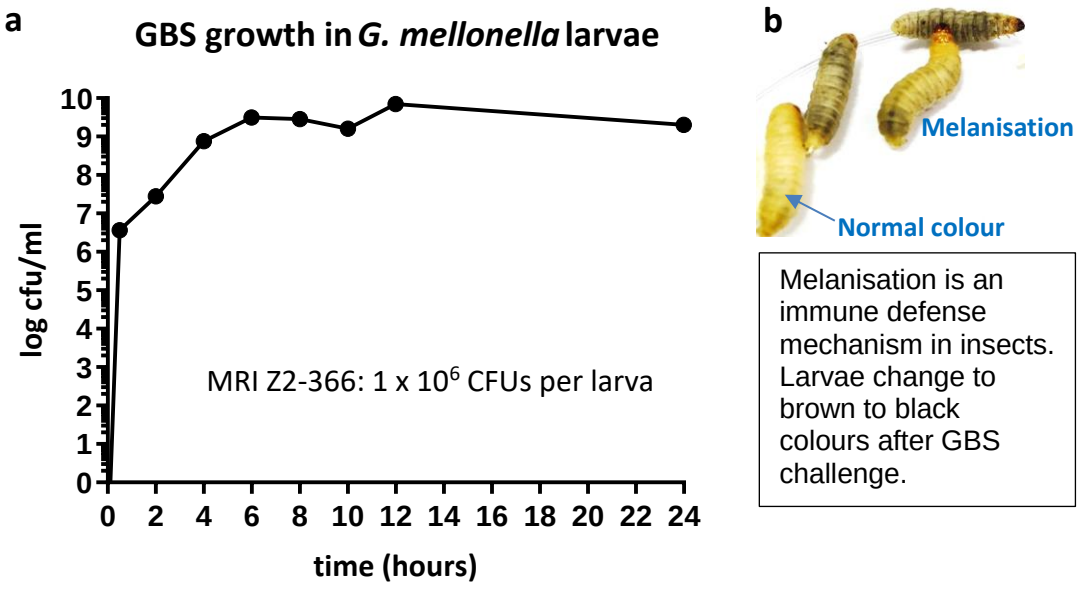


Figure 4-2 Group B *Streptococcus* (GBS) growth in *Galleria mellonella*
(a) Increase in the GBS concentration in *G. mellonella* larvae provides evidence of bacterial growth and shows that larvae can be used as infection model for bacteriocin/antibiotic testing. Example based on growth of GBS strain MRI Z2-366. Similar results were obtained with other strains listed in table 4-1. (b). Melanisation (development of dark colour) indicates that the larvae mount an immune response to GBS infection. Example based on response to MRI Z2-093 at 2 hrs after challenge. Similar results were obtained for other strains listed in table 4-1. At 24 h, all larvae were moribund or dead, regardless of which GBS challenge strain was used.

a

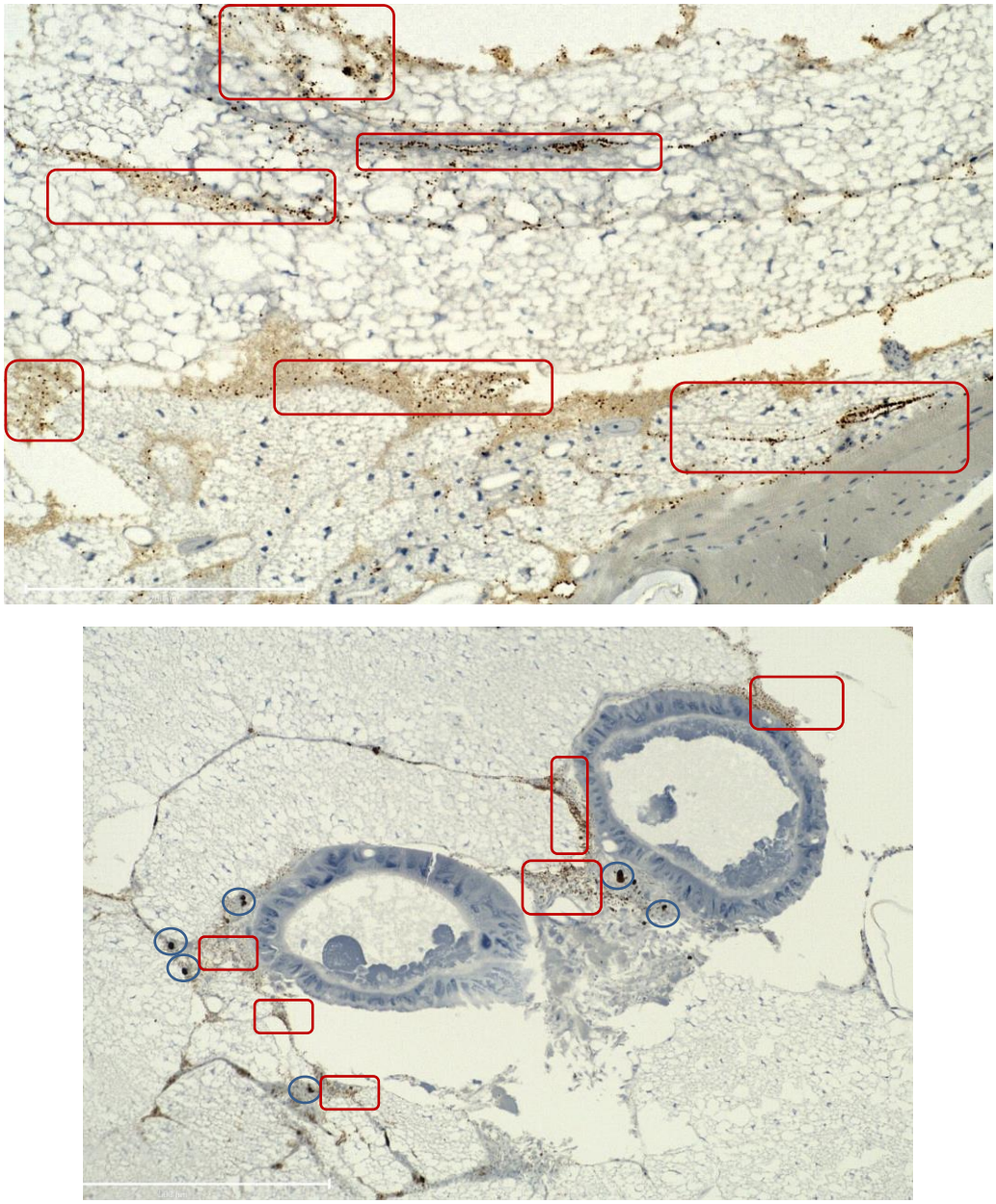


Figure 4-3 Multiplication of group B *Streptococcus* (GBS) in larval tissue
(a) GBS in larval tissue sections were detected by immunohistochemistry using anti-Streptococcus Group B antibody ab53584. 3,3'-diaminobenzidine (DAB) was a chromogenic substrate which results in a brown precipitate at the GBS cells (brown dots in red boxes). Innate immune response of insects plays a role in control of infection. Hemocytes are a cellular defense mechanism responsible for phagocytosis, nodulation and encapsulation. Insects produce several types of hemocytes. Phagocytic hemocytes engulf bacterial cells. For function of nodulation and encapsulation, hemocytes form an overlapping sheath around a target. Some hemocyte types contain cytoplasmic phenoloxidase precursors that likely play a role in melanization of hemolymph (Lavine and Strand 2002). Hemocytes (blue circles) can be seen as the large granular cells or small semigranular cells (García-García, García-García, and Rosales 2009).

b

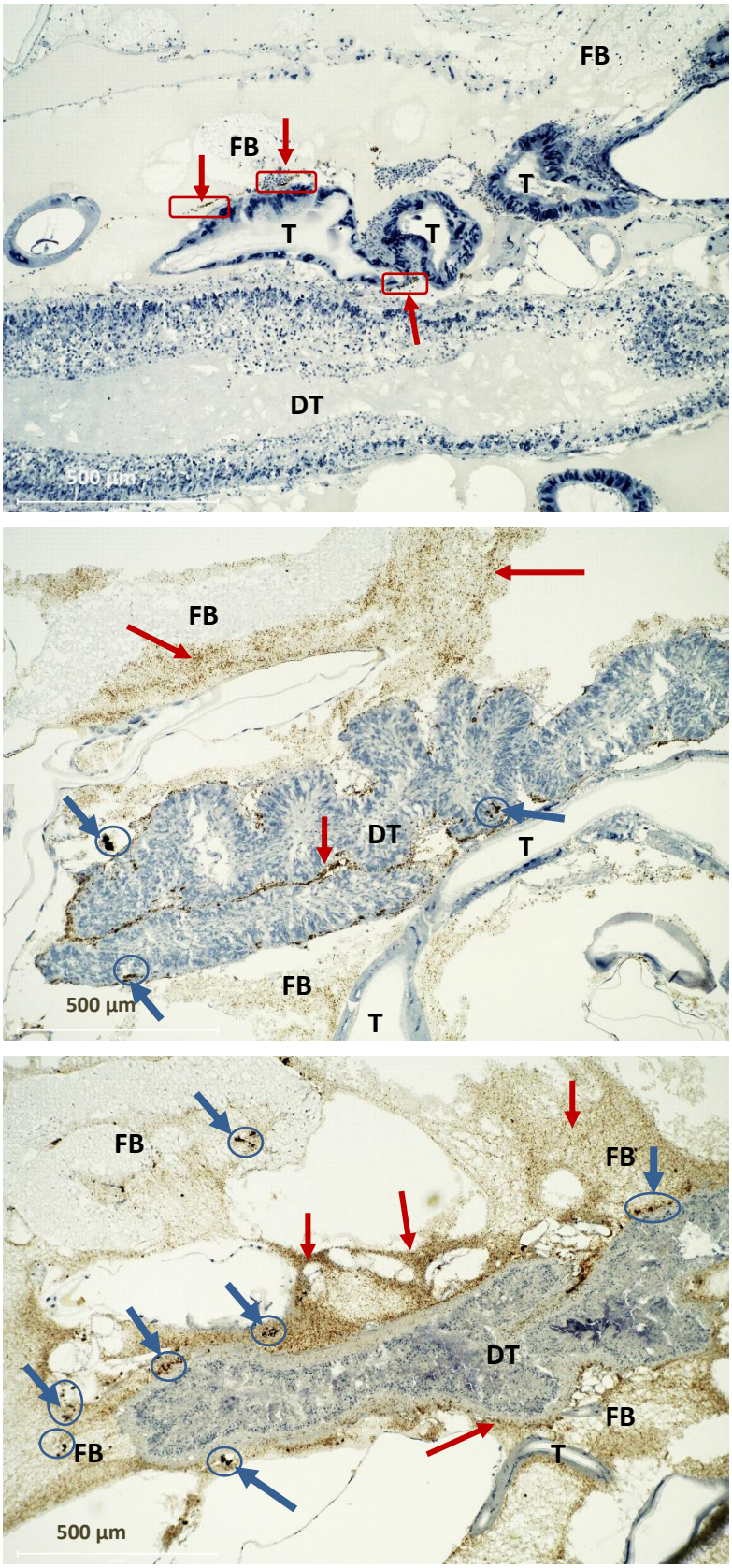


Figure 4-3 Multiplication of group B *Streptococcus* (GBS) in larval tissue
(b) Immunohistochemistry showed the presence of GBS in larval tissue sections at early stage after inoculation (0 h), late exponential (6 h) and stationary phase (12 h). GBS can be seen as brown staining (red arrows and boxes). Hemocyte infiltration (blue arrows and circles) indicates innate immunity is active during infection. DT; digestive tract, FB; fat body and T; trachea.

4.3.2 Optimal dose of agalacticin A & time of administration

4.3.2.1 Optimization of the dose of agalacticin A

GBS replication was strongly associated with mortality in *G. melonella* larvae. The *G. melonella* infection model was utilised to assess the *in vivo* efficacy of agalacticin A. Agalacticin A (10 µl) was initially injected at 2, 0.2 and 0.02 mg/ml into *G. mellonella* larvae 2 h post inoculation with MRI Z2-366 (ST283) and survival was monitored over 72 h post inoculation (Figure 4-4a). Dose-dependent survival was observed with survival at the lowest dose being similar to PBS-treated controls. However, increasing the dose to 5 mg/ml did not improve survival and so 2 mg/ml was selected as the optimal dose (Figure 4-4b).

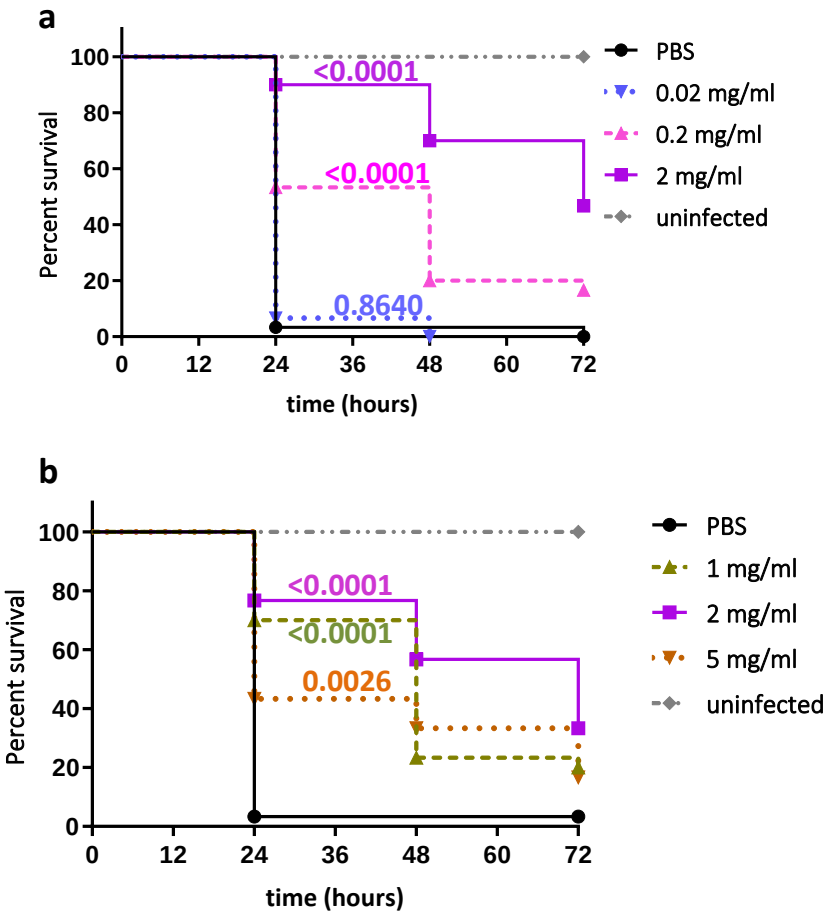


Figure 4-4 Optimisation of the dose of agalacticin A
(a) Survival of *Galleria mellonella* larvae depends on the dose of agalacticin A administered after challenge with group B *Streptococcus*. Concentrations less than 2 mg/ml have lower efficacy to rescue larvae. (b) Higher concentration of agalacticin A did not provide a better effect. Numbers indicate p-value based on comparison of treatment group to PBS group by Log-rank test.

4.3.2.2 Effect of time of administration on agalactacin A efficacy

Growth of GBS in *G. mellonella* (section 4.3.1) suggested that the number of GBS had increased by 2 h post inoculation and that exponential growth occurred at 2 to 6 hrs post inoculation. To determine the effect of changing the time of agalactacin A administration on treatment efficacy, larvae were challenged with MRI Z2-366 (ST283) and 10 µl of 2 mg/ml agalactinin A (20 ug per larva) were administered at either 2, 4, 6 or 8 h post-infection. Survival was monitored for 72 h post challenge (Figure 4-5a) and bacterial counts were also determined (Figure 4-5b). There was a clear effect of decreased survival with increasing the time between challenge and agalacticin A administration. Injection of agalacticin A at an early stage of bacterial multiplication, especially at 2 h, afforded the highest survival proportion with the lowest bacterial load.

Plating of homogenised larvae for bacterial counts (see section 2.6.6) showed that non-GBS microorganisms can grow on the selective chromogenic GBS agar, seen as blue colonies (Figure 4-5c). These colonies were tested by a group B-specific latex agglutination test (Slidex Strepto Plus B; bioMérieux, Marcy L'Étoile, France) and they were not GBS colonies. Because the non-GBS colonies did not interfere with GBS quantification, they were not investigated further.

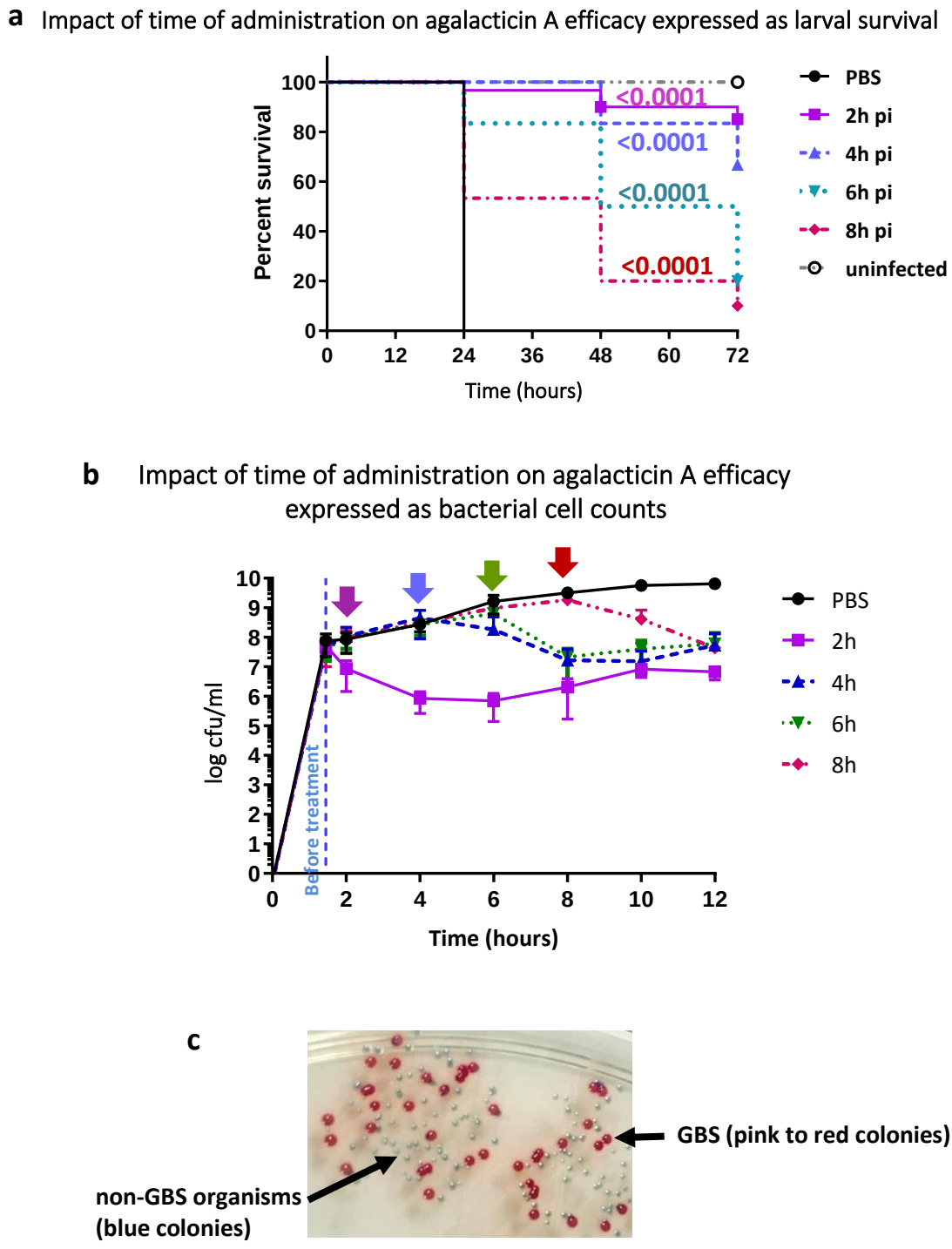


Figure 4-5 Optimization of the time of agalacticin A administration to protect *Galleria mellonalla* larvae from death due to group B *Streptococcus* (GBS) challenge

(a) Survival of larvae in response to agalacticin A administration at different times after GBS challenge. (b) Bacterial load of GBS recovered from homogenised larval samples after challenge with GBS and subsequent agalactin treatment. Arrows indicate time of agalacticin A injection at 2 h (purple), 4 h (blue), 6 h (green) and 8 h (red) post callenge. Agalacticin A can reduce bacterial load regardless of time of injection. However, administration at 2 h provides the highest survival proportion with the lowest bacterial load. (c) GBS colonies on the selective chromogenic agar (CHROMID Strepto B) appearing in pink to red and non-GBS microorganisms in blue colonies. Only pink or red colonies are counted to evaluate GBS counts.

4.3.2.3 Confirmation of functionality of agalacticin A

This was a collaboration with Catriona Thompson to determine the structure and active site of agalacticin A. The site-directed mutagenesis was based on the predicted active site of zoocin A. Zoocin A and agalacticin A are members of the M23 metalloprotease family. The active site of this family is conserved. Their catalytic mechanism involves a metal, i.e. a zinc ion in coordination with amino acid residues at the active site (Auld 2004; Grabowska et al. 2015). Thompson provided mutant agalacticin A for *in vivo* testing. The active site residue of the N-terminal catalytic domain was mutated from a histidine (H_{109}) residue to alanine (Thompson, unpublished). Activity of wild-type and mutant agalacticin A was checked *in vitro* on spot test and liquid culture and *in vivo* in the *Galleria* model. MRI Z2-366 was used in survival tests as described in section 2.6.5. Enzymatic activity of mutant agalacticin A was absent both *in vitro* and *in vivo* (Figure 4-6). This supports the idea that enzymatic activity of agalacticin A provides a therapeutic effect against GBS.

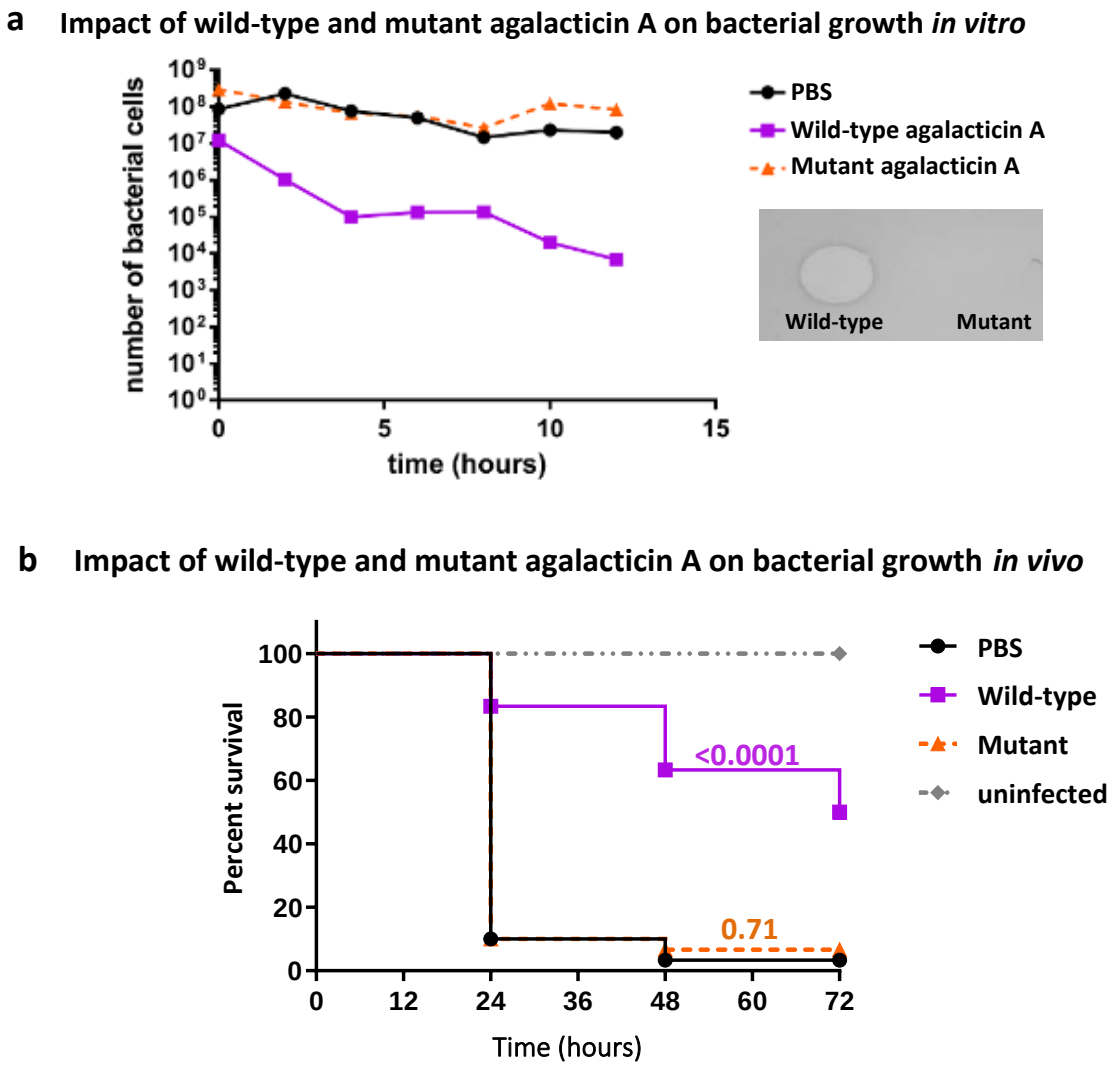


Figure 4-6 Biological activity of wild-type and mutant agalacticin A against group B *Streptococcus* (GBS)

Mutant agalacticin A was obtained from site-directed mutagenesis of the histidine residue in the active site of the N-terminal catalytic domain. (a) Incubation of GBS sequence type283 with wild-type and mutant agalacticin A in brain heart infusion broth shows that mutant agalacticin A lacks killing activity and induces no growth inhibition on spot test. (b) Administration of mutant agalacticin A does not provide protection against GBS infection in *Galleria mellonella* larvae. Modification of the histidine residue eliminates biological activity of agalacticin.

4.3.3 Comparison of agalacticin A to antibiotics

After optimization of the *G. mellonella* GBS infection model for testing agalacticin efficacy, a workflow was developed (Figure 4-7) to compare the efficacy of agalacticin A to ampicillin and erythromycin, which are representatives of intrapartum antibiotic prophylaxis (IAP) agents recommended by the Centers for Disease Control and Prevention (CDC) (Verani, McGee, and Schrag 2010).

Penicillin is a drug of choice in prevention of perinatal GBS disease in mothers and neonates and ampicillin is also a recommended agent. Ampicillin remains the preferred antibiotic to treat neonatal septicemia and meningitis. Ampicillin has broader antimicrobial activity than penicillin and is active against most gram-positive and some gram-negative bacteria, e.g. *Escherichia coli* (Wade and Benjamin 2011). An association between IAP and ampicillin resistance in newborns with *E. coli* or other non-GBS early-onset sepsis has been observed in the United States (Verani, McGee, and Schrag 2010). Erythromycin is an alternative antibiotic offered to penicillin-allergic mothers. For these reasons, we selected ampicillin and erythromycin in our study. Comparison of agalacticin A to antibiotics is performed by larval survival analysis and bacterial counts from homogenised larval samples on GBS selective agar.

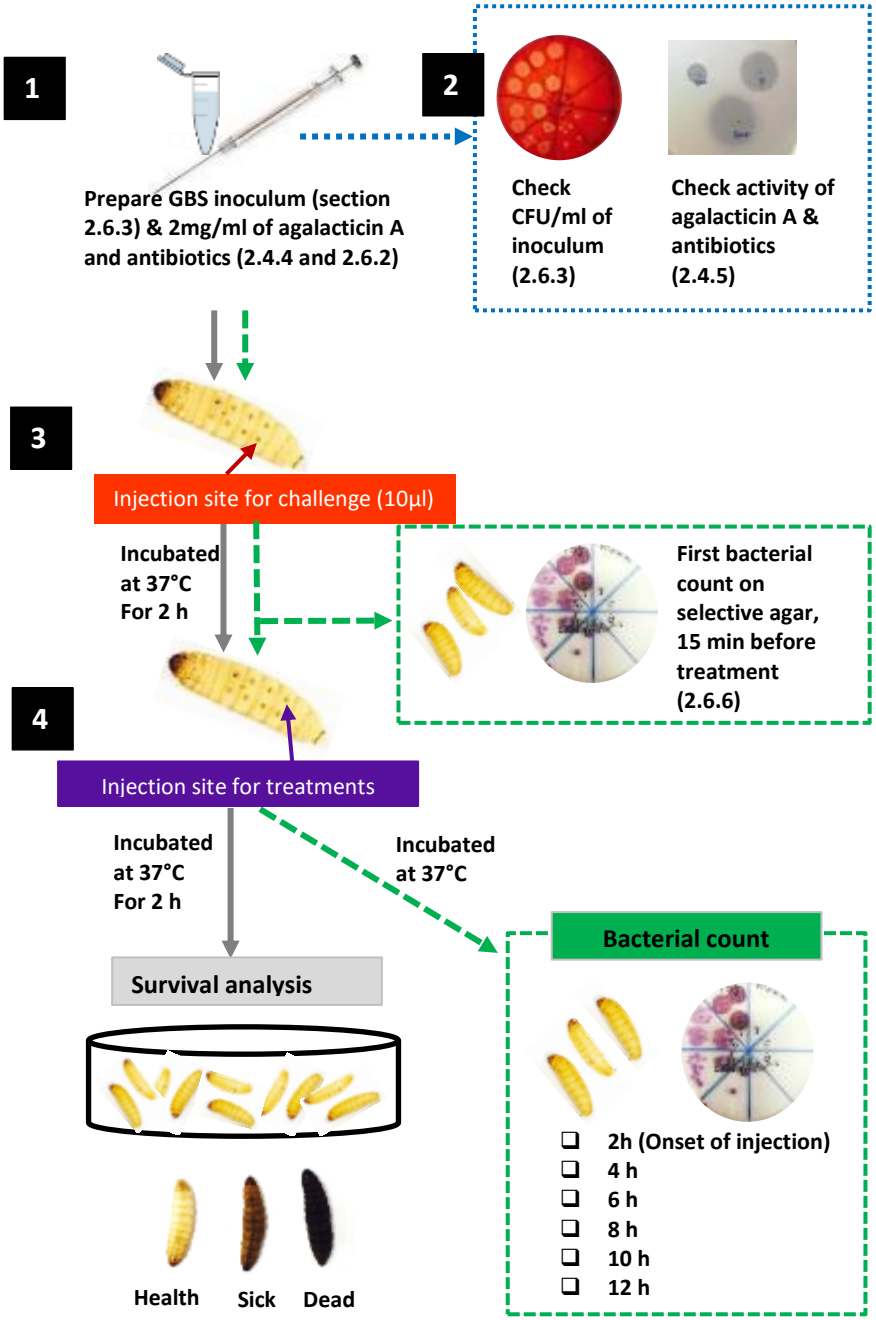


Figure 4-7 Workflow for comparison of agalacticin A to antibiotics in rescue of *Galleria melonella* larvae from group B *Streptococcus* (GBS) challenge
Procedures for larval survival analysis (solid grey line) and bacterial counts (green dashed line) were set up to compare the efficacy of agalacticin A to ampicillin and erythromycin, which are commonly used in intrapartum antibiotic prophylaxis. Steps shared between survival analysis and bacterial count are shown in a black box and numbers in parentheses refer to methods described in Chapter 2. Inoculum is prepared (step 1) and the number of colony-forming units (CFU) is checked on blood agar. During that time, agalacticin A and antibiotics are prepared at 2 mg/ml concentration and their activity is checked by spot test using the inoculum isolate (step 2). Inoculum (10 µl) is injected into the last right proleg and larvae are incubated at 37°C for 2 h (step 3) before treatment (10 µl) is injected in the last left proleg (step 4). The first bacterial count is performed 15 min before treatment to ensure that larvae have similar levels of infection. Larvae are collected after agalacticin A or antibiotic injection every 2 h until 12 h (counting time post challenge). For survival analysis, a group of 10 larvae is used in each treatment. Uninfected larvae injected with PBS, agalacticin A or antibiotics, respectively, are used as control to rule out death from agents' toxicity and trauma. Larvae are observed over 72 h to detect number of deaths.

4.3.3.1 Comparison of larval survival between agalacticin A and antibiotic treatments

Larvae were inoculated with hypervirulent GBS isolates (ST17 and ST283). The number of CFU in each inoculum is given in Tabel 4-1 and antibiotic susceptibility profiling is in Appendix ii. Agalacticin A, ampicillin and erythromycin at a concentration of 2 mg/ml were injected 2 h post inoculation and survival was assessed at 24, 48 and 72 h. Survival proportions of agalacticin A-treated larvae were similar to those for ampicillin and erythromycin-treated larvae except that ampicillin did not significantly increase survival of larvae infected with MRI Z2-132 (Figure 4-8). Low mortality was observed in Agalacticin A-treated groups across most challenge isolates. However, the success of agalacticin A in rescuing larvae from GBS infection was highly variable, as each test isolate showed a different response to agalacticin A and antibiotics.

ST17 (human isolates)	ST283 (fish isolates)

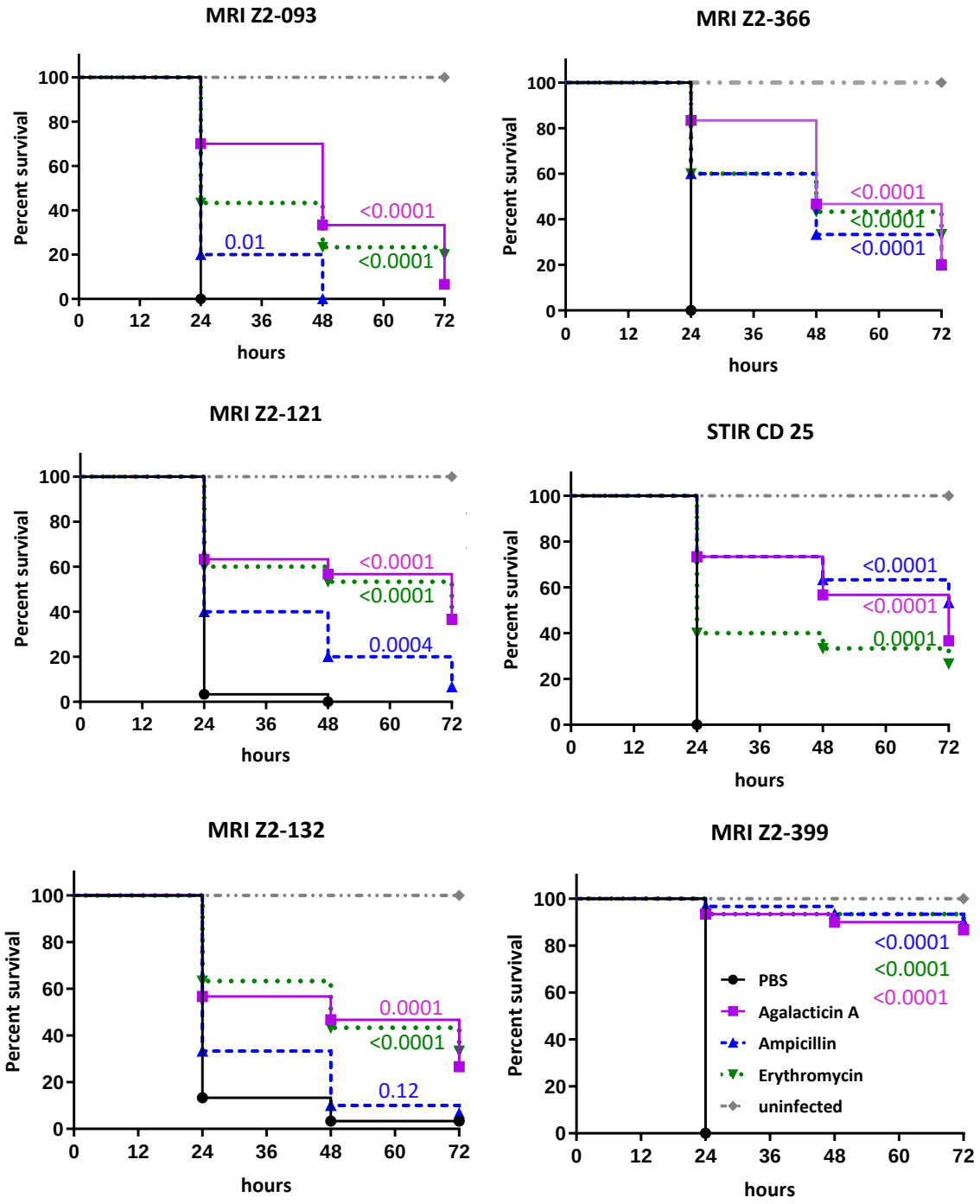


Figure 4-8 Impact of agalacticin A or antibiotic administration on survival of *Galleria mellonella* after challenge with different hypervirulent sequence types (ST) of group B *Streptococcus* (GBS)

To test efficacy of agalacticin A compared to antibiotics against hypervirulent GBS strains, ST283 (right) and ST17 (left) isolates are administered to groups of 10 larvae. Treatment is given in the early exponential phase of bacterial growth. Agalacticin A efficacy is similar to antibiotic efficacy in rescuing larvae from GBS challenge. The effect of agalacticin A is not uniform across GBS isolates. Numbers indicate p-values from comparison of treatment groups to PBS injected control groups by Log-rank test. PBS, black, agalacticin A; purple, ampicillin; blue; erythromycin, green and control; grey.

4.3.3.2 Comparison of bacterial counts between agalacticin A and antibiotic treatments

ST17 (MRI Z2-121) and ST283 (MRI Z2-366) were the test isolates used to evaluate bacterial load after each treatment. The number of CFU of GBS in larvae before treatment was not statistical different between groups based on the Kruskal-Wallis test (Table 4-2). Treatments were given at 2 h post inoculation and three larvae were collected immediately and used for bacterial count determination. In larvae treated with PBS, CFU count had increased compared to the pre-injection time point whereas CFU count had dropped immediately after injection in larvae treated with agalacticin or antibiotics (Table 4-2). Growth curves suggested agalacticin A served as bacteriolytic protein because the drop was immediate, whereas a bacteriostatic compound would only limit further growth and show gradual decline of CFU counts due to bacterial death. The GBS CFU count at 2 h post agalacticin treatment had dropped remarkably compared to pre-treatment values (Table 4-2 and Figure 4-9). The effect of ampicillin and erythromycin occurred later (for ST283) or more gradually (for ST17) (Figure 4-9). This suggests an early advantage of agalacticin A on larval survival due to a bacteriolytic effect. After 2 hrs post-treatment (4 hrs post-challenge) no further reduction in GBS numbers was seen in agalacticin A treated larvae and counts increased again from 4 to 10 h, possibly explaining high mortality of agalactin-treated larvae at 48 and 72 h, particularly among larvae challenged with MRI Z2-366.

Based on the difference in onset of impact of agalactin and antibiotic treatment, it was hypothesized that the combination of agalacticin A with antibiotics should improve survival. In addition, bacterial count data, particularly the increase in counts after the initial bacteriolytic effect, suggested that host factors may inactivate or break down agalacticin A. To explore these assumptions, combination treatment of agalacticin A with antibiotics and exploration of the fate of agalacticin A in the host were undertaken (section 4.3.4 and 4.3.5, respectively).

Table 4-2 Colony-forming unit (CFU) counts for group B *Streptococcus* in *Galleria mellonella* before treatment and immediately after treatment.

Isolates	Time points relative to treatment	Number of colony-forming units (CFU/ml)			
		PBS	Agalacticin A	Ampicillin	Erythromycin
MRI Z2-121	Before	1.11E+08	8.90E+07	3.55E+07	9.68E+07
	After	8.10E+08	4.22E+07	2.51E+08	2.11E+08
MRI Z2-366	Before	6.28E+06	1.75E+07	8.16E+06	1.30E+07
	After	1.33E+07	4.60E+06	5.94E+06	8.51E+06

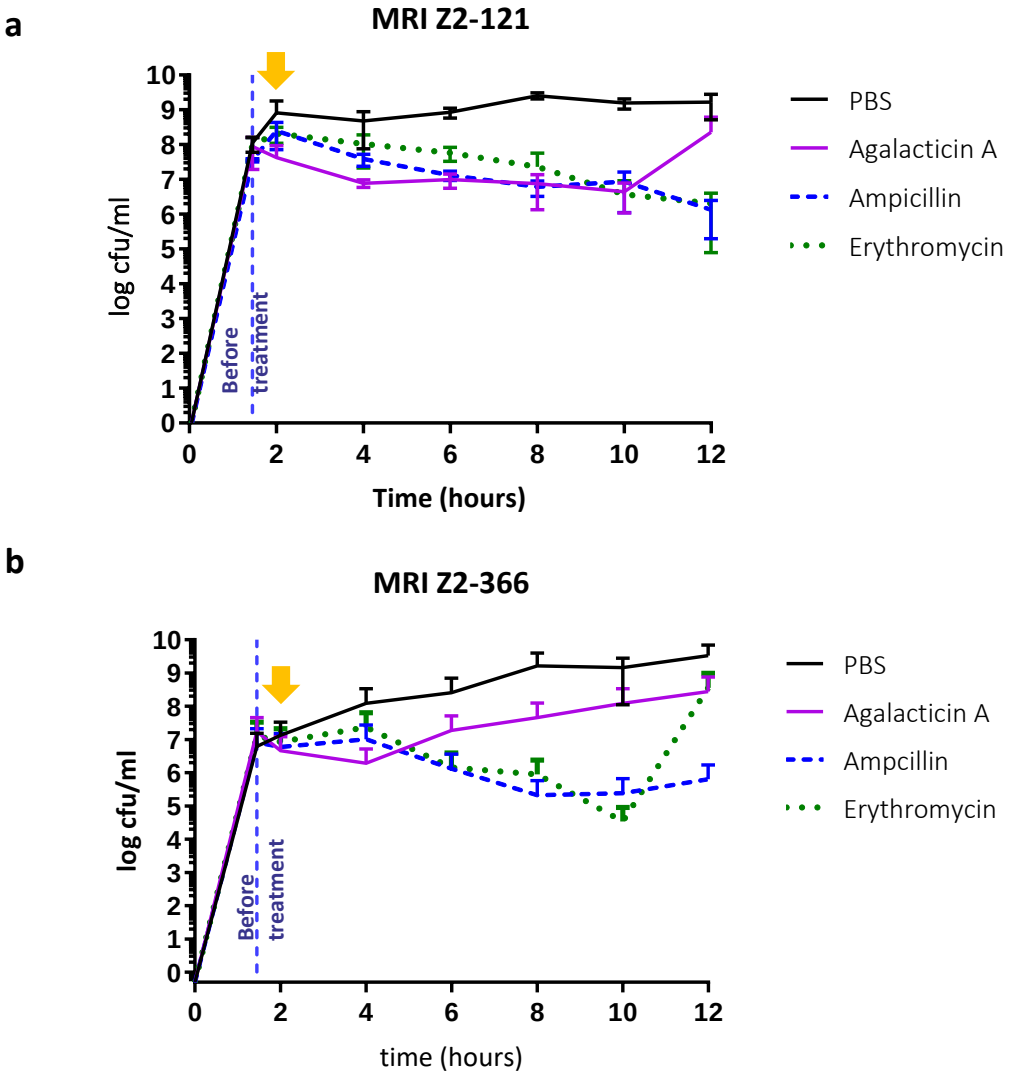


Figure 4-9 Group B *Streptococcus* (GBS) bacterial counts after challenge and treatment of *Galleria mellonalla* larvae with agalacticin A or antibiotics
Homogenised samples from three larvae were plated at each time point to evaluate CFU counts in response to PBS and treatments. Blue dashed line indicates the bacterial count immediately prior to treatment. Yellow arrows indicate the time of administration of treatment. GBS counts before and immediately after treatment are provided in Table 4-2. Agalacticin A causes an immediate drop in CFU count, suggesting a bacteriolytic effect. Ampicillin and erythromycin exert their antibacterial effect more slowly. From 4 hrs post challenge, CFU counts in agalacticin A-treated larvae follow the same trend as those in PBS-treated controls. In contrast, the shape of the CFU curve differs between PBs-treated controls and antibiotic-treated groups.

4.3.4 Use of agalacticin A in combination with broad spectrum antibiotics

The effect of agalacticin A on *G. mellonella*'s survival was not uniform but varies depending on bacterial isolates and its duration of action is quite short. A combination of bacteriocin with antibiotics was hypothesized to increase the effect of agalacticin A and also reduce the amount of antibiotic used. Although those specific hypotheses were not tested, a preliminary study was conducted to explore the potential of combining agalacticin and antimicrobial agents in treatment of GBS infection. Fractional inhibitory concentration (FIC) was not determined in this study so synergistic or additive effects or antagonism are not defined. Rather, the combination of agalacticin A with ampicillin or erythromycin at ratio of 1:1 of 2 mg/ml concentration was compared to each single agent.

Combination of agalacticin A with either ampicillin or erythromycin resulted in numerically higher survival of ST283 challenged larvae than use of bacteriocin or drug alone (Figure 4-10). In contrast, combination of ampicillin and erythromycin, used as control, resulted in lower survival. For ST17-challenged larvae, improved survival was seen after agalacticin + erythromycin treatment but not after agalacticin + ampicillin treatment. For ST283-challenged larvae, survival was lowest for treatment with the combination of antimicrobials. Those results are preliminary and the sample size was small, limiting statistical power, but they are sufficiently encouraging to support further evaluation of combination treatment.

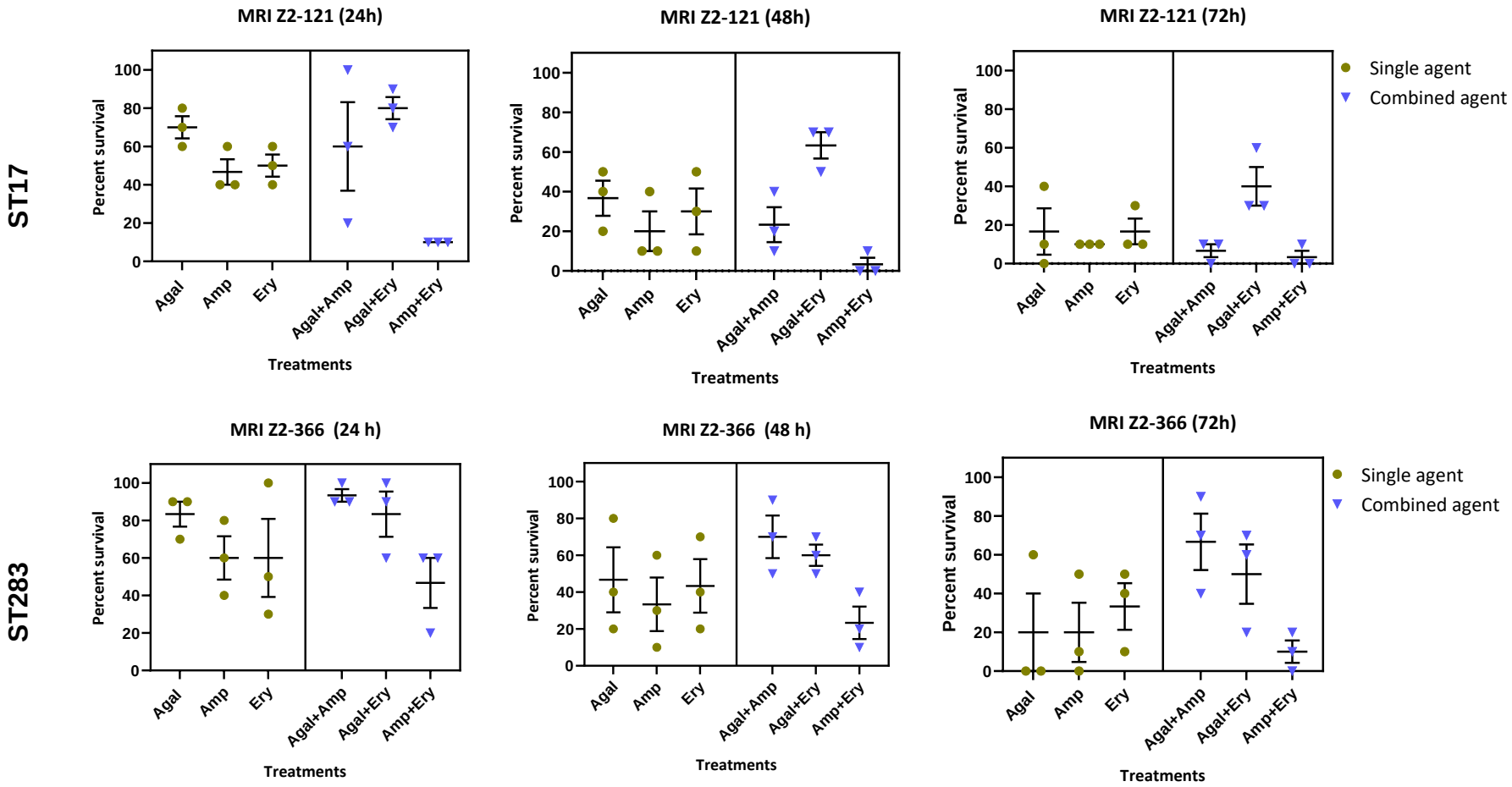


Figure 4-10 Comparison of single agent and combinations of agalacticin or antibiotic for treatment of group B *Streptococcus* infection in *Galleria mellonella.*
Hypervirulent strains ST283 and ST17 were used to challenge 10 larvae per treatment group and survival was observed at 24 h (left), 48 h (middle) and 72 h (right column) post challenge. Left side of graph represents treatment with a single agent and the right side represents treatment with combined agents. Three experiments were performed for each isolate and results for all replicate are shown with symbols. agal = agalacticin A, amp = ampicillin and ery - erythromycin

4.3.5 Distribution and elimination of agalacticin A in the host

4.3.5.1 Degradation or elimination of agalacticin A

The analysis of bacterial counts after treatment of GBS-infected larvae with agalacticin A, ampicillin and erythromycin indicated that the *in vivo* activity of agalacticin was short-lived relative to the small molecule antibiotics. This suggests that agalacticin A may be rapidly degraded by proteases or inactivated or eliminated in some other way through interaction with host factors. To test this hypothesis, Western blot and IHC using an anti-agalacticin A antibody (section 2.6.8) were performed to understand how agalacticin A is distributed and eliminated in larvae.

The loss of agalacticin A in larval tissue with time was determined by Western Blot. Protein was extracted by ReadyPrep Protein Extraction Kit (Bio-Rad) (section 2.6.8 b.) from groups of larvae (n = 3 at each time point) at 0, 2, 4, 6 and 8 h after agalacticin A administration. Levels of agalacticin A were visualised by Western Blot which indicated a reduction in amount of detectable agalacticin A with time (Figure 4-11a).

In addition, immunohistochemistry was used to detect the presence of agalacticin A in larval tissue over time in groups of larvae (n = 3 at each time point) at 0, 2, 4 and 6 h after agalacticin A administration. Visibility of agalacticin A-specific staining decreased over time, consistent with results from the Western Blot (Figure 4-11b). Anatomy and physiology of larvae were discussed with Professor Julian Dow, University of Glasgow and a conceptual model of agalacticin elimination was developed.

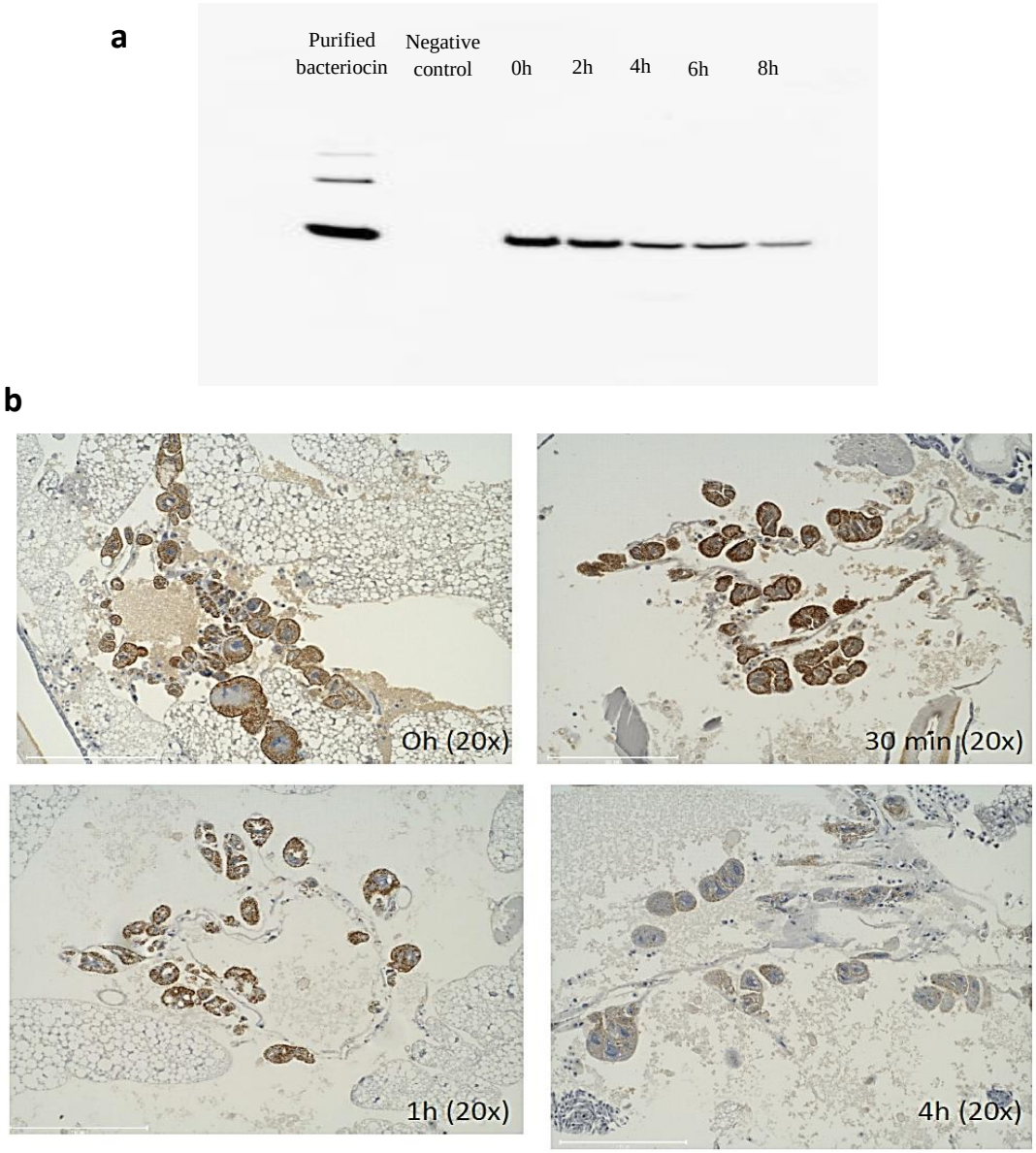


Figure 4-11 Concentration of agalacticin A in *Galleria mellonella* larval tissue after single administration

(a) Agalacticin A extracted from homogenised larval samples at 0,2,4,6 and 8 h after administration was visualised by Western Blot. Band intensity corresponds to amount of agalacticin A, showing a gradual reduction in agalacticin A detection with time. (b) Immunohistochemistry sections presented a faded signal of agalacticin A (brown staining) related to time post-inoculation which is consistent to finding from Western Blot. Tissue sections were microscopically examined by the EVOS® imaging systems with the same magnification.

4.3.5.2 Distribution of agalacticin A

To determine the distribution of agalacticin A in larval tissue, sections of larvae fixed at 0 h, 30 min, 1, 2, 4 and 6 h after administration of agalacticin A were microscopically examined. Agalacticin A had a highly restricted distribution and was found in the cells adjacent to the dorsal vessel (Figure 4-12). Agalacticin was not detected in the body cavity. To detect the change in distribution of agalacticin A with time, longitudinal sections of larvae from different time points after challenge were examined (Figure 4-13). Agalacticin was predominantly detected in the rear part of the larvae's body at 1 h post-administration, in the middle of the body at 2 hrs, and towards the front of the body at 4 hrs. This progression from rear to front matches the direction of the pumping action of the tubular heart of caterpillars. Removal of agalacticin A was clearly seen at 6 h as a reduction in the level of specific staining.

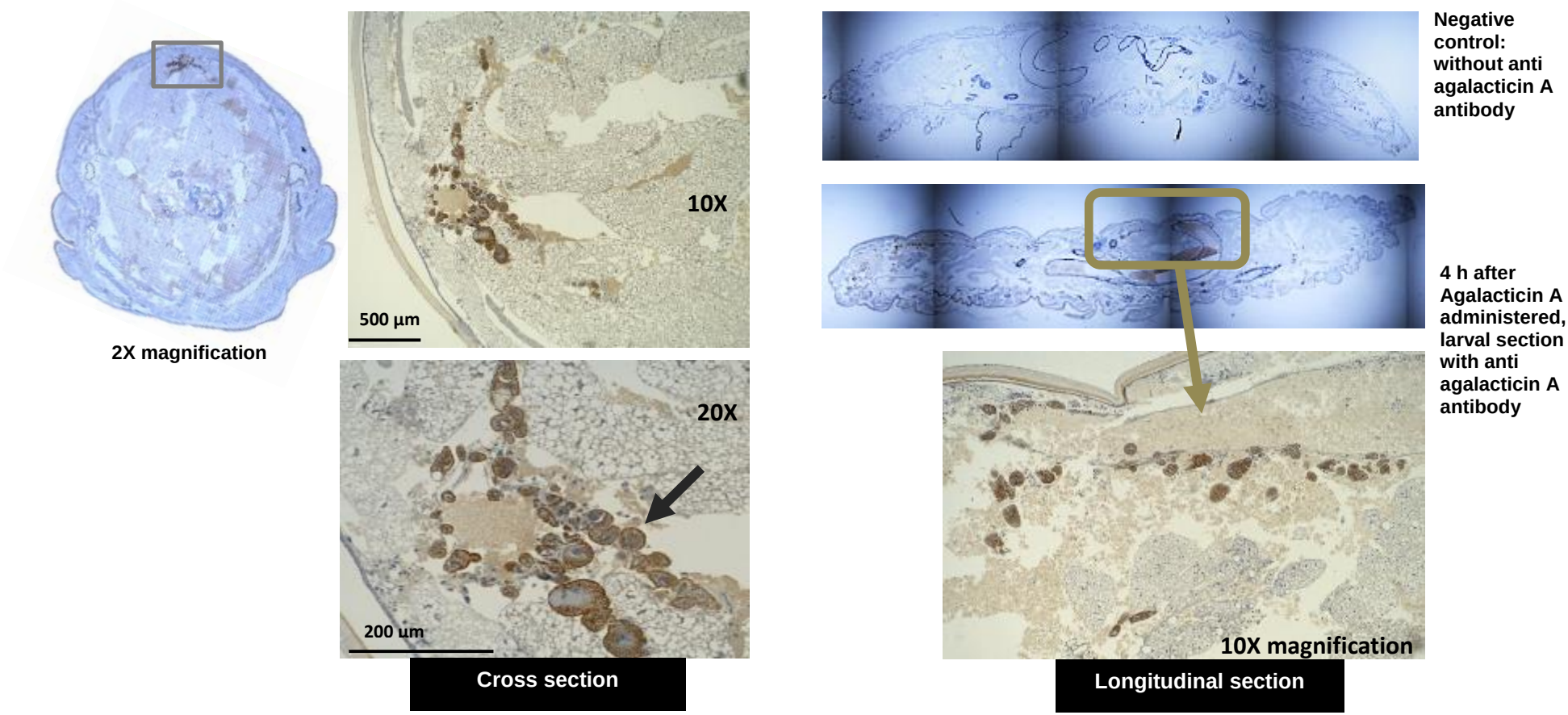


Figure 4-12 Immunohistochemistry (IHC) detecting agalacticin A in *Galleria mellonella* larvae
Larvae were injected with agalacticin A and three larvae were collected each time point (30 min, 2, 4 and 6h after administration). Larval samples were prepared in cross (left) and longitudinal (right) sections for IHC with anti agalacticin A antibody from rabbits. Left, detection of agalacticin A shortly after injection. Black arrow indicates agalacticin A, stained in brown, is trapped in cells around dorsal vessel, visible at the top in the 2x magnification and to the left in the 10x and 20x magnification.

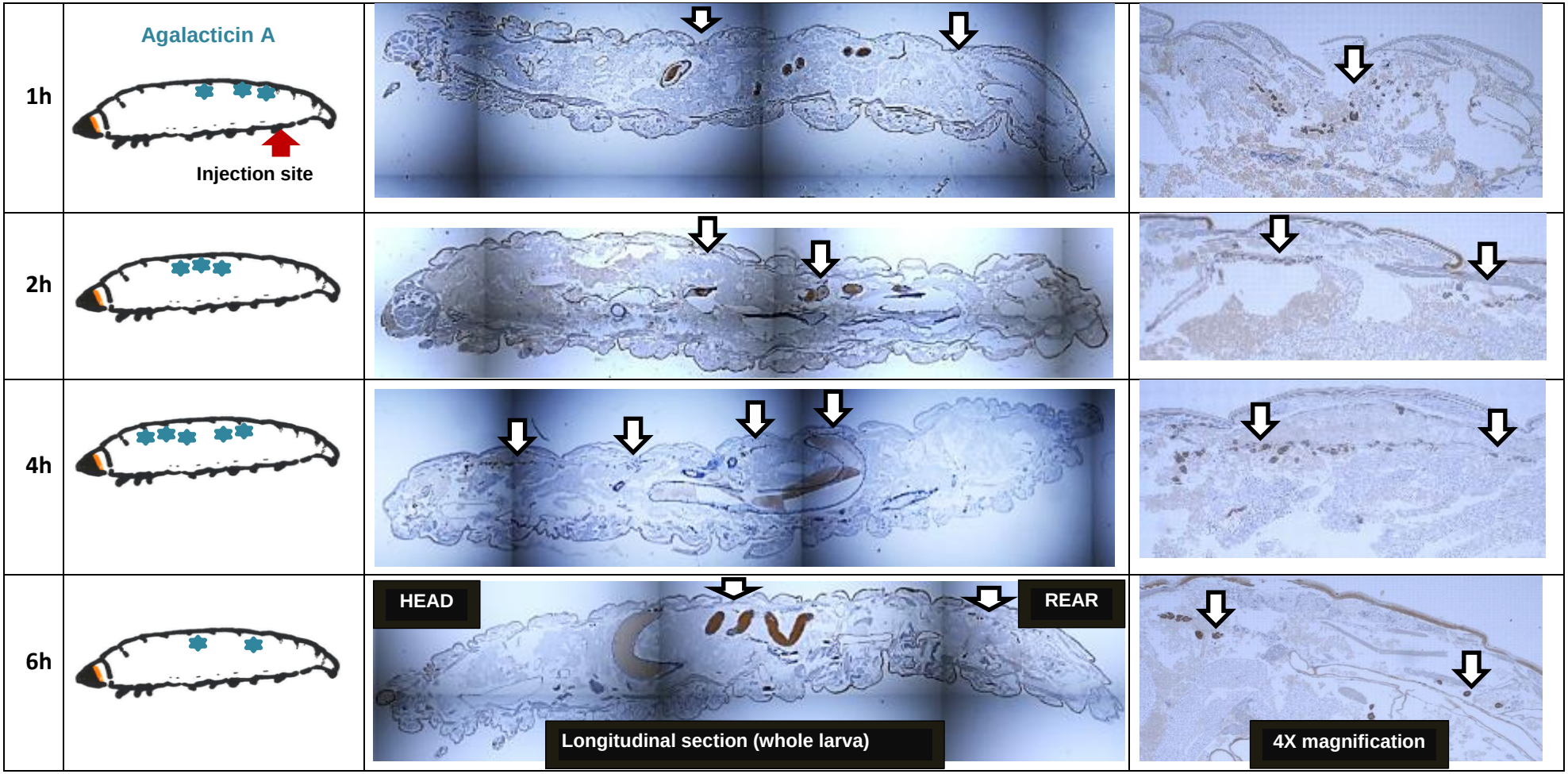


Figure 4-13 Distribution of agalacticin A in *Galleria mellonella* larvae over time as detected by immunohistochemistry
Presence of agalacticin A over time shown in blue stars (left) and white arrows (middle and right). Schematic drawing depicts location of agalacticin A post administration. Scanning of the whole larval sections showed that agalacticin A was distributed from rear to head over time, with agalacticin A trapped in cells shown at higher magnification in the right column.

4.4 Discussion

4.4.1 Overall results

This study has shown that *G. mellonella* is a useful host for studying the efficacy of antibiotics in treatment of GBS infection. During the course of this work, we reported that mortality in the *G. melonella* GBS infection model is dose-dependent and that hypervirulent ST283 and ST17 strains show increased virulence in this model (Six et al. 2019). *G. mellonella* has previously been used to investigate virulence factors in other bacterial species, including variations in capsule structure of *Streptococcus pneumoniae* strains (Evans and Rozen 2012), a cell wall anchored protein, M-types of *Streptococcus pyogenes* (Group A Streptococcus) (Loh et al. 2013) and hypervirulent and non-virulent strains of *Streptococcus suis*, which is a zoonotic pathogen (Velikova, Kavanagh, and Wells 2016). Results also demonstrate that agalacticin A has antistreptococcal activity *in vivo*. The bacteriolytic effect of this bacteriolysin was deduced from bacterial counts recovered after treatment of GBS-challenged wax moth larvae.

Initial assessment of combination of agalacticin A with conventional antibiotics suggests that combination treatment may be useful to reduce the volume of antibiotic needed to kill GBS. Combined bacteriocin and antibiotic treatment may offer advantages in therapeutic effect and the need for less antibiotic to be used, which could reduce selective pressure for antimicrobial resistance (Houndt and Ochman 2000). Groups of larvae treated with the combination of ampicillin and erythromycin, used as control, have the lowest percent survival, probably because the bacteriostatic activity of the macrolide is antagonistic to the action of ampicillin which inhibits cell wall synthesis during bacterial cell division (Lundgren et al. 2000). Combination of agalacticin A with ampicillin and erythromycin has a beneficial effect or no effect on survival compared to treatment with a single compound. In contrast, the activity of zoocin A, a member of the M23 metalloprotease was inhibited by penicillin (Heath et al. 2004). Therefore, additional drug combinations and interactions must be considered in further studies of antibiotics combined with agalacticin A.

Based on bacterial counts from challenged and treated larvae, and Western Blot and IHC on treated larvae, the duration of agalacticin A action is limited to 6 hrs. Agalacticin A has a highly restricted distribution in larvae and appears to be transported via the dorsal vessel. The observed distribution of agalacticin in *Galleria* larvae matches the distribution of

nephrocytes in *Drosophila*, suggesting that agalacticin may be concentrated in nephrocytes. Elimination of agalacticin by nephrocytes could explain the observed decrease in bioavailability over time, and the short duration of treatment effect described in section 4.3.5 and 4.3.3, respectively. Insect nephrocytes are aligned with the dorsal vessel and heart tubes (Zhang, Zhao, and Han 2013). They regulate haemolymph composition by filtration, followed by endocytosis, and metabolise toxic materials, and thus fulfil a renal-like function (Denholm and Skaer 2009). Proteins, foreign substances or toxins enter the nephrocyte's filtration system to be degraded or reused (Na and Cagan 2013). A mechanism explaining the linkage between reduction in detectable agalacticin A, its distribution in the larval body and insect physiology is proposed in Figure 4-14.

Short bioavailability of bacteriocins has been reported in the *G. mellonella* model before. Epidermicin NIO1 is a Class II bacteriocin with antistaphylococcal properties and has 50 min of action post administration in *G. mellonella* larvae (Gibreel and Upton 2013). The insect excretory system has anatomical and functional similarity to the vertebrate kidney. Nephrocytes and malpighian tubes are anatomically comparable to podocytes of glomeruli and renal tubes, respectively (Weavers et al. 2008; Denholm and Skaer 2009). Insect nephrocytes, like glomerular podocytes in the vertebrate kidney, form a size- and charge-selective barrier (Weavers et al. 2008). Bacteriocins are small molecules and may be taken up and metabolized faster than complex molecules (Weavers et al. 2008). For example, epidermicin NIO1 is small peptide containing 51 amino acid residues and a molecular mass of 6,074 Da (Sandiford and Upton 2012). Agalacticin A is eliminated more slowly than epidermicin, NIO1, possibly because it is a larger molecule (299 amino acids, 32 kDa) but faster than antibiotics, which have complex structures, e.g. ampicillin (phenyl ring) (Boles, Girven, and Gane 1978) and erythromycin (a multi-branched, lactone ring substituted with an amino and a nitrogen-free 6-deoxy sugar) (Martin et al. 1975). This may explain why antibiotics still had an effect on GBS in challenged larvae after agalacticin stopped working. The removal of proteins and bacteriocins by a kidney–like structure in *G. mellonella* suggested that agalacticin A is likely to be eliminated by the glomerular filtration in vertebrates.

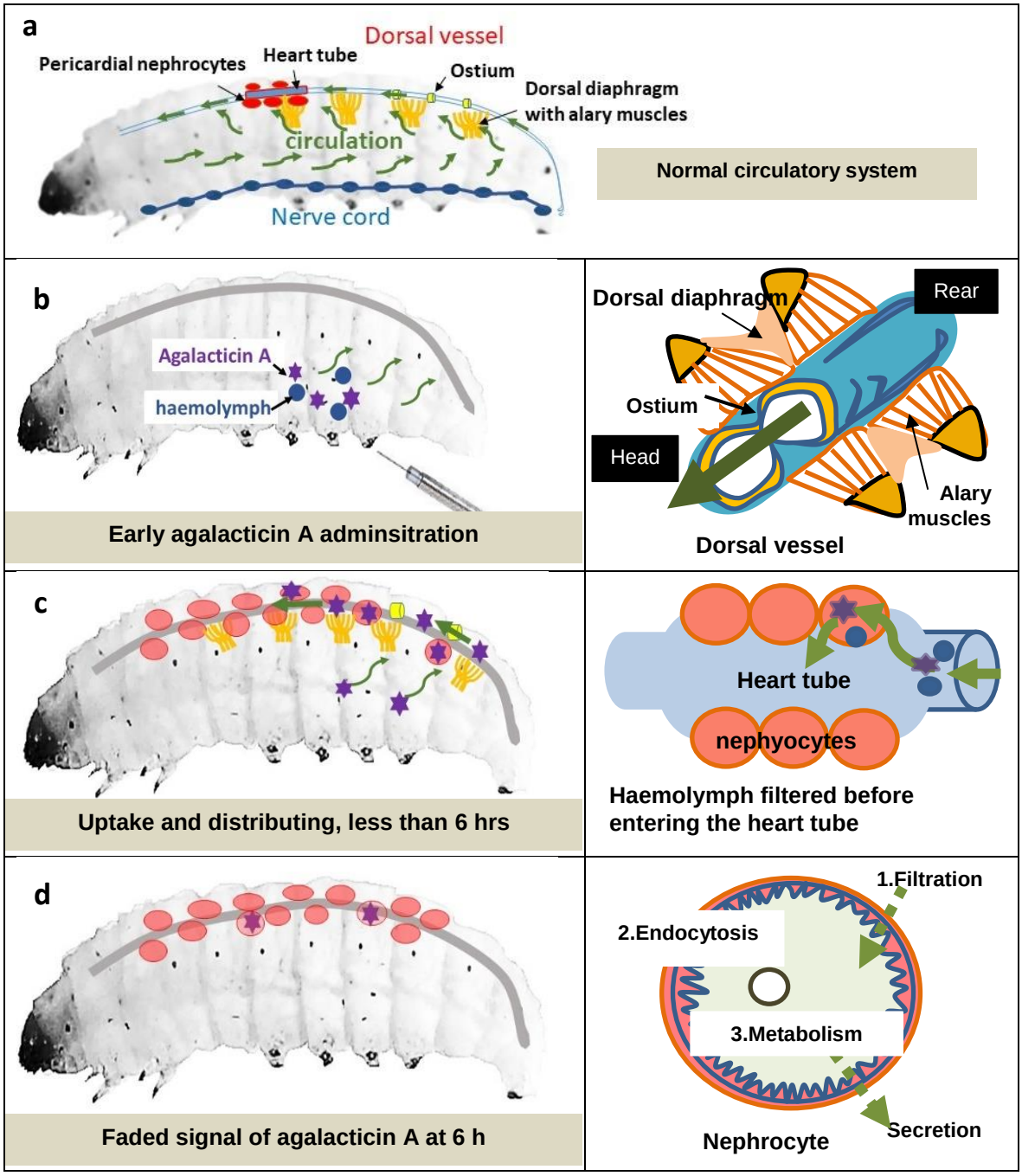


Figure 4-14 Proposed model for distribution and elimination of agalacticin A via *Galleria mellonella* nephrocytes

(a) Insect circulatory system; haemolymph flows backwards from the haemocoel and is pumped forwards into the dorsal vessel through ostia (Wirkner, Togel, and Pass 2013). (b) After agalacticin A administration, flow of haemolymph containing agalacticin A is actively forced by special pumping organs referred to as dorsal vessel and heart. The wall of the dorsal vessel consists of muscle fibrils and is contractile and pumps haemolymph to the head (Wirkner, Togel, and Pass 2013; Denholm and Skaer 2009). (c) Haemolymph and agalacticin A is filtered by the pericardial nephrocytes before it enters the heart tube (F. Zhang, Zhao, and Han 2013) and pumped out to the anterior part of the body. Trapping of agalacticin A in cells as detected by IHC may be due to accumulation of agalacticin A during the nephrocyte filtration. (d) Visibility of agalacticin A decreased over time, which may be from the function of nephrocyte to regulate haemolymph composition. Nephrocytes selectively take up molecules by basement membrane filtration as shown in the diagram. Molecules are endocytosed or metabolised before being secreted back to haemolymph (Denholm and Skaer 2009). The model proposes that Agalacticin A is filtered and endocytosed in nephrocytes causing degradation. The elimination of agalacticin A reduces its efficacy over time. Agalacticin A (purple stars), nephrocytes (red circles), haemolymph flow (green arrows), haemolymph (blue circles), muscle fibrils (yellow lines), ostia (yellow tubes).

4.4.2 Conclusion and future work

Agalacticin A proved to efficiently kill GBS in *G. mellonella* larvae. The role of nephrocytes in the distribution and elimination of bacteriocin should be confirmed by IHC using against the protein marker of nephrocyte's slit diaphragm, e.g. nephrin, NEPH1 (Denholm and Skaer 2009). Processing of agalacticin A by the host, especially renal clearance should be further explored in murine and piscine models. Modification of the structure of agalacticin A may limit its elimination and expand its bioavailability. Combination of agalacticin A with antibiotics may be advantageous in clinical settings and mitigate the risk of antibiotic resistance from selective drug pressure. The combination of agalacticin A with common antibiotics should be studied to ascertain safety in patients who receive other drugs. Synergistic or additive effects or antagonism of drugs with agalacticin A should be determined using the fractional inhibitory concentration (FIC) index (Botelho 2000; Bhusal, Shiohira, and Yamane 2005; Meletiadis et al. 2010). This could be done *in vitro* and in animal models prior to evaluation in humans. Comparisons of results obtained in the wax moth larvae with those from mammalian models should be conducted for comfirmation that the *G. mellonella* model is a reliable predictor for treatment efficacy.

In summary, the *G. mellonella* model may be a useful candidate in pre-screening bacteriocin before proceeding to tests in mammalian models. This study suggests that the *G. mellonella* model can demonstrate renal drug elimination similar to the mammalian models. Agalacticin A seems promising as novel GBS treatment. However, advanced drug delivery systems or modified chemical properties will be required for clinical application to widen the stability of agalacticin A.

Chapter 5 Identification of phenotypic markers of niche adaptation using metabolic profiling

5.1 Introduction

Availability of nutrients in the host environment plays a role in adaptation or specialisation of bacterial species or strains to an ecological niche, which may include host species or organ systems. Evolutionary forces select for genetic mechanisms that provide bacteria with a survival advantage in specific niches. For example, *Streptococcus uberis* has many metabolic regulators in its genome. Flexibility of carbohydrate utilization allows *S. uberis* to grow in the bovine gut and mammary glands and to survive in the environment, e.g. on pasture (Ward et al. 2009). In contrast, the *Streptococcus pyogenes* (Group A Streptococcus: GAS) genome contains limited diversity of the sugar transport and utilization apparatus. The spectrum of substrates utilized by GAS is narrower and GAS is more niche-restricted than *S. uberis*, surviving in the human host but not in the environment (Ward et al. 2009). The carbohydrate metabolism in GAS is involved in its survival in the nutrient-limited environment of the human oropharynx and saliva, and the catabolite control protein A (CcpA), a key regulator of carbohydrate metabolism influences the modulation of virulence factors (Shelburne et al. 2008).

Compared to many other streptococci, *S. agalactiae* has a large spectrum of hosts. However, highly virulent or successful GBS clones have emerged in specific hosts. In humans, clonal complex (CC) 17 has been recognized as the main cause of invasive neonatal infections since 1960 (Anthony and Okada 1977; Sørensen et al. 2010). CC67 is known as a bovine-specific lineage (Sørensen et al. 2010) and the closely related CC61 is a bovine adapted clone spreading throughout Portuguese dairy herds since the early 1990s (Almeida et al. 2016). CC552 is only found in cold-blooded species such as fishes and frogs (Rosinski-Chupin et al. 2013; Delannoy et al. 2016). *S. agalactiae* can be a harmless commensal bacteria in the gastrointestinal tract of humans and animals (Manning et al. 2004). To become pathogenic, GBS strains modify genes for functional adaptation or virulence factors to fit within the host restricted conditions (Maurelli 2007). For example, bovine GBS has a lactose metabolism operon (Lac.2) as an accessory operon to grow in the lactose-rich environment in the bovine udder whilst most human isolates lack this ability (Lyhs et al. 2016; Richards et al. 2013). The genome of bovine GBS strains contains an unusually high proportion of insertion sequences compared to human isolates, indicating Lac.2 and other genomic islands were acquired through lateral gene transfer (LGT), whereby some of those mobile genetic elements originate from other *Streptococcus* species that are found in cattle (Richards et al. 2011). The human neonatal clone ST17 has a virulence factor called the surface-anchored hypervirulent GBS adhesin (HvgA)

(Tazi et al. 2010). HvgA confers meningeal tropism in neonates (Landwehr-Kenzel and Henneke 2014). Tazi and colleagues suggested that HvgA may act as mediator for GBS to adhere and translocate through the intestinal wall and across the blood brain barrier (BBB) in late-onset GBS disease (LOD) (Tazi et al. 2010, 2012). Moreover, glucose level has an influence on a two-component system (TCS) called CovSR (Control of virulence Sensor and Regulator) of CC19 GBS (Di Palo et al. 2013). The CovS/R is the global regulatory system involved in GBS virulence (Lamy et al. 2004). Expression of GBS virulence factors, e.g. HvgA of CC17 (Landwehr-Kenzel and Henneke 2014) and the *cyl* operon of CC23 (Firon et al. 2013) are controlled by The CovS/R system.

Both acquisition of novel gene traits and reductive evolution (loss or inactivation of genes) may enable bacteria to adapt to a niche (Maurelli 2007). Genes can be inactivated by point mutation, insertion, or deletion when they no longer serve the pathogenic lifestyle (Rosinski-Chupin et al. 2013; Maurelli 2007). Frameshift and nonsense mutations causing pseudogenes are reported to be involved in the adaptation of GBS to fish (Delannoy et al. 2016). Pseudogenization also contributes to adaptation to cattle, as described for the capsular operon of CC61 (Almeida et al. 2016). Genes encoding virulence determinants in human GBS are absent in ST260, e.g. laminin-binding protein (*lmb*), surface protein rib and C-alpha protein (Delannoy et al. 2016). The *cyl* locus, which is associated with hemolysis, is not absent but incomplete in ST260 and ST261 (Delannoy et al. 2016). These examples show that some of the major virulence factors and mechanisms involved in niche adaptation are different between *S. agalactiae* populations from different host species.

Delannoy et al. (2016) provided evidence that all fish-associated GBS strains (CC7, CC283 and CC552) shared the open reading frames (ORFs) in locus 3. This locus is predicted to be involved in carbohydrate transport including galactose metabolism. Locus 3 contains the *gal* gene cluster (*gal*M, *gal*K, *gal*E, and *gal*T) to utilize galactose via the Leloir pathway (Grossiord et al. 1998). Based on genomic analysis, galactose utilization and the Leloir pathway may be an important mechanism for GBS adapted to fish hosts. Zadoks and colleagues investigated the role of locus 3 using *in vitro* testing and fish challenge models and showed that the knock-out mutants of locus 3 were less virulent than wild type strains, or even non-pathogenic (Zadoks, personal communication). However, the mechanism that translates presence of locus 3 in the genome to virulence in fish is unknown. The observation of phenotypes could potentially provide insight into pathways or virulence factors of piscine GBS strains, including but not limited to the role of locus 3.

In an attempt to find alternatives to antibiotics, vaccines can offer a promising strategy in GBS control and eradication. *In silico* prediction has been used to identify potential immunogenic proteins and their encoding genes in GBS from human and animal origins with the aim of finding potential vaccine targets (Pereira et al. 2013). The functions of the predicted proteins were unknown, and their antigenicity was not proven (Pereira et al. 2013). Phenotypic testing, e.g. nutrient utilization, can offer investigators a way of assessing gene expression and functionality of gene products, and can help to avoid missing metabolic pathways relevant to the virulence factors in pathogenicity or with related roles in immunogenicity. As described in earlier chapters, lactose (milk sugar) utilisation is an example of metabolic adaptation in bovine GBS, which lives predominantly in the bovine milk producing organ. In another streptococcus species associated with bovine mastitis, *Streptococcus uberis,* metabolic pathways have been the specific focus of vaccine development. *Streptococcus uberis* is unable to synthesise all amino acids required for its growth, a phenomenon known as auxotrophy (Kitt and Leigh 1997). Mammary glands have limited free amino acids and do not appear a logical environment for growth of *S. uberis.* Bovine milk, however, contains plasminogen and *S. uberis* produces plasminogen activator (PauA) to convert plasminogen to plasmin in milk. Plasmin is a serine protease which induces host inflammatory response resulting in the breakdown of milk protein into free amino acids that *S. uberis* can use for its growth (Leigh 1993). PauA was therefore explored as potential vaccine target, with promising initial results, although it was never commercialised (Leigh et al. 1999).

To identify metabolic pathways or phenotypic traits that could be explored as potential vaccine targets, or to link genotypic features such as Locus 3 to functional traits, large-scale screening of isolates is needed. The Biolog system is a phenotypic screening system that has been used for microbial identification (Klingler et al. 1992). Moreover, it can detect phenotypic diversification of bacteria responding to different metabolites and environments (Plata, Henry, and Vitkup 2014). Biolog GEN III includes chemical sensitivity tests and carbon sources to evaluate metabolic pathways and viability of bacterial species (detail was given in the section 1.3.2.2). Metabolic profiling using the Biolog system could be of use to identify metabolic pathways associated with GBS from particular hosts and to provide insight into mechanisms of competitive advantage or niche restriction. This, in turn, could contribute to discovery or refinement of vaccine candidates or pathways that lend themselves to metabolic or dietary manipulation.

5.2 Aims and objectives

The aim of this study was to identify phenotypic markers of niche adaptation using metabolic profiling with the Biolog GEN III assay. The overview of host association is shown in Figure 5-1 and the major CC and their host species used in this study are in Table 5-1. Workflow in the identification of phenotypic markers by the Biolog system is shown in Figure 5-2.

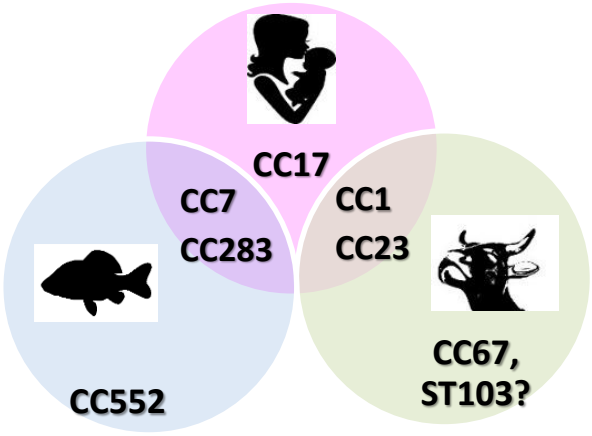


Figure 5-1 Host specialization of group B *Streptococcus* (GBS)
GBS populations in three host species. Host-specific clonal complexes (CC) are CC17 (human), CC552 (fish) and CC67 (cow). ST103 has been described to be a bovine-adapted GBS (Zadoks et al. 2011) but it was recently reported in humans in China (Wang et al. 2018; Li et al. 2018) so its status as bovine-associated strain is uncertain.

Table 5-1 Major clonal complexes (CC) of group B *Streptococcus* used in this study, including their host species and predominant clinical manifestation.

Clinical manifestation	Host species	Clonal complex (CC)	cross – species transmission	References
Asymptomatic colonization, carriage	Humans	CC1, CC19, CC23		Van Der Mee-Marquet et al. 2008 Lyhs et al. 2016
Invasive neonatal disease	Humans	CC17[a]		Poyart et al. 2008
Bovine mastitis	Dairy cattle	CC1, CC19, CC23 CC67[a] CC103[b]	human-to-animal transmission may occasionally occur	Mahmmod et al. 2015 Sørensen et al. 2010 Lyhs et al. 2016
Found in stranded aquatic mammals[c]	Dolphin, seal	CC7 (dolphin)[c] CC23 (seal)[c]	Anthropogenic environmental contamination	Delannoy et al. 2013
Streptococcosis in cold-blooded species	Fish species, bullfrog	CC7 CC283 CC552[a]	CC7: Anthroponotic CC283: Foodborne	Delannoy et al. 2013

a. Host-specific

b. Primarily described in cattle (Zadoks et al. 2011) but also reported in humans (Wang et al. 2018; Li et al. 2018), so its status as bovine-associated strain is uncertain.

c. GBS isolated from the post-mortem, a systemic infectious process was unknown.

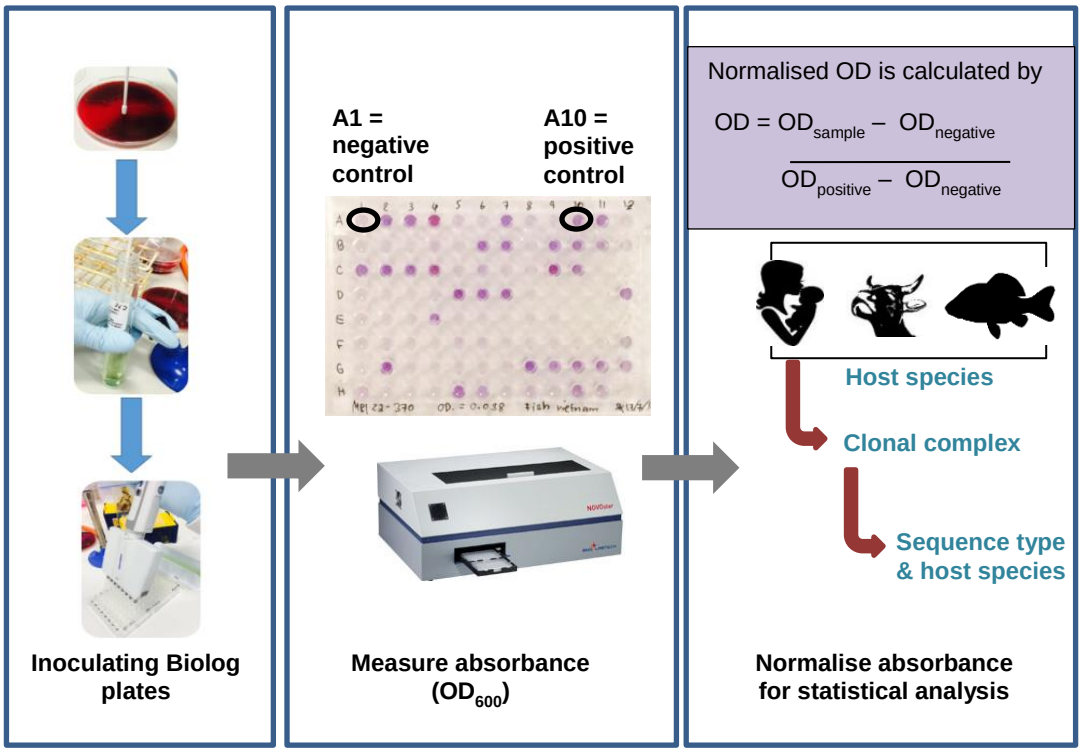


Figure 5-2 Workflow of metabolic profiling using Biolog
Biolog GENIII system carbon source and biochemical assays were used for metabolic profiling of group B *Streptococcus* (GBS). GBS colonies were resuspended in inoculating fluid and 100 µl bacterial suspension was loaded in each well, including control wells A1 (negative) and A10 (positive). Plates were incubated at 37°C overnight and absorbance measured at OD_{600}. Standardized absorbance was calculated using the formula in the box, and compared between host species, clonal complexes and sequence types, as appropriate.

5.3 Results

5.3.1 Overview of Biolog results

Eighty-eight isolates (29 bovine, 17 human and 42 piscine) were tested for their use of sugars (well A1-9, B1-9 and C19), sugar alcohol (D1-5), hexose-phosphate (D6-7), amino acids (D8-9 and E1-9), hexose acids (F1-9) and carboxylic acids, esters and fatty acids (G1-9 and H1-9). Based on normalized OD600, carbon utilisation was similar between groups (host, CC) for many substrates but some substrates were observed to be used differently by different host species or CC. All GBS isolates had high mean OD for 14 specific carbon sources, which are mainly sugars including the derivatives of glucose (N-acetyl-D-glucosamine and N-acetyl-β-D-mannosamine) (Table 5-2; mean normalized absorbance at least 1.0), whereas they all have low mean ability to use 42 other carbon sources such as saccharides found in plants, sugar alcohols, L-amino acids and carboxylic acids (Table 5-3). OD-values for substrates with high or low mean utilization across isolates were normally distributed (Appendix vi), and very few outliers were observed (e.g. 3-methyl-glucose use by a single fish isolate, and D-galactonuric acid use by a single bovine isolate). Statistical significance of differences between host species did not necessarily indicate biologically meaningful differences. For compounds that were not or barely used, differences between hosts were sometimes significant, e.g. for L-pyroglutamic acid, which had a mean adjusted OD of zero for human and bovine isolates and a mean adjusted OD below zero for fish isolates, indicating non-use across host species (Appendix vi).

Only very few substrates were consistently associated with a single host. Specifically, lactose and cellobiose utilisation were associated with the bovine host (for details, see section 5.3.2). For a subset of substrates consisting of twelve carbon sources, variability of OD values was observed, showing high and low users within host species (Figure 5-3). CC-associated phenotypes were identified in CC17 and CC283 (Table 5-4, with details in section 5.3.3).

Table 5-2 Carbon sources that were utilized by *Streptococcus agalactiae* from at least one of its three major hosts.

Well	High carbon source utilization	host species				Statistical analysis[a]
		human	bovine	piscine	p value	
Sugars						
A2	Dextrin	+++	+++	+++	0.09	One Way ANOVA
A3	D-maltose	+++	+++	+++	0.14	One Way ANOVA
A4	Trehalose[b]	+++	+++	+++	0.06	Kruskal-Wallis test
A5	Cellobiose	0	+++	0	**<0.0001******	One Way ANOVA
A7	Sucrose	+++	+++	+++	**0.006****	One Way ANOVA
B2	Lactose[b]	0	+++	0	**<0.0001******	One Way ANOVA
B4	β-methyl-D-glucoside[b]	+++	+++	+++	**0.027***	Kruskal-Wallis test
B5	D-salicin[b]	+++	+++	+++	**0.0007*****	Kruskal-Wallis test
B6	N-acetyl-D-glucosamine	+++	+++	+++	0.30	One Way ANOVA
B7	N-acetyl-β-D-mannosamine	++	++	+++	**0.0006*****	One Way ANOVA
B9	N-acetyl-neuraminic acid	++	++	++	**0.003****	One Way ANOVA
C1	α-D-glucose	+++	+++	+++	0.82	One Way ANOVA
C2	D-mannose	+++	+++	+++	0.22	One Way ANOVA
C3	D-fructose	+++	+++	+++	0.54	One Way ANOVA
C4	Galactose	+++	+++	+++	0.18	One Way ANOVA
C9	Inosine	+++	+++	+++	0.97	One Way ANOVA
Hexose-PO_4						
D6	D-glucose-6PO_4	+++	+++	+++	0.11	One Way ANOVA
D7	D-fructose-6-PO_4[b]	+++	+++	+++	0.16	Kruskal-Wallis test
Amino acids						
E4	Arginine	++	++	++	0.62	One Way ANOVA
Hexose acid						
F1	Pectin	+	+	+	0.20	One Way ANOVA
F5	D-glucuronic acid	+/0	+/0	+/0	**0.01***	One Way ANOVA
Carboxylic acids, esters and fatty acids						
G2	Methyl pyruvate	++	++	++	0.12	One Way ANOVA
G8	L-malic acid[b]	++	++	+++	**0.003****	Kruskal-Wallis test
G9	Bromo-succinic acid	+/0	+	+	**<0.0001******	One Way ANOVA
H5	α-keto-butyric acid	++	++	++	**0.002****	One Way ANOVA
H6	Acetoacetic acid	+	+	+	0.04	One Way ANOVA

+++; Mean normalised absorbance is more than 1.0 (exclusive)
++ ; Mean normalised absorbance is 0.5 (exclusive) to 1.0 (inclusive).
+ ; Mean normalised absorbance is more than 0 (exclusive) to 0.5 (inclusive).
0; Mean normalised absorbance is 0 or below.

[a] Normalised absorbance based on carbon utiliszation assays plotted by Graphpad Prism8 (Figure 5-3 and Appendix vi) is statistically compared based on analysis of variance for carbon sources with normal OD-distribution and on non-parametric Kruskal-Wallis ANOVA for bimodal distributions. If the Statistical significance presents as *, P ≤ 0.05; **,P ≤ 0.01; ***, P ≤ 0.001 and ****, P ≤ 0.0001.

[b] Bimodal distribution

Table 5-3 Carbon sources that were not (0) or hardly (+) utilized by *Streptococcus agalactiae* from any of its three major hosts.

Well	Low carbon source utilization	host species		
		human	bovine	piscine
A6	Gentibiose	0	0	0
A8	Turanose	0	0	0
A9	Stachyose	0	0	0
B1	Raffinose	0	0	0
B3	Melibiose	0	0	0
B8	N-acetyl-D-galactosamine	0	0	0
C5	3-methyl glucose	+/0	+/0	+/0
C6	D-fucose	0	0	0
C8	L-rhamnose	0	0	0
D1	D-sorbitol	0	0	0
D2	D-mannitol	0	0	0
D3	D-arabitol	0	0	0
D4	Myo-inositol	0	0	0
D8	D-aspartic acid	0	0	0
D9	D-serine	0	0	0
E1	Gelatin	0	0	0
E2	Glycyl-L-proline	0	0	0
E3	L-alanine	0	0	0
E5	L-aspartic acid	0	0	0
E6	L-glutamic acid	0	0	0
E7	L-histidine	0	0	0
E8	L-pyoglutamic acid	0	0	0
E9	L-serine	0	0	0
F2	D-galacturonic acid	+/0	+/0	+/0
F3	L-galactonic acid lactone	+/0	+/0	0
F4	D-gluconic acid	+/0	+/0	0
F6	Glucuronamide	+/0	+/0	+/0
F7	Muic acid	0	+/0	0
F8	Quinic acid	0	0	0
F9	D-saccharic acid	0	+/0	0
G1	p-hydroxy-phenylacetic acid	0	0	0
G3	D-lactic acid methyl ester	0	0	0
G4	L-lactic acid	0	0	0
G5	Citric acid	0	0	0
G7	D-malic acid	0	0	0
H1	Tween 40	0	0	+/0
H2	ɣ- amino-butyric acid	0	0	0
H3	α-hydroxy-butyric acid	0	0	0
H4	β-hydroxyl-D,L-butyric acid	0	0	0
H7	Proprionic acid	0	0	0
H8	Acetic acid	+/0	+/0	+/0
H9	Formic acid	0	0	0

0; normalised absorbance (OD_{600}) is below 0.1 and +; normalised absorbance is of 0.1 to 0.5.

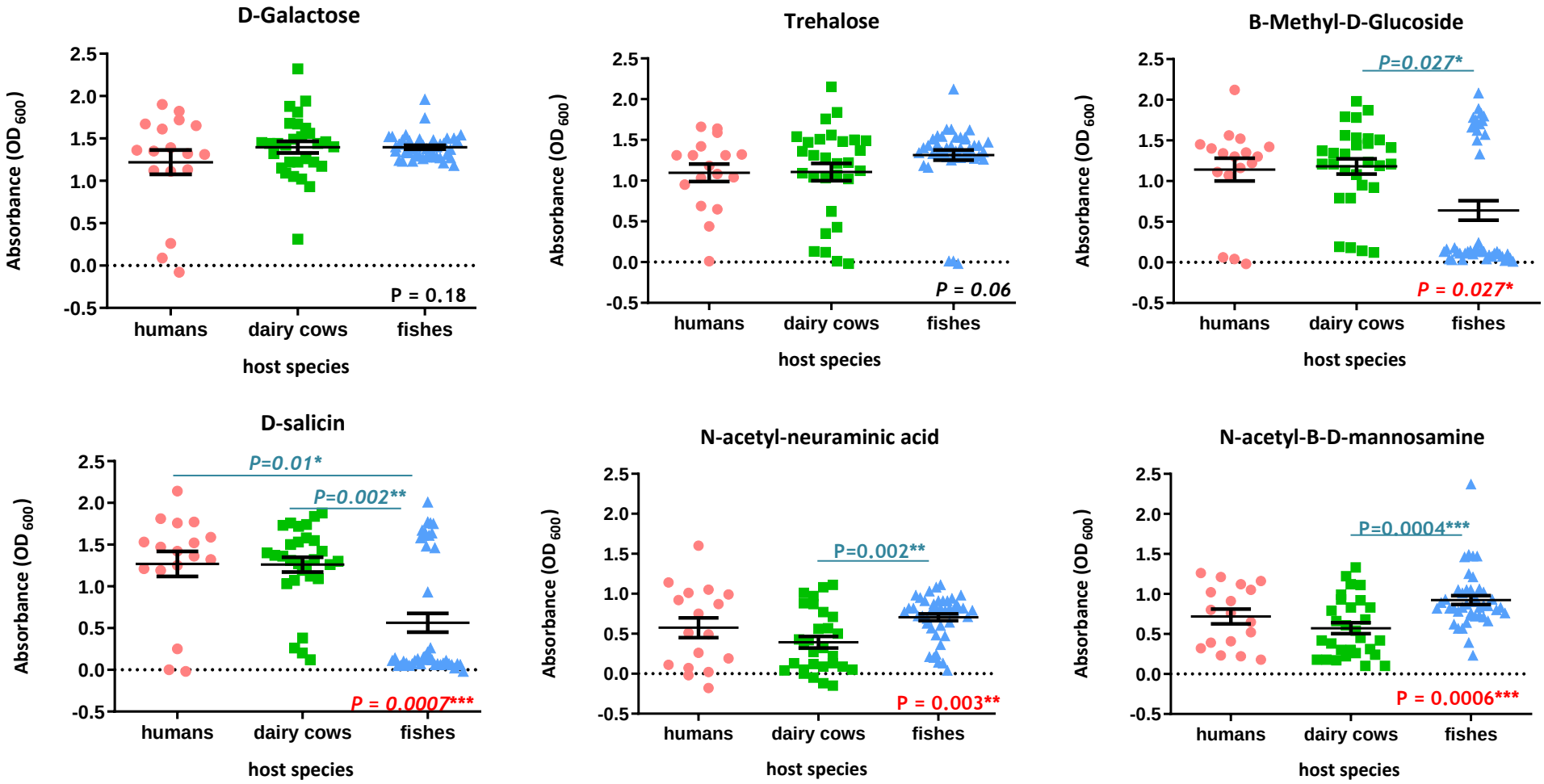


Figure 5-3 Variability in carbon source utilization within and between group B *Streptococcus* (GBS) from three host species.
Normalised absorbance based on carbon utilisation assays plotted by Graphpad Prism8. Statistical comparison based on Analysis of Variance for carbon sources with normal OD-distribution (p-value in bold) and on non-parametric Kruskal-Wallis ANOVA for bimodal distributions (p-value in italics). Number on the right side of graph is p-value for comparison across 3 host groups. Numbers above plots are comparisons between two host species.

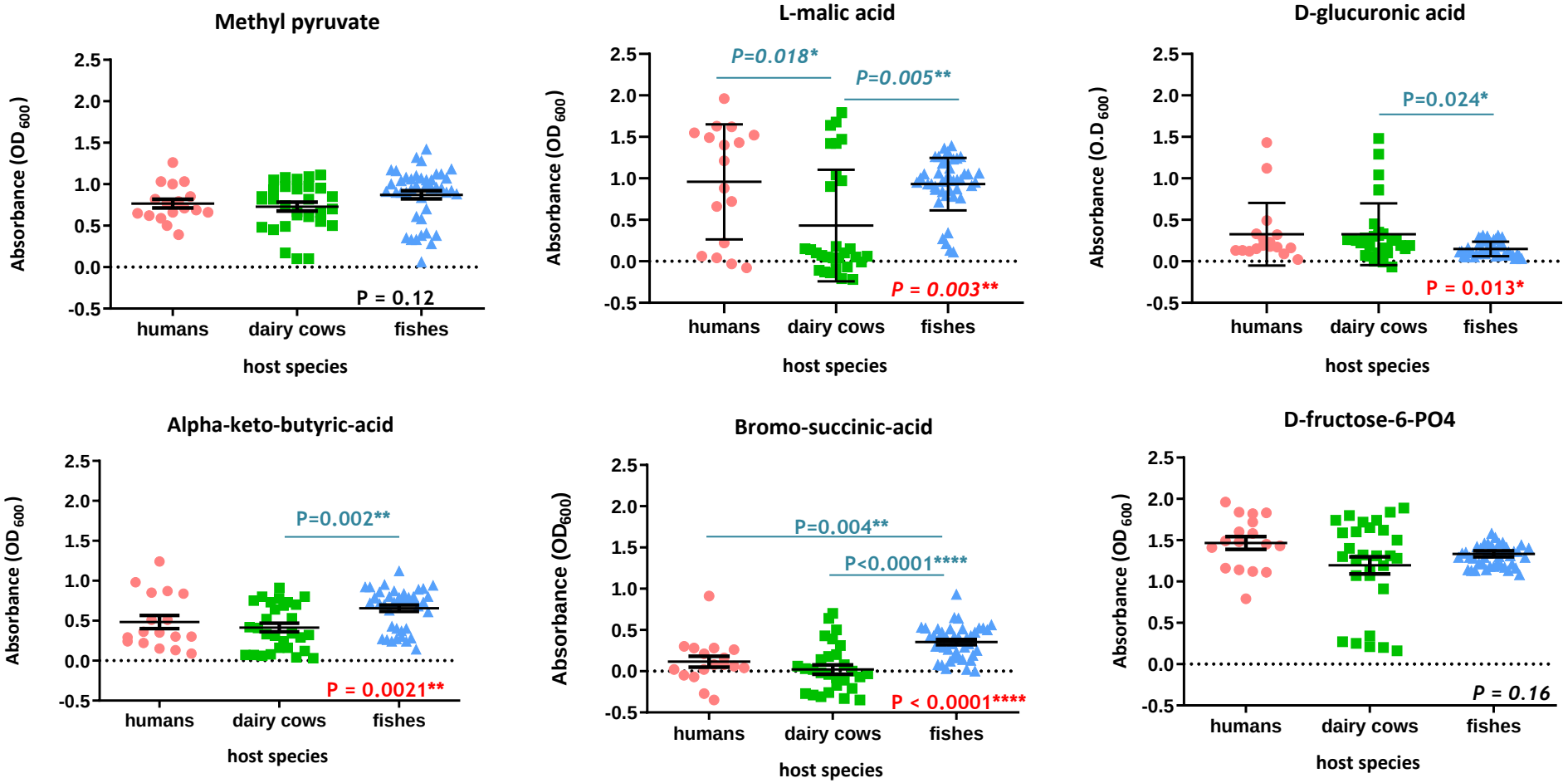


Figure 5-3 Variability in carbon source utilization between group B *Streptococcus* isolates from three host species (continuation)

Table 5-4 Carbon source utilization by *Streptococcus agalactiae* isolates from different host species and clonal complexes.

Carbon sources	Assays	% Utilization[a] human	bovine	fish
Sugars	trehalose	76%	79%	100%
	B-methyl-D-glucoside	82%	86%	33%
	salicin	82%	86%	29%
	N-acetyl-B-D-mannosamine	59%	48%	55%
	N-acetyl neurominic acid	65%	52%	74%
	galactose	82%	97%	100%
hexose-PO4	D-fructose-6PO4	71%	79%	100%
hexose acid	D-glucuronic acid	18%	14%	0%
Carboxylic acids, esters and fatty acids	methyl pyruvate	100%	76%	74%
	L-malic acid	59%	38%	88%
	alpha-keto butyric acid	29%	41%	71%
	Bromo succinic acid	35%	31%	74%

Carbon sources	Host-associated			Multiple hosts													
	CC17 (n=3)	CC67 (n=2)	CC103 (n=7)	CC1 (n=6)		CC7 (n=14)		CC8 (n=7)		CC19 (n=4)		CC23 (n=7)		CC196 (n=4)		ST632 (n=2)	CC283 (n=30)
	Human	Bovine	Bovine	Human	Bovine	Bovine	Fish	Human	Bovine	Human	Bovine	Human	Bovine	Human	Bovine	Bovine	Fish
B-methyl-D-glucoside	0/3	0/2	6/7	3/3	3/3	2/2	12/12	4/4	3/3	2/2	2/2	3/3	3/4	2/2	2/2	2/2	2/30
D-salicin	0/3	1/2	6/7	3/3	3/3	2/2	12/12	4/4	3/3	2/2	2/2	3/3	3/4	2/2	2/2	2/2	0/30
N-acetyl neuraminic acid	3/3	0/2	4/7	3/3	2/3	2/2	4/12	3/4	3/3	1/2	1/2	0/3	1/4	1/2	1/2	0/2	27/30
L-malic acid	2/3	0/2	1/7	1/3	1/3	1/2	7/12	2/4	2/3	2/2	0/2	3/3	2/4	0/2	2/2	0/2	30/30
alpha-keto butyric acid	0/3	0/2	3/7	1/3	2/3	1/2	1/12	1/4	3/3	0/2	0/2	0/3	0/4	2/2	0/2	2/2	29/30
Bromo succinic acid	2/3	0/2	0/7	1/3	1/3	0/2	2/12	3/4	3/3	0/2	1/2	1/3	1/4	1/2	1/2	0/2	29/30

[a] Isolates were considered to utilise a substrate if the normalised absorbance in the Biolog GENIII assay was 0.5 or above.

5.3.2 Host-associated phenotypes

Lactose metabolism is specific to bovine GBS strains (Richards et al. 2013). Lactose utilization was chosen as positive control to assess the performance of the Biolog system. All bovine isolates (29/29) used lactose but none of human and fish isolates did. Even human isolates belonging to CCs that also include bovine isolates, e.g. CC23, CC1 and CC19, were unable to ferment lactose (Figure 5-4a). In addition, mean cellobiose utilisation was significantly higher in bovine isolates than in human or fish isolates, although there was overlap between populations from different hosts for this substrate (Figure 5-4b). Lactose profiling confirmed the usefulness of Biolog in detection of phenotypic variability in the GBS populations.

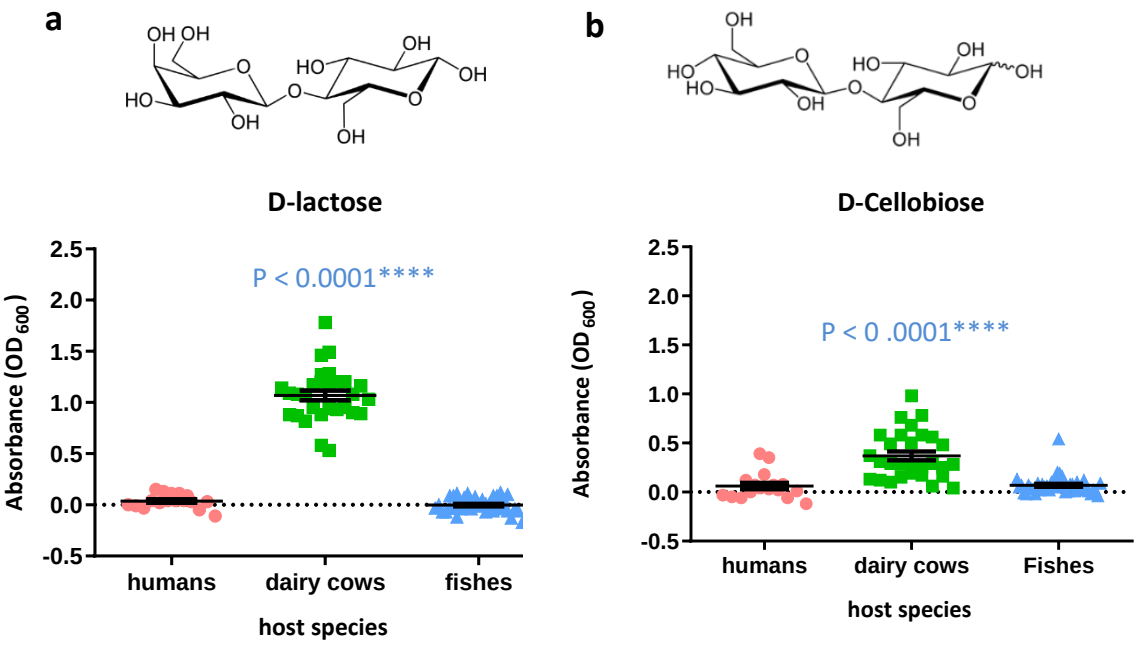


Figure 5-4 Phenotypic markers of bovine *Streptococcus agalactiae* identified by Biolog testing
(a) Normalized absorbance in Biolog assay for lactose, showing utilisation by bovine isolates but not by isolates from humans or fishes. (b) Normalized absorbance in Biolog assay for cellobiose, showing utilisation by some isolates from all hosts but higher mean values for bovine isolates.

For several other substrates, significant differences were detected between isolates from different host species. For example, average use of beta-methyl-glucoside and D-glucoronic acid were lowest among fish isolates and average use of N-acetyl neuraminic acid, N-acetyl-B,D-mannosamide, and bromo-succinic acid was highest among fish isolates (Figure 5-3). However, those differences were small compared to the differences observed for lactose or cellobiose, and/or the distribution of OD values within the group of fish isolates was bimodal. Because there are two CCs in the fish isolates, analysis at CC-level was conducted.

5.3.3 Clonal complex-associated phenotypes

Distinct fish-associated phenotypes were not detected in the comparison between host species but bimodal distributions were observed for some substrates in isolates from the fish group (Figure 5-3). For D-salicin, β-methyl-D-glucoside and L-malic acid utilization, fish isolates included high and low users. All fish isolates belonged to CC7 (ST7 and its variant ST 500) or CC283 (ST283 and its variant ST491), the only CCs that are found in fish that can grow at 37°C. CC283 fish isolates had low ability to use D-salicin and β-methyl-D-glucoside but all CC7 fish isolates used both substrates. To ensure D-salicin and β-methyl-D-glucoside are clonal complex-associated phenotypes, all clonal complexes regardless of host species were explored for their ability to use these substrates. CC283 and CC17 were found to share this phenotype, i.e. loss of ability to use D-salicin ($P < 0.0001$) (Figure 5-5a). The pattern of utilization of β-methyl-D-glucoside was similar to D-salicin use but CC67 also has low ability to use this substrate (Figure 5-5b). Loss of D-salicin catabolism appears to be a unique phenotypic marker of the only known hypervirulent strains in humans, ST17 (hypervirulent in neonates) and ST283 (hypervirulent in adults).

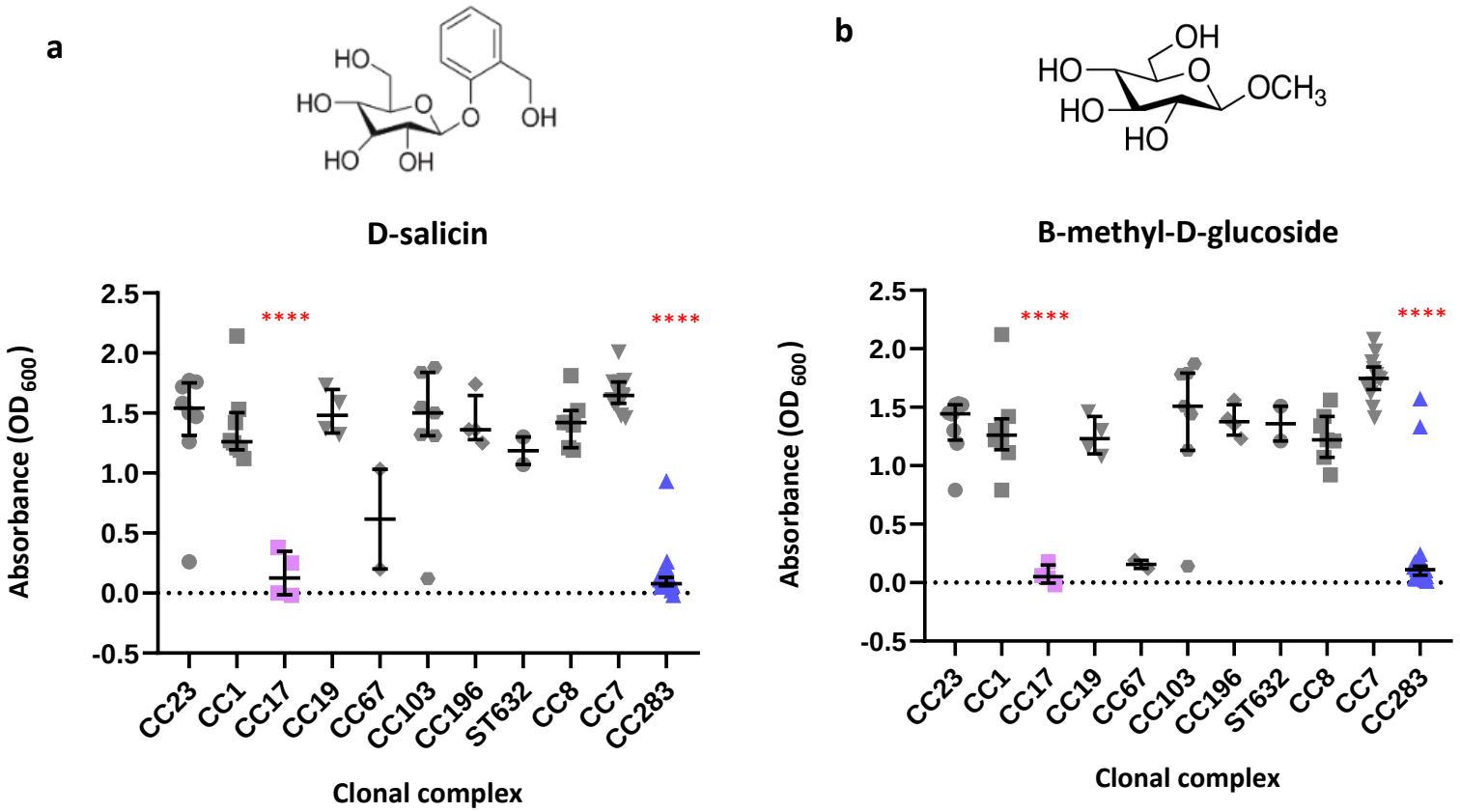


Figure 5-5 Beta-glucoside utilization of group B *Streptococcus* from different clonal complexes (CC) or sequence types (ST)
D-salicin and β-methyl-d-glucoside are glucosides with β-glycosidic bonds. (a) Absorbance in D-salicin utilization assay, showing lack of use in hypervirulent strains from CC17 and CC283. (b) Absorbance in β-methyl-D-glucoside utilization assay, showing low use in CC7, CC283 and bovine-associated CC67. CC17 isolates (pink); CC283 isolates (blue). Red stars mean $P < 0.0001$ based on non-paramatric Kruskal-Wallis statistic. Because of the existence of outliers and extreme values, the median and interquartile ranges are used to summarize a typical value and the variability, respectively. The absorbance values were ranked and divided into quartiles. The median (50th percentile) is the middle bar, while the 25th (one quarter) and 75th (third quarter) percentiles are shown by the lower and upper bars. The difference between the lower and upper bars is the interquartile range, displaying the spread of the absorbance from the typical value.

5.4 Discussion

5.4.1 General results

Phenotypic profiling is helpful in understanding differences in metabolic pathways that may be involved in niche adaptation. This study has shown similarity and divergence in carbon utilization of GBS isolates originating from different host populations. Lactose profiling confirmed that the Biolog GEN III is a powerful tool in the identification of phenotypic markers. However, Biolog GEN III was not successful in detecting phenotypes of fish-specific strains which grow at 28°C, i.e. those belonging to CC552. However, the previous genomic studies by Delannoy and colleagues found the same putative host-adaptation genes involved in carbohydrate catabolism in all fish-associated GBS strains (CC7, CC283 and CC552). Hence, phenotypes or metabolic pathways that are common to all fish isolates should also be detected through testing of CC7 and CC283, which could be followed by confirmation for CC552 under modified conditions. However, even looking at just two of the three CCs found in fish, no common phenotype separating fish isolates from human and bovine isolates was detected and this would not be resolved by inclusion of CC552 in Biolog testing.

5.4.2 Host associated phenotypes

The Biolog system could successfully detect lactose use, a phenotypic marker of niche adaptation in bovine GBS isolates. Phenotypic markers of GBS isolated from other host species were not detected from metabolic profiling. The findings about lactose utilization are congruent with previous studies. Lactose metabolism is an important adaptation to the bovine mammary gland. The lactose operon (Lac.2) in bovine GBS appears to be acquired via lateral gene transfer (LGT) from other mastitis pathogens such as *Streptococcus uberis* and *Streptococcus dysgalactiae* subsp. *dysgalactiae* (Richards et al. 2011). In addition, cellobiose was found to be highly used in bovine GBS isolates which may be related to cellulose and hemicellulose which are the major components of plant cell walls that breakdown into cellobiose by the enzymatic activity of cow rumen microorganisms (Del Pozo et al. 2012).

Based on genomic analysis, locus 3, which is common to all fish-associated GBS strains (CC7, CC283 and CC552), contains a gene cluster to utilize galactose via the Leloir pathway (Delannoy et al. 2016). Because of that, galactose use was hypothesized to be a phenotypic marker for GBS adapted to fish hosts. However, phenotypical analysis in this study found

galactose can be highly utilized by most GBS isolates regardless of host species. Moreover, based on findings from Zadoks and colleagues, knock-out mutants of locus 3 have the same sugar utilization as wild-type strains, including the same use of galactose (Zadoks, personal communication). Galactose profiling from Biolog and the locus 3 knock-out suggest that the Leloir pathway, which was unique to fish-associated GBS isolates in the genomic study, may have a different role in fish. This result was unexpected but it shows the importance of phenotyping and metabolic profiling for functional analysis.

5.4.3 Clonal complex associated phenotypes

Loss of catabolism of D-salicin and other β-glucosides (β-methyl-D-glucoside) was observed in CC283 and CC17. Piscine CC283 has been recognized as a hypervirulent zoonotic clone causing streptococcal meningitis and osteoarthritis in nonpregnant adults after transmission through raw fish consumption (Ip et al. 2006; Kalimuddinet al. 2017). CC17 is the dominant GBS strain in human neonatal invasive infections, especially meningitis (Da Cunha et al. 2014). Based on earlier Biolog studies, Domelier and colleagues also reported differences in β-methyl-D-glucoside use between groups of strains. They classified isolates from neonatal cerebrospinal fluid as high-risk (HR) group and isolates from vaginal and gastric samples as low-risk (LR) group. The HR group showed lower ability to use β-methyl-D-glucoside than the HR group at 49% and 76%, respectively. For salicin use, no significant difference was detected between HR and LR groups (Domelier et al. 2006). It is possible that the HR group contained more ST17 than the LR group, because ST17 is associated with neonatal meningitis. Based on our results, a difference in both β-methyl-D-glucoside and salicin use would be expected between HR and LR, which was not observed. Without genotyping data for the LR and HR groups, it is difficult to know how to explain those results.

In addition, CC67 isolates appeared to be low β-glucoside users, especially for β-methyl-D-glucoside. CC67 is a bovine-specific strain that is closely related to CC17 (Sørensen et al. 2010). The genetic relationship may explain the similarity in metabolic profile. One of two CC67 isolates was also salicin negative but there are no documented cases of CC67 in humans, not even in low- and middle-income countries where CC67 is common in cattle and milk is often consumed raw, e.g. in Colombia (Cobo-Angel et al. 2018). CC67 may not be hypervirulent, or the sal^- phenotype may not be common in CC67. With only 2 isolates included in the current study, it is difficult to generalize about CC67.

5.4.4 Further work

The inability of β-glucoside utilization, especially for D-salicin, suggests that the hypervirulent strains ST17 and ST283 share virulence factors or pathogenic mechanisms. Investigation of CC283 and CC17 will be described in Chapter 6.

Chapter 6 Potential mechanism for hypervirulence of non-salicin using Group B *Streptococcus* (ST17 and ST283)

The bioinformatic analysis of amplicon sequencing of the hyaluronidase (*hyl*B) gene was conducted by Chiara Crestani, Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow.

6.1 Introduction

GBS are commensal bacteria of intestinal and genitourinary tracts in humans and animals but some lineages are adapted to specific hosts and have become hypervirulent, e.g. ST17 in neonates (Sørensen et al. 2010) and ST283 in adults (Ip et al. 2006). Metabolic profiling indicated the potential of ST-specific phenotypes, with use of salicin in all STs apart from ST17 and ST283 (chapter 5, section 5.3.3). Studies on hydrolysis of salicin from serotype Ia, Ib, II, III and non-typable human GBS isolates showed that lack of hydrolysis of salicin was almost exclusively detected among serotype III strains (Kjems, Perch, and Henrichsen 1980) and specifically in CC17 (Sørensen et al. 2010). In GBS from cattle, salicin negative isolates are common among CC67 and singletons, and there is no strong relationship with serotype (Sørensen et al. 2010). Prior to our study, no data was available on salicin metabolism in GBS from fishes.

Salicin is a phenolic glycoside found in plants, e.g. willow bark, poplar trees and the black haw plant. The name salicin is derived from its presence in willow, which has the scientific genus name *Salix*. Hydrolysis of salicin produces salicylic alcohol which can be converted into salicylic acid and acetylsalicylic acid, known as aspirin (Figure 6-1) (Hedner and Everts 1998). Salicin inhibits the inflammatory response by down-regulation of pro-inflammatory cytokines (Li et al. 2015).

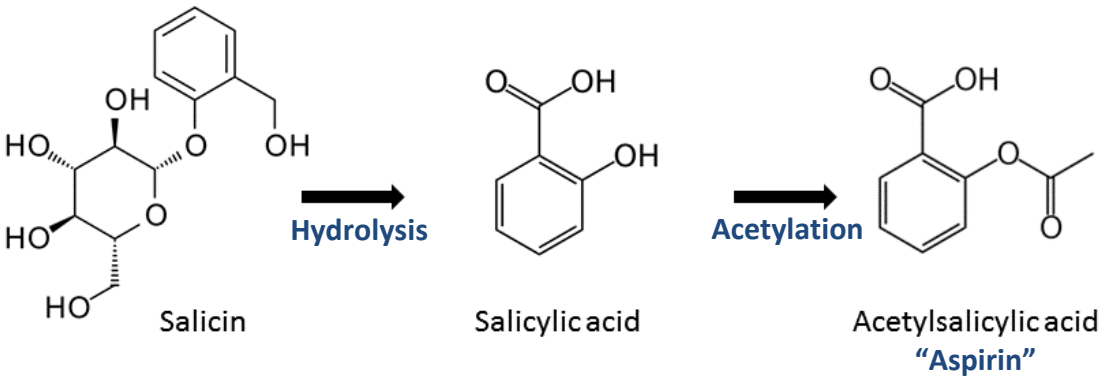


Figure 6-1 Bioconversion of salicin into acetylsalicyclic acid (aspirin)
Salicin is a plant-derived glycoside and aspirin precursor and has been used in treatment of rheumatic fever and as an analgesic agent (Rodnan and Benedek 1970). Salicin is hydrolysed to D-glucose and salicylic alcohol (saligenin) by the β-galactosidase from mucosa of small intestine and/or the β-glucosidase of intestinal flora (Akao et al. 2002). Oxidation of salicylic alcohol produces salicylic acid and further acetylation to acetylsalicylic acid known as aspirin. Source, naturespoisons.com

If bacteria use salicin, the salicin level is reduced and the inflammation is not inhibited. This allows the inflammatory process to clear infection. In contrast, if bacteria cannot use salicin (ST17 and ST283), the level of salicin may continue to be high enough to suppress an inflammation. This may be an advantage to preventing inflammatory diseases induced by lipopolysaccharides (LPS) of Gram-negative bacteria (Li et al. 2015) but it might lead to negative outcomes if the inflammatory response is required to remove bacterial infection. Indeed, some studies suggest that activation of the inflammasome is needed to protect the host from GBS infection (Costa et al. 2012). The potential mechanism of action of salicin in gram-positive infections is unknown.

This study hypothesizes that D-salicin is a structural analogue to a host tissue component, especially hyaluronic acid/hyaluronan (HA) which contain β-glycosidic linkages. A glycosidic linkage is a covalent bond that connects a carbohydrate (sugar) molecule to another group, which may also be a carbohydrate. In contrast to an α–bond, a β-glycosidic bond occurs when the two carbons that are linked have different as opposed to the same stereochemistry. Enzymes can usually break one type of glycosidic bond only (Figure 6.2). Hyaluronidase plays an important role in pathogenesis of GBS infections in humans. *Streptococcus agalactiae* produces hyaluronidase which specifically cleaves β-glycosidic bonds of HA. HA disaccharides as products from hyaluronidase activity can block the pro-inflammatory response, enabling GBS to escape host immune detection (Kolar et al. 2015) (Figure 6.3). Based on structural similarity of the glycosidic bonds in salicin and HA disaccharadise, loss of the ability to use salicin in ST17 and ST283 may be a marker for loss of ability to degrade HA. This would enhance the action of GBS hyaluronidase by preventing degradation of HA disaccharides. The lack of enzymatic activity to cleave β-glycosidic linkages may lead to stability of HA disaccharides binding to Toll-like receptors and may facilitate bacterial spread.

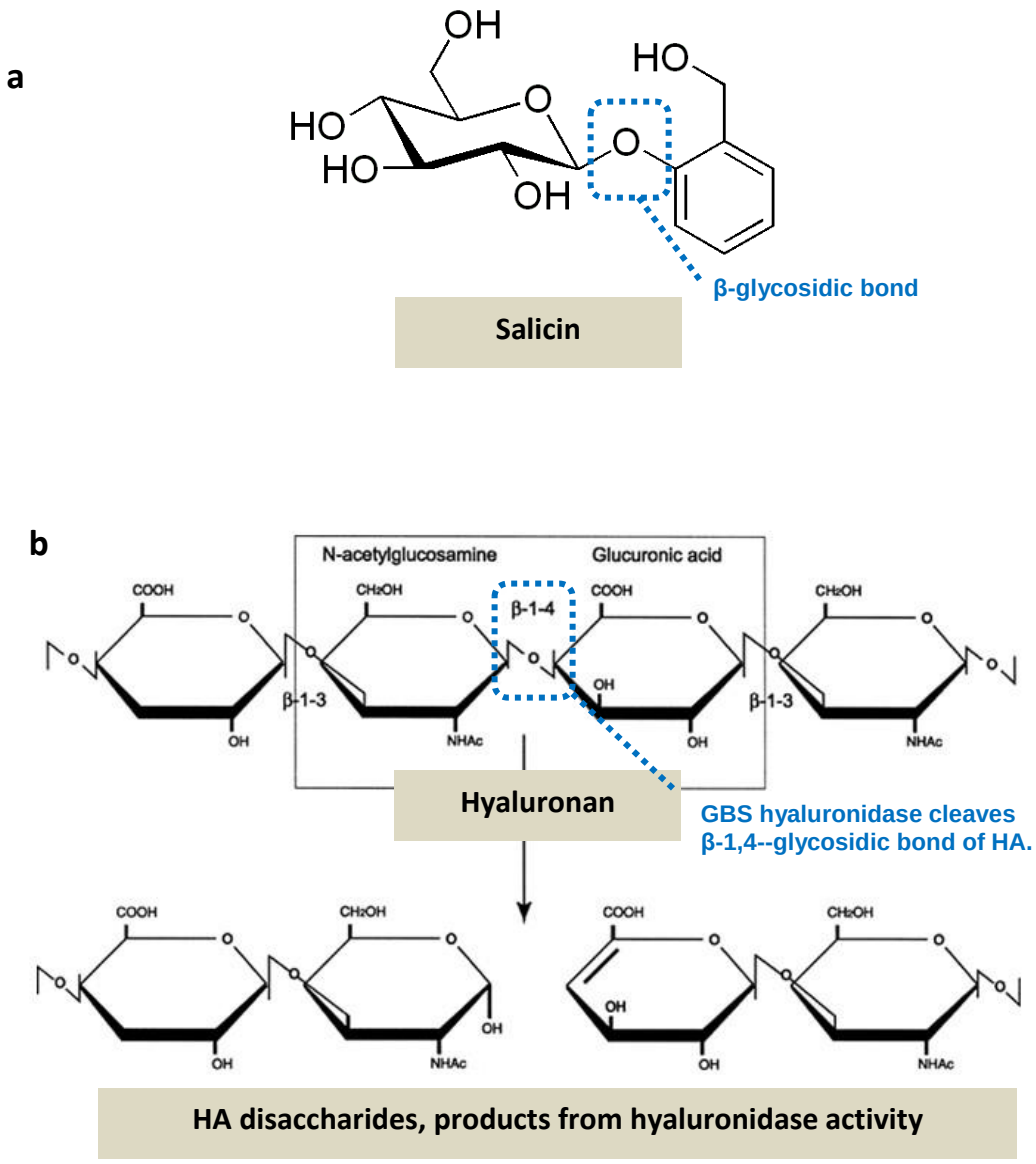


Figure 6-2 Structure of salicin and hyaluronan
(a) Salicin is a β-D-glucopyranoside. The β-D-glucose and salicylic alcohol moieties are bonded by β-1,1′-d-glycosidic bond (Mahdi 2014). (b) Hyaluronic acid (HA) or hyaluronan is glycosaminoglycan found in extracellular matrix especially of soft connective tissues and fluids (Laurent and Fraser 1992). HA is a large linear polymer composed of alternating residues of β-D-(1→3) glucuronic acid and β-D-(1→4)-N-Acetylglucosamine. Depolymerisation of HA can occur by either infectious or non-infectious tissue injury. *Streptococcus agalactiae* produces hyaluronidases which specifically cleave the β-1,4-glycosidic bonds of HA. GBS hyaluronidase is believed to cleave HA polymer in a continuous direction along the HA chain until the whole chain is degraded. The products of GBS hyaluronidase are unsaturated HA disaccharide (S. Li and Jedrzejas 2001). Reprinted by permission from Oxford University Press. FEMS Microbiology Letters: Hyaluronidases of Gram-positive bacteria Hyaluronidases of Gram-positive bacteria, (Hynes, Wayne L.; Walton, Sheryl Lynne), [copyright] 2000.

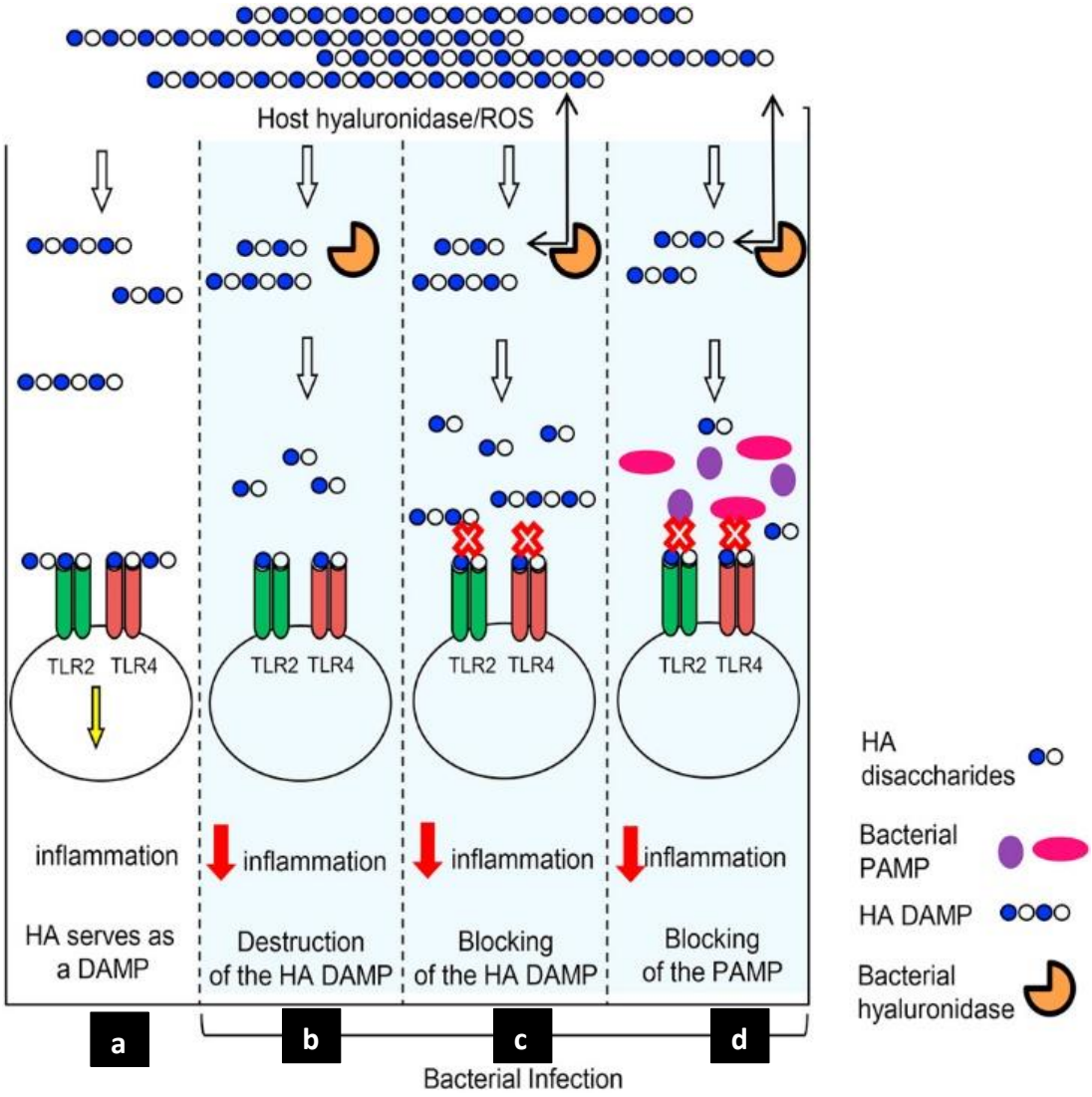


Figure 6-3 Group B *Streptococcus* (GBS) evades host immunity by degrading hyaluronan, proposed by Kolar et al., 2015.

(a) After non-infectious tissue injury, high molecular-weight hyaluronan (HMWHA) is cleaved by host hyaluronidases into low molecular-weight hyaluronan (LMWHA). Release of HA fragments from damaged or stressed cells plays a role in danger-associated molecular patterns (DAMPs) and helps to induce sterile inflammation (C. Mueller 2012) leading to tissue remodelling after injury (Lopresti and Brown 2015). Host innate immunity can detect the presence of gram-positive bacteria through pathogen-associated molecular patterns (PAMPs) via Toll-like receptor, TRL2 (C. Mueller 2012), which also recognises DAMPs (Lopresti and Brown 2015). Kolar *et al*. also proposed models for a mechanism of GBS hyaluronidase as a spreading factor. The products of GBS hyaluronidase are HA disaccharides that may either disrupt the pro-inflammatory signalling of LMWHA or impede PAMP signalling. (b) GBS hyaluronidase converts pro-inflammatory HA fragments, products from host hyaluronidase or reactive oxygen species (ROS) to HA disaccharides. Change in HA size causes loss of biological activity and DAMP property. (c) Blocking of the HA DAMP, HA disaccharides act as TLR2/4 agonists that do not allow LMWHA binding. (d) Blocking of the PAMP and binding of HA disaccharides to TRL2/4 could further block PAMP stimulation. Therefore, GBS hyaluronidase can utilize host tissue components to create anti-proinflammatory effects and to evade the host innate immune system. Reprinted by permission from Elsevier. Cell Host & Microbe: Group B *Streptococcus* Evades Host Immunity by Degrading Hyaluronan, (Stacey L. Kolar, Pierre Kyme, Ching Wen Tseng, Antoine Soliman, Amber Kaplan, Jiurong Liang, Victor Nizet, Dianhua Jiang, Ramachandran Murali, Moshe Arditi, David M. Underhill, George Y. Liu), [copyright] 2015.

6.2 Aims and objectives

Salicin utilization phenotypes and association with hyaluronidase production may provide more information on specific virulence mechanisms of hypervirulent lineages ST17 and ST283. The aim of this study was to further explore hyaluronidase use in hypervirulent and non-hypervirulent GBS strains. Specifically, phenotypic expression of hyaluronidase was assessed and for selected isolates the underlying genotype was determined. A brief synthesis of results will be the basis for suggestions for further research.

6.3 Results

6.3.1 Hyaluronidase activity assay

Hyaluronidase activity of 87 GBS isolates (28 bovine, 17 human and 42 fish) was determined on solid medium (Figure 6-4) using the method described in section 2.8.1. Turbidity produced by the conjugation of bovine serum fractions with non-depolymerized HA in acidic conditions aids in the detection of hyaluronidase activity. Digested HA products are not precipitated which can be visualised as a clear zone (Smith and Willett 1968). The zone diameter from experiments in triplicate was measured and isolates that did not produce a clear zone were categorized as a hyaluronidase-negative (hyl^-) phenotype (Figure 6-4a). Based on comparison between host species (Figure 6-5), activity of hyaluronidase in human and bovine isolates is the same ($P > 0.9$, Kruskal-Wallis test). Fish isolates have higher hyaluronidase activity than human ($P = 0.0002$) or bovine ($P < 0.0001$) GBS, although this result is limited to CC7 and CC283 isolates because CC552 was excluded from analysis. The list of GBS with hyl^- phenotype is in Table 6-1. From phenotypic findings, all of CC19 (4/4), 25% (2/8) of CC1 and 7% (1/14) of CC7 have no hyaluronidase activity.

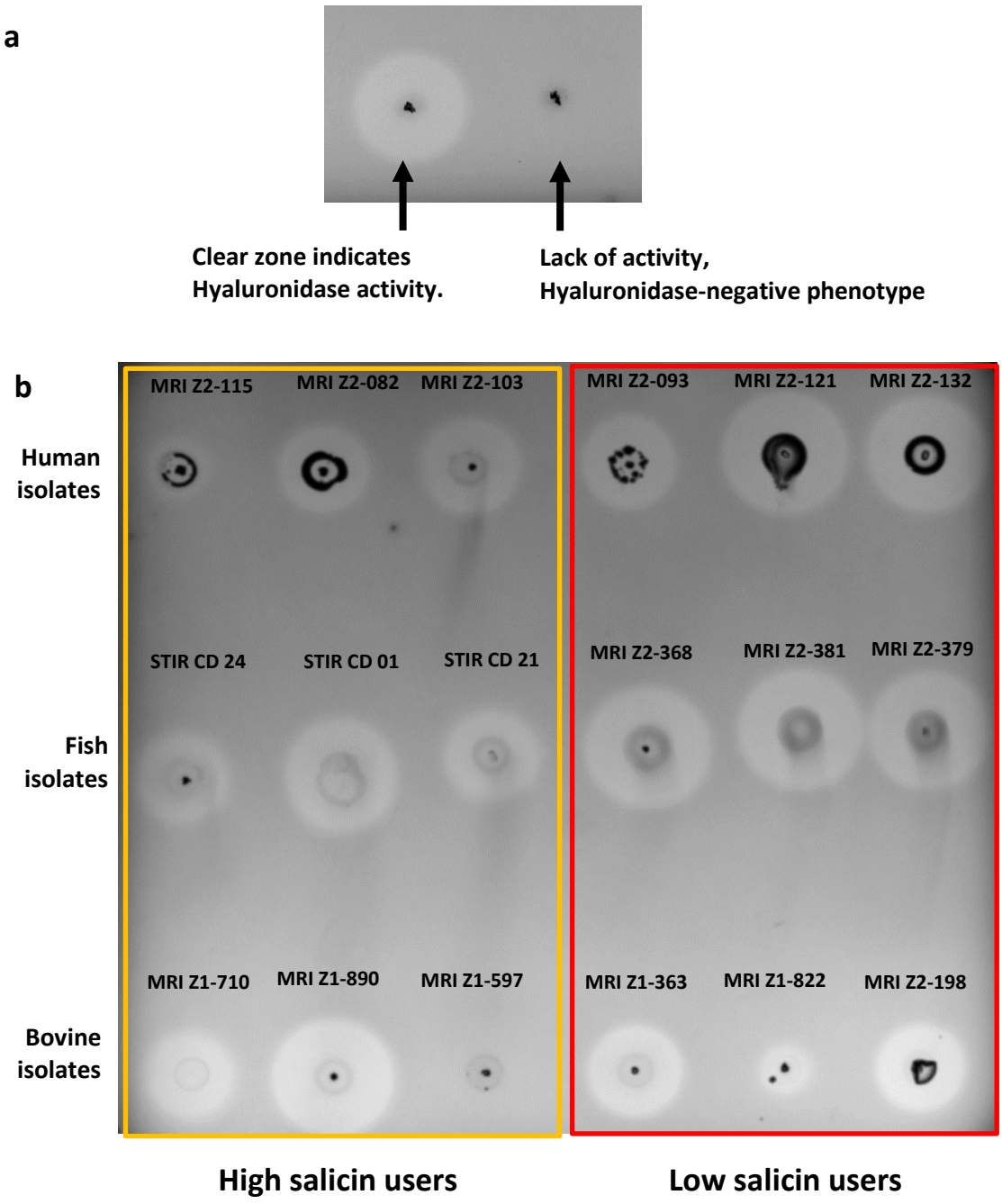


Figure 6-4 Hyaluronidase activity assay of group B *Streptococcus* (GBS) isolated from humans and animals

(a) BHI agar containing 400 µg/ml hyaluronic acid (HA) and 10% (wt/vol) bovine Serum Albumin Fraction V were spotted with 2 µl liquid culture of test isolates and incubated overnight before examination of the enzymatic activity of hyaluronidase. The nondegraded HA conjugated with the albumin is precipitated in acetic acid. Hyaluronidase-positive GBS produces hyaluronidase, which cleaves the HA polymer into fragments that can be washed away by acid, visible as a clear zone. No clear zone is seen around the non-hyaluronidase producers. (b) Hyaluronidase activity and zone diameter from human (first row), fish (middle) and bovine isolates (last row). Diameter was measured across the bacterial colony and lysis zone for high salicin users (left) and low users (right).

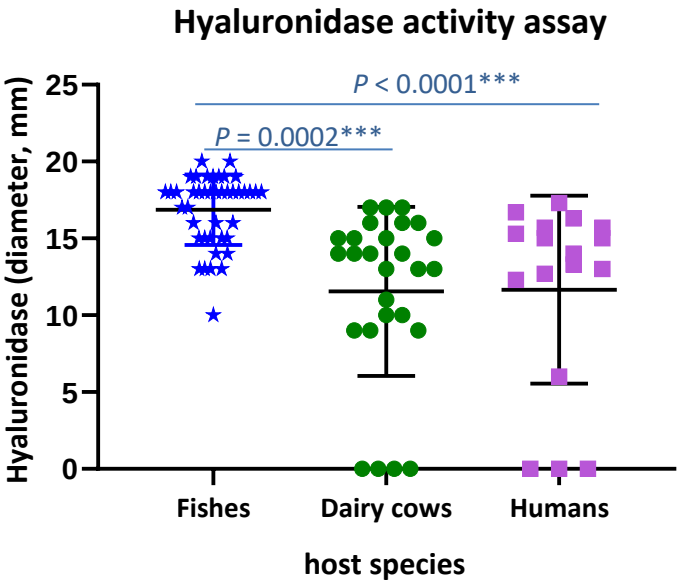


Figure 6-5 Hyaluronidase activity assay of Group B *Streptococcus* (GBS) from three host species
Zone diameter is used to quantify the activity of hyaluronidase and compared between GBS from humans and animal species. Statistical analysis was conducted using non-parametric Kruskal-Wallis test to account for outliers (hyaluronidase-negative isolates).

Table 6-1 List of *Streptococcus agalactiae* isolates, including serotype and sequence type (ST, identical to clonal complex (CC) for this set) which lack hyaluronidase activity

GBS isolates	Host	serotype	ST/CC
MRI Z1-597	Dairy cow	ND	19
MRI Z1-586	Dairy cow	ND	7
MRI Z1-811	Dairy cow	ND	19
MRI Z2-062	Dairy cow	V	1
MRI Z2-101	Human	III	19
MRI Z2-089	Human	II	1
MRI Z2-102	Human	III	19

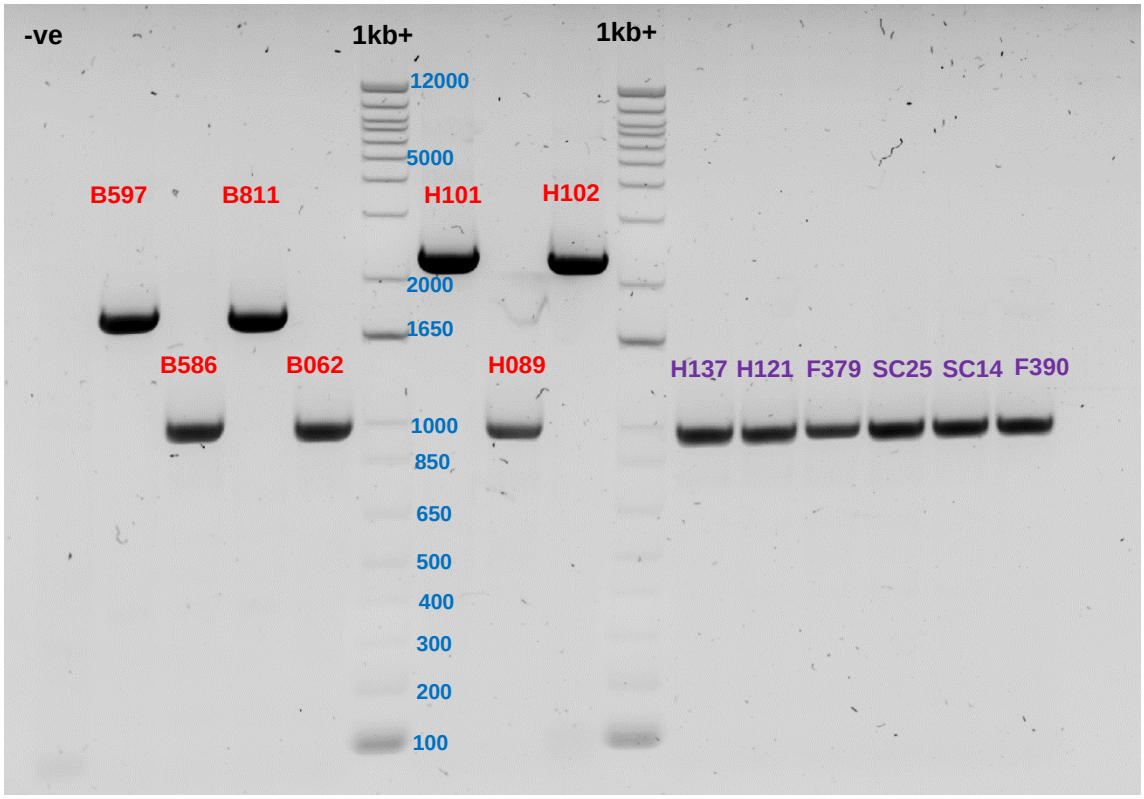
6.3.2 Determination of Hyaluronidase gene by PCR

To detect the presence of the *hylB* gene encoding hyaluronidase (Gase, Ozegowski, and Malke 1998; Sukhnanand et al. 2005), published PCR primers (Sa-hylF and Sa-hylR) and conditions to amplify a 950-bp fragment of *hylB* were used (Sukhnanand et al. 2005). Seven isolates with a hyl^- and 13 isolates with a hyl^+ phenotype were selected for PCR (Table 6-2). In a hyl^- phenotype, four out of seven isolates (MRI Z1-597, MRI Z1-811, MRI Z2-101 and MRI Z2-102) had a larger PCR amplicon than expected. Absence of hyaluronidase activity may be due to insertion of the transposable element IS*1548* in *hylB* (Sukhnanand et al. 2005), so the larger PCR amplicons in CC19 GBS isolates suggested presence of the insertion element (Granlund et al. 1998; Sukhnanand et al. 2005) (Figure 6-6).

Table 6-2 Selected group B *Streptococcus* (GBS) isolates for PCR amplification of the *hylB* gene

GBS isolates	Host	CC	Hyaluronidase assay (diameter; mm)	The *hylB* amplicon length (bp)[a]
MRI Z1-597	**Dairy cow**	**19**	**0.0**	**> 1650**
MRI Z1-811	**Dairy cow**	**19**	**0.0**	**> 1650**
MRI Z2-101	**Human**	**19**	**0.0**	**>2000**
MRI Z2-102	**Human**	**19**	**0.0**	**>2000**
MRI Z1-586	Dairy cow	7	0.0	~ 1000
MRI Z2-089	Human	1	0.0	~ 1000
MRI Z2-062	Dairy cow	1	0.0	~ 1000
MRI Z2-198	bovine	67	13.00	~ 1000
MRI Z1-050	bovine	103	13.00	~ 1000
MRI Z1-363	bovine	17	14.33	~ 1000
MRI Z1-354	bovine	7	16.67	~ 1000
MRI Z1-808	Bovine	1	16.33	~ 1000
MRI Z2-093	human	17	15.00	~ 1000
MRI Z2-132	human	17	17.33	~ 1000
MRI Z2-130	human	196	15.00	~ 1000
MRI Z2-189	human	8	12.33	~ 1000
STIR-CD-14	fish	283	16.67	~ 1000
MRI Z2-384	fish	283	17.33	~ 1000
STIR-CD-02	fish	7	15.33	~ 1000
STIR-CD-26	fish	7	16.33	~ 1000

[a] Expected size is 950-bp.



***hylB* PCR amplicon**

Figure 6-6 PCR amplicons of *hyl*B gene of group B *Streptococcus* (GBS) isolates
GBS isolates were selected based on phenotypic hyaluronidase activity. The first seven lanes represent hyaluronidase-negative isolates (red label) and the rest represent hyaluronidase-positive isolates (purple). The expected size of the *hylB* PCR product is approximately 1000 bp but larger PCR fragments were found in CC19. Despite being phenotypically hyaluronidase negative, the *hylB* PCR amplicon of isolates from CC1 (B062, H089) and CC7 (B586) was of the expected size. Subsequent amplicon sequencing showed that the larger fragments contain the *IS1548* insertion sequence. The amplicon size of *hylB* from human CC19 isolates (H101, H102) is larger than from bovine CC19 isolates (B597, B811). Amplicon sequencing showed that the complete IS1548 sequence was present in human isolates, whereas a truncated IS1548 sequence was present in the bovine isolates, explaining the difference in amplicon size (Crestani, personal communication). Host species shown in letter: B, bovine; H, human and F or SC, fish isolates. Number after host species means specimen ID in MRI Z2/Z1 (for B, H or F) or STIR-CD (for SC) bacterial collections.

6.3.3 Association between hyaluronidase activity and salicin hydrolysis

To explore the association between salicin use and hyaluronidase activity, the standardized absorbance in the salicin utilization assay from the Biolog GENIII system (Chapter 5) was plotted against the zone diameters of hyaluronidase activity assays. Raw data is provided in Appendix vii. The major clusters based on salicin utilization/hyaluronidase activity are 8% sal^+/hyl^- (7/87), 50% sal^+/hyl^+ (44/87) and 41% of sal^-/hyl^+ (36/87) phenotypes (Figure 6-7). There was no sal^-/hyl^- group. The sal^+/hyl^- group consists of CC19, 1 and 7 with almost the same proportion of human and bovine isolates. Each of those CCs also includes sal^+/hyl^+ isolates. The hypervirulent strains, CC17 and CC283 are sal^-/hyl^+ phenotype. Thus, loss of ability to hydrolyse salicin is associated with hypervirulent lineages but not with loss of enzymatic activity of hyaluronidase. Two non-CC17, non-CC283 isolates were sal^-/hyl^+, i.e. one CC67 isolate and one CC103 isolate. Both CCs are primarily found in cattle and they have not been associated with hypervirulence in humans. Other isolates within CC67 and CC103 were sal^+/hyl^+.

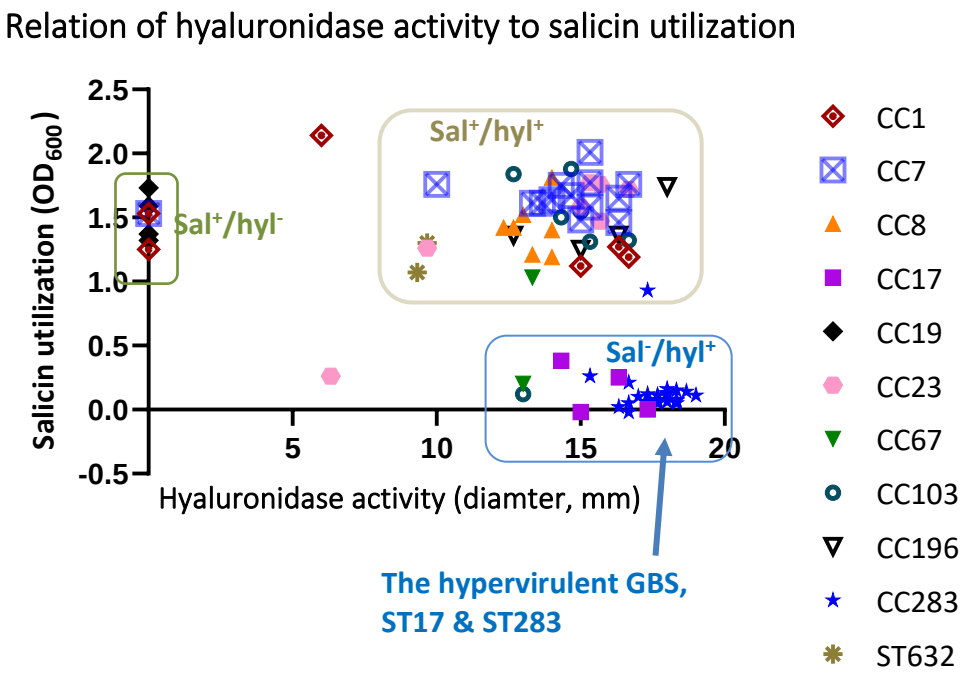


Figure 6-7 Hyaluronidase activity and salicin utilization in group B *Streptococcus* (GBS)
The zone diameter from the hyaluronidase activity assay from section 6.3.1 and the OD-value obtained in salicin assays using Biolog GENIII system (Chapter 5) were plotted against each other. A non-salicin user was defined by the OD- value below 0.5. If there was no clear zone in hyaluronidase assay that isolate was categorised as the hyl^-.

6.4 Discussion

6.4.1 Hyaluronidase phenotypes and the *hylB* gene

Hyaluronidase is a virulence factor for GBS to evade host immune detection (Kolar et al. 2015). The variability of hyaluronidase phenotypes has been observed in several studies. Kjems, Perch, and Henrichsen studied the hyaluronidase production in invasive and non-invasive strains of Danish human *S. agalactiae*. Most (75%) of GBS produced hyaluronidase but serotype III were divided into two lineages, hyaluronidase positive and negative (Kjems, Perch, and Henrichsen 1980). Granlund et al. identified genotypes associated with observed differences in hyaluronidase expression of human isolates. They found an insertion sequence (IS) of 1317 nucleotides in *hylB*, designated IS*1548*, which leads to the loss of hyaluronidase activity. IS*1548* is almost exclusively found among non-hyaluronidase producers. IS*1548* was present in non-invasive GBS strains but the hypervirulent clones lacked it (Granlund et al. 1998). An additional copy of IS*1548* located downstream of s*cpB* and upstream of *lmb* is a common feature for all human GBS isolates in which the IS*1548* is found (Granlund et al. 1998).

This study found that the majority of GBS are hyaluronidase producers, including CC17 and CC283 isolates, both of which belong to serotype III. This is consistent with the work by Granlund et al. and Sørensen et al., who described that most serotype III (Granlund et al. 1998) isolates, including CC17 (Sørensen et al. 2010), express hyaluronidase. Sørensen et al. found that CC19 and CC1 have no hyaluronidase activity and the findings of this study are in accordance with those results.

In comparative studies of human and bovine GBS isolates from the USA, average hyaluronidase activity of human isolates was lower than in bovine isolates (Sukhnanand et al. 2005), but that was not observed with our isolate collection. The hyaluronidase phenotype appears to be CC-associated rather than host-specific or serotype-dependent, and differences between studies may be due to differences in the set of isolates that was included in the study. In Sukhnanand's study, isolates were representative of the distribution in the respective host populations whereas in this thesis, isolates were selected based on ST and not necessarily representative of the natural distribution in the population. Sukhanand and colleagues did not detect any *hylB* genes with IS*1548* insertions among 52 bovine isolates, although they did detect them in 8 of 52 human isolates (Sukhnanand et al. 2005). Here, IS*1548* was detected in bovine isolate MRI Z1-597,

which belongs to CC19. The difference between the current study and Sukhnanand's study may be related to the CC distribution of bovine GBS in different countries. In the USA, CC61/67 is the predominant bovine lineage (Springman et al. 2014). Our isolates mostly came from Northern Europe, where CC67 is almost non-existent but CC19 is occasionally found in cattle (Lyhs et al., 2016; Zadoks et al., 2011). There was one exception to the proposed rule that hyaluronidase use is CC-specific rather than host-specific: all piscine CC7 isolates were hyaluronidase-positive. The study included one bovine CC7 isolate, and this was the only CC7 isolate that was hyaluronidase-negative. CC7 is very rare in cattle (Lyhs et al., 2016; Zadoks et al., 2011), and was specifically selected for the current study to see whether there were host-specific rather than CC-specific differences between piscine and non-piscine GBS in the Biolog assay (Chapter 5).

6.4.2 Hyaluronidase/salicin phenotypes

The association between hyaluronidase production and hydrolysis of salicin was first studied by Kjems, Perch, and Henrichsen in 1980. Characteristic of GBS in different serotypes were used to classify them into three biotypes: sal^+/hyl^+, sal^+/hyl^- and sal^-/hyl^+. Salicin-negative isolates were all hyaluronidase-positive and most of serotype III were sal^-/hyl^+ (Kjems, Perch, and Henrichsen 1980). This study has shown that the Sal^-/hyl^+ phenotype is specifically associated with the hypervirulent clades CC17 and CC283, and not with CC19, which also belongs to serotype III. Salicin is a β-glucoside and its use could be regulated by a β-glucoside operon. B-glucoside operons have been well studied in *Escherichia coli* that was speculated to provide positive selection for growth at low temperature outside of the host (Neelakanta, Sankar, and Schnetz 2009) and prevent formation of toxic metabolites that could inhibit cell growth (Reynolds, Felton, and Wright 1981). B-glucoside operons in *Bacillus subtilis* have been studied with a focus on their role in the phosphoenolpyruvate-dependent phosphotransferase system (PTS) and carbon catabolite repression to support cell growth after glucose exhaustion (Krüger and Hecker 1995). The β-glucoside utilization and operon of GBS has not been described.

GBS can evade the host immune system and disseminate in the body thanks to the action of hyaluronidase. In non-infectious tissue injury, HA fragments generated by host hyaluronidases play a role in danger-associated molecular patterns (DAMPs) and bind to Toll-like receptor (TLR2), inducing a pro-inflammatory response (C. Mueller 2012) to clear cell debris, remodel tissue and remove infectious agents (Lopresti and Brown 2015). GBS hyaluronidase impedes pro-inflammatory signalling by destruction of HA fragments to HA disaccharides (Kolar et al. 2015). Both salicin and products from GBS hyaluronidase have anti-inflammatory properties. Salicin may be directly involved in host immune evasion via the antipyretic/anti-inflammatory effects. The hypervirulent clones may leave salicin uncleaved to exploit its anti-inflammatory effect, thus avoiding bacterial clearance by host immunity. However, because the sal^-/hyl^+ phenotype has been observed in several studies, it is postulated here that the lack of salicin utilization may be linked to the action of hyaluronidase. In an attempt to identify a potential mechanism for hypervirulence of non-salicin users, interaction of salicin and hyaluronidase pathways is proposed.

D-salicin is hypothesized to be a structural analogue to hyaluronan as they both contain β-glycosidic bonds. The hypervirulent clones' lack of enzymatic activity to cleave the β-glycosidic bond of salicin may indicate an inability for degradation of HA

disaccharides, a product from GBS hyaluronidase, because HA disaccharides also have a β-glycosidic bond. If the enzyme cleaving salicin has activity, it may also break down HA disaccharides to monosaccharides, leading to restoration of the pro-inflammatory signalling pathway. In this scenario, inability to use salicin is postulated to be a marker of inability to hydrolyse HA disaccharides, rather than a compound that is directly involved in HA metabolism. Schematic representation of the hypothesis on salicin linked to hyaluronidase is shown in Figure 6-8.

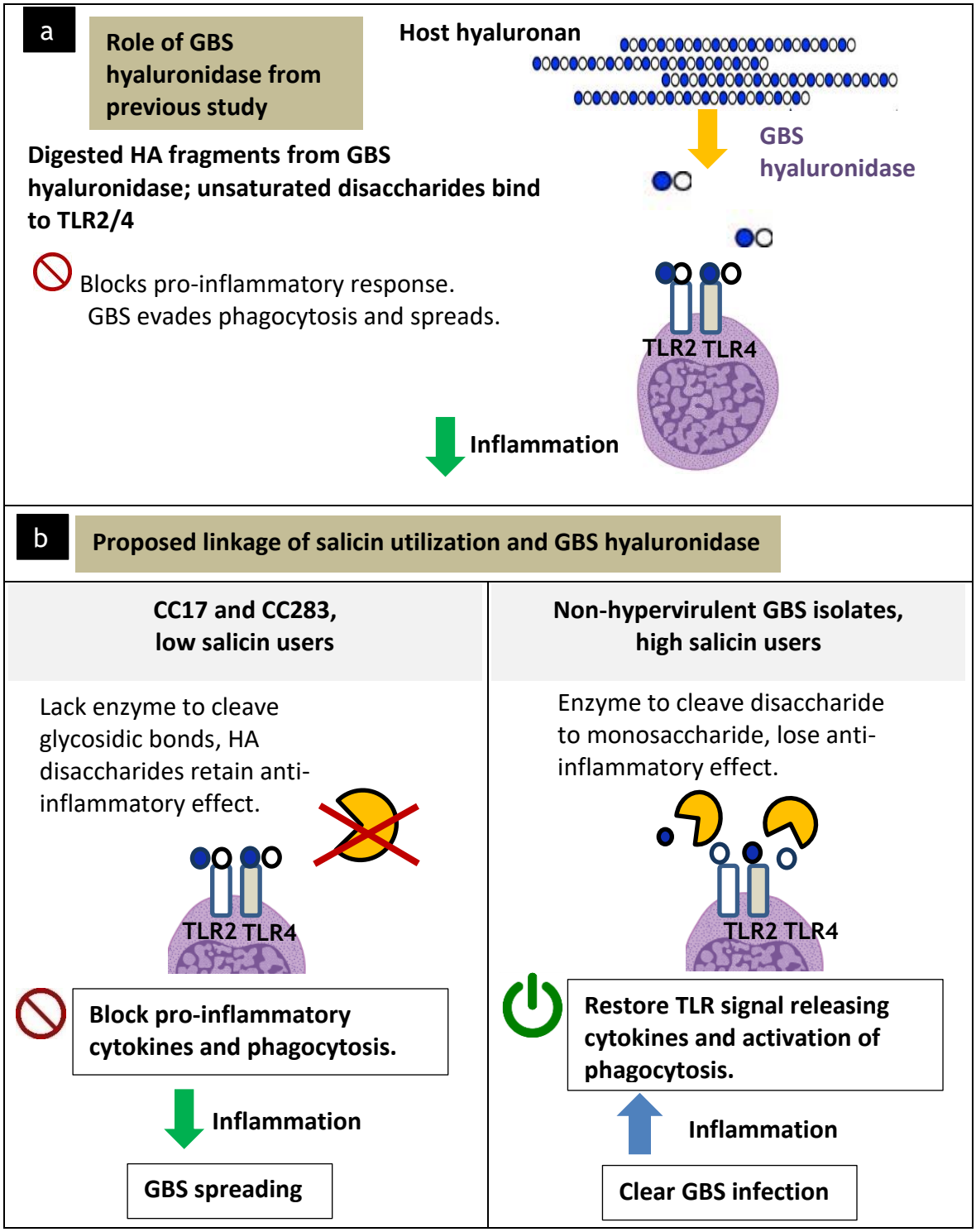


Figure 6-8 Schematic representation of the hypothesis on the association between salicin use and hypervirulence through the hyaluronidase pathway.
The association between salicin non-use and hypervirulence is hypothesized to be involved in the sal^-/hyl^+ phenotype of hypervirulent strains CC17 and CC283. Depolymerisation of hyaluronan (HA) in host tissue gives HA fragments that bind to Toll-like receptor and activate a pro-inflammatory response. GBS hyaluronidase breaks down HA fragments into HA disaccharide blocking the pro-inflammatory signalling pathway, letting GBS escape host immune detection. If GBS produce enzymes to cleave the β-glycosidic bond of salicin that means the β-glycosidic bond of HA disaccharide may also be cleaved resulting in the loss of immune evasion. CC17 and CC283 have no enzyme to utilize salicin, and this lack of enzyme may help the stability of the HA disaccharide structure, maintaining anti-inflammatory properties and contributing to hypervirulence.

6.4.3 Suggestions for future work

Hyaluronidase (also called hyaluronate lyase) is encoded by *hylB* gene (Lin et al. 1994). Loss of hyaluronidase activity occurs from the insertion sequence *1548* (Sukhnanand et al. 2005). The presence of IS*1548* in hyl- isolates should be examined by PCR and DNA sequencing (Granlund et al. 1998; Rolland et al. 1999) to gain insight in the genetic background of hyaluronidase inactivation. To investigate the proposed model of the association between salicin, hyaluronidase and hypervirulence, a knockout mutant of the salicin operon could be investigated *in vitro* and *in vivo*. Other than murine and fish infection model, *Galleria mellonella* larvae may be a useful model to initially investigate sensing of invading microbes (Altincicek and Vilcinskas 2006; Altincicek et al. 2007). *G. mellonella* recognizes the degradation of extracellular matrix components (ECM), i.e. collagen, by its endogenous enzyme to generate products with immune-stimulatory activity (Altincicek and Vilcinskas 2006) In addition, the peptidoglycan recognition protein (PGRP) in an component of innate immunity is conserved from insects to humans (Kang et al. 1998).

The relation of salicin to the other virulence factors, the C5a peptidase and Lmb protein should be studied. The *scp*B gene encodes C5a peptidase (ScpB) which is a surface associated serine protease having a bifunction for inactivation of the complement component (C5a) and for mediating bacterial binding to fibronectin (Beckmann et al. 2002). Other than hyaluronan, GBS can use the host extracellular matrices (ECM), e.g. fibronectin and laminin as anchoring point for adhesion and invasion of epithelial cells (Hull, Tamura, and Castner 2008) in promoting colonization, and central nervous system invasion (Shabayek and Spellerberg 2018). Fibronectin has a multi-domain structure providing binding sites for other extracellular matrixs including heparin/heparan sulfate which are glucosamine-containing glycosaminoglycans similar to hyaluronan (Mulloy and Forster 2000). Binding of glycosaminoglycan to the heparin/heparan sulfate binding sites of fibronectin was reported to increase the formation of insoluble fibronectin and the binding affinity is size-dependence like hyaluronan (Raitman et al. 2018). The ScpB mediates GBS only binding to insoluble fibronectin but not to the soluble form (Beckmann et al. 2002). The ST283 GBS infection has septic arthritis as a clinical manifestation (Wang et al. 2018). Fibronectin is abundant in synovial fluid, and a high level of fibronectin is correlated with the progression of joint destruction (Barilla and Carsons 2000). Bovine isolates generally lack the *scpB* and *lmb* genes, and so does the piscine CC552 isolate STIR-CD-17, but they are recognized as virulence genes in human isolates (Sørensen et al. 2010; Delannoy et al.

2016; Beckmann et al. 2002). Inability of salicin utilization may be involved with the stability of glycosaminoglycan binding to fibronectin binding sites to enhance the action of the ScpB.

In addition, GBS strains associated with meningitis expressed more Lmb protein than other isolates (Al Safadi et al. 2010). The scpB-lmb intergenic region is a hot spot for integration of mobile genetic elements (MGEs), GBSi1 and IS*1548* (Al Safadi et al. 2010) which are located in the promoter region of the lmb gene. Invasive GBS isolates carrying IS*1548* in the scpB-lmb intergenic region showed high laminin binding ability (Shabayek and Spellerberg 2018) and all GBS strains harbouring IS*1548* had another copy located downstream of the C5a peptidase gene (Granlund et al. 1998).The distribution of MGEs according to the structure of scpB-lmb intergenic region, clonal complexes and their interaction with the host extracellular matrix should be determined to find a potential mechanism of salicin's involvement in GBS virulence.

In summary, the potential mechanism for hypervirulence of non-salicin users in *S. agalactiae* remains unknown but the specific phenotype is identified in the hypervirulent GBS, CC17 and CC283. Inactivation of genes may provide functional adaptation and host restriction (Rosinski-Chupin et al. 2013). Non-use of salicin in *S. agalactiae* may be an important evolutionary mechanism in the emergence of hypervirulent clones.

Chapter 7 Final discussion

7.1 Concluding remarks

This study has made important contributions to the GBS research field, providing novel information about a selective GBS treatment and a unique metabolic feature only associated with hypervirulent GBS strains. Bacteriocin was proven to have efficacy against target bacteria without impacting on key components of the microbiome. A special feature of ST17 and ST283 GBS – life threatening GBS strains – is their inability to utilize salicin. While its pathway and mechanism remain unknown, a brief synthesis of results and hypothesized mechanisms may open opportunities for other researchers to investigate this unique feature further. Elimination of bacteriocin in *G. melonella* may extend the use of *G. melonella* in the field of drug modification, i.e. to determine the duration of treatment and half-life of compounds. Moreover, the translations of this work in One Health and aquaculture are described in the following sections.

7.2 The emergence of a zoonotic clone warrants vigilance for public health and the fish industry

Streptococcus agalactiae has evolved from a commensal to a pathogenic bacterium that is causing a large (CC17) or rising (CC283) burden of infectious disease in humans. Invasive GBS disease in non-pregnant adults has been clinically reported in low and middle-income countries in Southeast Asia, including cases from Lao PDR in 2000 (Barkham et al. 2018) and Thailand in 1999-2009 (Bunyasontigul et al. 2009), and in high income countries in Asia, including Hong Kong (1993 – 2012) (Ip et al. 2006; Ip et al. 2016) and Singapore (2015) (Tan, Lin, Foo, Koh, et al. 2016). Infections with GBS ST283 have caused a quiet epidemic in Southeast Asia since the end of the 20th century (Barkham et al. 2019). There is a lack of detailed information about the scale of the problem because confirmation of the causative agent and identification at strain level is only practiced in some countries or hospitals in the region. The consumption of raw freshwater fish has been proven to have been the source of the ST283 outbreak in Singapore (Kalimuddin et al. 2017) and fish production, harvest or consumption practices are thought to contribute to its occurrence throughout Southeast Asia (Barkham et al. 2019). The presence of ST283 and its single locus variant (SLV), ST491 in farmed fish have been detected in several Asian countries, including Malyasia (Barkham et al. 2019), Thailand (Delannoy 2013; Kayansamruaj et al. 2018), and Vietnam (Delannoy 2013). More recently, serotype III isolates of GBS from Brazil have been confirmed to be ST283 (Pereira, Tavares, and Figueiredo 2019). Import of live fish from Singapore to Brazil in 2014 was the potential

source of introducing ST283 to the Brazilian fish industry (Pereira, Tavares, and Figueiredo 2019). The spread of ST283 from Asia to South America indicates the possibility of global expansion of this hypervirulent GBS clone.

The emergence of a zoonotic GBS has raised the matter of food safety and turned GBS in aquaculture from an animal health problem into a One Health problem. To produce a safe and efficient fish supply, the potential routes of transmission should be identified, e.g. contaminated water sources or introduction of healthy looking carrier fish. In Singapore, the problem was linked to normal looking fish sold at market, and in Malaysia, carriage of ST283 in apparently healthy fish has been shown (Barkham et al 2019). Implementation of good aquaculture practices (Kleter and Marvin 2009) can promote fish health and reduce risk of GBS transmission within and between farms. Fish and its products should be hygienically handled from fish farm to table (Reilly and Kaferstein 1998) to prevent the contamination with ST283 in the food chain. However, consumption behaviour may also play a role as major factor in human transmission. Culture and food hygiene may be potential risks of foodborne diseases. For example, several local dishes are eaten raw in Northern and Northeastern (Isan) region of Thailand. Using fresh pig/bovine blood or bovine bile mixed with cooked food is common in these regions. Freshly squeezed lime juice over raw meat or seafood is a traditional way of preparing Isan dishes and some people may misunderstand that food is cooked because flesh appears to be pale colour. Outbreaks of *Streptococcus suis* infection in human in the Northern Thailand were linked to eating raw pork or uncooked pig blood (Fongcom et al. 2009). Thai BBQ & Hot pot is famous in Thailand (Figure 7-1a), cross contamination can happen from mixing of raw and cooked meat, fish or seafood. Not only freshwater fishes but also prepared seafood with multiple ingredients may contain *S. agalactiae* (van der Mee-Marquet et al. 2009).

In addition, social media has influence on eating behaviours related to food safety. Food channels can be positive and negative conveyors. Recipes with uncooked food may promote the emergence of foodborne diseases. In 2018, curing raw egg yolks with fish sauce (Figure 7-1b) and eating them raw was famous in Thailand. This recipe has been shared nationwide on social media. There have been no reports on salmonellosis resulting from this fashion but it may be an underestimation due to lack of reporting. However, social media can be an important tool for food safety-advocacy. In Singapore, people received warning messages about yu-sheng dishes from family, friends and colleagues before the official government advisory had been issued (Dr Swaine Chen, personal communication). Consumer education and awareness and risk communication (Wilcock et al. 2004)

on raw fish/food consumption and food hygiene should be promoted. For GBS control and prevention, disease surveillance and monitoring of a zoonotic GBS should be holistically conducted in public health and aquaculture.

(a) Thai BBQ & Hot pot

(b) Curing raw eggs

Figure 7-1 Potential risk of foodborne disases from eating behaviours
(a) BBQ-Hot pot is a famous Thai cuisine in all regions of Thailand, pot is shared by several people and chopsticks are used for picking meat, fish or seafood. Cross contamination may happen by a mix of cooked and raw food during grilling or boiling or food may be contaminated by chopsticks. Source: https://th.theasianparent.com. (b) Curing raw eggs in fish sauce has been the most favourite recipe for sharing via social media in Thailand. Safety scheme like the British Lion mark to reduce *Salmonella* in eggs has not been strictly practiced in Thailand.

7.3 The potential application of agalacticin A in clinical settings and aquaculture

Agalacticin A provides efficacy against GBS regardless of serotype or sequence type and selectively inhibits some closely related bacterial species. These properties may offer a promising therapeutic agent for ST283 GBS in adults in Southeast Asia. ST283 GBS infection has common clinical presentations of meningitis (10-35%), endocarditis (4.5-10%) and septic arthritis (23-39%) (Barkham et al. 2019). Meningitis cases showed poor outcomes such as encephalopathy, focal neurological deficits, and/or seizures (Tan et al. 2017) and mortality rate was 3.4% (Kalimuddin et al. 2017). Fast action of Class III bacteriocin may be of interest for ST283 GBS endocarditis and septic arthritis and may be meningitis. For treatment of bacterial meningitis in adults, agalacticin A must be delivered across the blood-brain barrier (BBB) and its distribution and concentration must be high enough (Domingo et al. 1997) to reach sites of infection (Tan et al. 2017). Modification of the structure of agalacticin A may expand clinical pharmacokinetics. Combination of agalacticin A with antibiotics may be advantageous in reducing the risk of neurological sequelae. In addition, agalacticin A may be of interest as a therapeutic agent in perinatal and infant diseases because it is unlikely to give adverse outcomes on the microbiome and subsequent child development due to its narrow spectrum. Studies of the microbiome should be conducted on murine models of vaginal tract commensal carriage and on fish commensal carriage. 16S rRNA gene sequencing could be used to detect the impact of agalacticin A on other commensal microorganisms (Bernardini et al. 2017; Ding et al. 2018; van Kessel et al. 2011; Standen et al. 2015).

In the aquaculture setting, *S. agalactiae* kills the large size fish leading to big economic losses and waste of all time, feed and other resources invested in the fish. Administration of agalacticin A may reduce mortality in fish production. However, the route of administration in mass production is a challenge. Biotechnology such as the combination of the agalacticin A gene with the biosynthesis machinery of the model lantibiotic nisin may be used to produced agalacticin A on a large scale (van Heel et al. 2016). Moreover, the source of GBS and routes of transmission in fish production (see production cycle in Appendix viii) must be identified so that use of agalacticin A can be targeted to those sources before economic damage occurs. Carrier fingerlings were reported to be a source of *S. agalactiae* in Malaysia (Amal et al. 2013). Presence in fingerlings may be due to vertical transmission in the hatcheries (Pasnik, Evans, and Klesius 2007). Selective decontamination of fish eggs in a hatching jar by agalacticin A could be tried. Previous studies have shown that incorporation

of Generally Recognized As Safe (GRAS) antimicrobials such as bacteriocins, e.g. nisin into plastic films can enhance microbial safety of cold-smoked salmon from *Listeria monocytogenes* (Ye, Neetoo, and Chen 2008; Neetoo et al. 2008). To mitigate the risk of ST283 in fish products, food packaging impregnated with agalacticin A for fish fillets (Woraprayote et al. 2018) may be invented.

Agalacticin A can be eliminated by host metabolism by different mechanisms, e.g. liver enzymes or gill elimination. which may vary among species of fish (Feng, Jia, and Li 2008). Vertebrate models, including murine and fish models, should be used and findings from the Galleria model as described in this thesis should be compared to those from other host species to validate the Galleria model for pharmacokinetic studies. Drug delivery systems should be explored to improve the efficacy and stability of agalacticin A (Fahim, Khairalla, and El-Gendy 2016). For ST283 GBS infection, especially the potential treatment of human meningitis, drug transport across the BBB and distribution to site of infection should be explored. A mouse model of ST283 infection has recently been published, but it is not clear whether this includes meningitis (Yang et al. 2019). A tilapia challenge model has been established (Zadoks, personal communication) and this would be useful for exploring the distribution of agalacticin A in fish. Immunohistochemistry with anti-agalacticin A antibody, as used in Chapter 4, may detect and trace agalacticin A distribution and could show whether it enters the brain. In aquaculture, water salinity plays a significant role in the excretion pathway, and the elimination of antibiotic in seawater tilapia was more rapid than that in freshwater tilapia (Feng, Jia, and Li 2008). Some chemical properties of agalacticin A should be improved according to the purpose of application, e.g. passing through BBB in human medicine or resistance to environment conditions in aquaculture.

7.4 Clonal complex-associated phenotypes

The preliminary study on salicin utilization and hyaluronidase production of GBS from different CC has shown sal^-/hyl^+ phenotype is associated with the hypervirulent GBS strains. Non-salicin hydrolysis is predominantly found in CC17 and CC283, which are hypervirulent GBS strains, and occasionally in bovine strains (Chapter 5 and 6). Mechanisms underlying the lack of salicin hydrolysis are unknown and should be examined. Salicin is a β-glucoside and its use could depend on the presence and function of a β-glucoside operon. The β-glucoside operons have been well studied in *Escherichia coli*. The β-glucoside systems, i.e. the *bgl* (aryl-β-D-glucoside) operon, and the *bgc* (aryl-β-D-glucosides and cellobiose) operon are cryptic because these genes cannot be expressed, or induced, in wild-type *E. coli* Kl2 (Reynolds, Felton, and Wright 1981). The inactivation of *bgl* operon was believed to be involved in self-protection, because β-glucosides are abundant in nature and hydrolysis of these substrates produces a toxic compound, i.e. aglycone (Ismail and Hayes 2005). The cryptic *bgl* operon is not induced by some β-glucosides to prevent formation of toxic metabolites that could inhibit cell growth (Reynolds, Felton, and Wright 1981). The *bgc* operon was described later and found to be most prevalent in an extraintestinal-pathogenic *E. coli*, but rare in commensal strains (Neelakanta, Sankar, and Schnetz 2009). Expression of the *bgc* operon may be temperature regulated (Neelakanta, Sankar, and Schnetz 2009) which suggests that it may provide an advantage for growth at low temperature outside of the host. However, studies of β-glucoside systems in streptococci are limited.

If non-hydrolysis of salicin and the sal^-/hyl^+ are proven and confirmed as phenotypic markers of CC17 and CC283, it may provide a cost-effective tool for laboratory settings in low and middle-income countries that can provide initial screening of CC283 for fish farms. In Thailand, some farms are contract farms and a big company distributes fish fingerlings to those small farms. A laboratory may be set up in that province close to the farm cluster, and such laboratories could provide GBS diagnostics. Alternatively, fish samples may be sent to the local government offices, the Department of Livestock or the Department of Fisheries. However, the sal-/hyl+ can occasionally be found in CC67 and CC103 (Chapter 5) and in rare STs (Sørensen et al. 2010) and other isolates in serotype III (Kjems, Perch, and Henrichsen 1980), so genotypic confirmation of strain identity may be necessary. The shared trait of CC67 may be from its genetic relationship to CC17 (Sørensen et al. 2010). Invention of rapid amplification of DNA with minimal equipment requirement, e.g. loop-mediated isothermal amplification (LAMP) assay (Suebsing et al. 2013) can provide

accuracy and can be applied in small field laboratories (Ke et al. 2014). This method has been described for GBS in fishes (Suebsing et al. 2013) and could potentially be modified to detect ST283 specifically. Primers for ST283 have already been developed (Barkham et al. 2019).

To increase the global and domestic food supply, the Food and Agriculture Organization of the United Nations (FAO) has also promoted aquaculture development in Africa, especially since 1989. In 2003, Sub-Saharan Africa contributed 0.13 and 13.6 percent to total World and Africa aquaculture production, respectively (FAO 2005). According to Barkham et al., the emergence of ST283 occurred around the same time as the expansion of aquaculture (Barkham et al. 2019). The lesson learnt from ST283 emerging in Southeast Asia may provide knowledge and understanding of zoonotic or hypervirulent bacterial diseases in fin-fish farming that is essential for sustainable aquaculture development in sub-Saharan Africa. To date, GBS in Africa appears to belong to CC552 (Verner-Jeffreys et al. 2018), which doesn't pose a zoonotic risk, but introduction of broodstock or fingerlings from Asia may lead to introduction of ST283. This has already happened for ST283 in Brazil (Pereira, Tavares, and Figueiredo 2019) and the global distribution of CC552 itself is also attributed to trade in tilapia (Kawasaki et al. 2018). In addition, more fish-pathogenic or zoonotic GBS strains may emerge as production intensifies in new regions of the world.

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Appendices

Appendix i

Bacterial strains (section 2.2.1, 2.3, 2.5 and 3.3.1)

Table A- 1 *Streptococcus agalactiae* strains

Specimen ID	Host species	Source	Country of origin	Farm	Serotype	CC	ST	Reference	Experiments					
									Identification of bacteriocin producing strains	Spectrum of bacteriocin	*Galleria* challenge	Biolog assay	Hyaluronidase activity assay	*hyl*B gene
MRI Z1-050	bovine	mastitis	Denmark	ND	Ia	103	103	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	✔
MRI Z1-158	bovine	mastitis	Denmark	ND	Ia	23	23	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	
MRI Z1-354	bovine	mastitis	Denmark	ND	Ia	7	7	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	✔
MRI Z1-363	bovine	mastitis	Denmark	ND	III	17	17	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	✔
MRI Z1-586	bovine	mastitis	Denmark	ND	ND	7	7	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	✔
MRI Z1-597	bovine	mastitis	Denmark	ND	ND	19	19	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	✔
MRI Z1-600	bovine	mastitis	Denmark	ND	ND	23	23	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	
MRI Z1-707	bovine	mastitis	Denmark	ND	ND	1	1	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	
MRI Z1-710	bovine	mastitis	Denmark	ND	ND	314	314	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	
MRI Z1-715	bovine	mastitis	Denmark	ND	ND	103	103	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	
MRI Z1-717	bovine	mastitis	Denmark	ND	ND	19	19	Prof. Ruth N. Zadoks,	✔	✔				
MRI Z1-719	bovine	mastitis	Denmark	ND	ND	23	23	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	
MRI Z1-803	bovine	mastitis	Denmark	ND	ND	314	314	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	
MRI Z1-808	bovine	mastitis	Denmark	ND	ND	1	1	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	✔
MRI Z1-811	bovine	mastitis	Denmark	ND	ND	19	19	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	
MRI Z1-822	bovine	mastitis	Denmark	ND	ND	23	23	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	

Specimen ID	Host species	Source	Country of origin	Farm	Serotype	CC	ST	Reference	Experiments					
									Identification of bacteriocin producing strains	Spectrum of bacteriocin	*Galleria* challenge	Biolog assay	Hyaluronidase activity assay	*hylB* gene
MRI Z1-851	bovine	mastitis	Denmark	ND	ND	196	196	Prof. Ruth N. Zadoks,	✔	✔				
MRI Z1-858	bovine	mastitis	Denmark	ND	ND	103	314	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	✔
MRI Z1-872	bovine	mastitis	Denmark	ND	ND	103	314	Prof. Ruth N. Zadoks,	✔	✔				
MRI Z2-001	bovine	mastitis	Finland	ND	III	632	632	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-002	bovine	mastitis	Finland	ND	III	632	632	Lyhs et al. 2015	✔	✔				
MRI Z2-005	bovine	mastitis	Finland	ND	ND	632	632	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	
MRI Z2-007	bovine	mastitis	Finland	ND	ND	103	103	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	
MRI Z2-041	bovine	mastitis	Finland	ND	Ib	8	8	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-044	bovine	mastitis	Finland	ND	Ia	103	103	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-045	bovine	mastitis	Finland	ND	Ib	8	8	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-053	bovine	mastitis	Finland	ND	Ib	8	8	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-060	bovine	mastitis	Finland	ND	IV	196	196	Lyhs et al. 2015	✔	✔				
MRI Z2-062	bovine	mastitis	Finland	ND	V	1	1	Lyhs et al. 2015	✔	✔		✔	✔	✔
MRI Z2-065	bovine	mastitis	Finland	ND	IV	196	196	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-077	human	UTI	Finland	ND	V	1	462	Lyhs et al. 2015	✔			✔	✔	
MRI Z2-081	human	skin and soft tissue infection	Finland	ND	Ia	23	23	Lyhs et al. 2015	✔	✔		✔	✔	✔
MRI Z2-082	human	skin and soft tissue infection	Finland	ND	Ib	8	8	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-089	human	female carriage	Finland	ND	II	1	1	Lyhs et al. 2015	✔	✔		✔	✔	✔
MRI Z2-093	human	UTI	Finland	ND	III	17	17	Lyhs et al. 2015	✔	✔	✔	✔	✔	✔

Specimen ID	Host species	Source	Country of origin	Farm	Serotype	CC	ST	Reference	Experiments					
									Identification of bacteriocin producing strains	Spectrum of bacteriocin	*Galleria* challenge	Biolog assay	Hyaluronidase activity assay	*hyl*B gene
MRI Z2-098	human	female carriage	Finland	ND	IV	196	196	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-101	human	skin and soft tissue infection	Finland	ND	III	19	19	Lyhs et al. 2015	✔	✔		✔	✔	✔
MRI Z2-102	human	skin and soft tissue infection	Finland	ND	III	19	19	Lyhs et al. 2015	✔	✔			✔	
MRI Z2-103	human	UTI	Finland	ND	Ia	23	23	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-106	human	female carriage	Finland	ND	III	19	19	Lyhs et al. 2015	✔	✔				
MRI Z2-115	human	skin and soft tissue infection	Finland	ND	VI	1	1	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-120	human	UTI	Finland	ND	IV	196	196	Lyhs et al. 2015	✔	✔				
MRI Z2-121	human	UTI	Finland	ND	III	17	17	Lyhs et al. 2015	✔	✔	✔	✔	✔	✔
MRI Z2-126	human	UTI	Finland	ND	Ia	23	23	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-130	human	female carriage	Finland	ND	IV	196	196	Lyhs et al. 2015	✔	✔		✔	✔	✔
MRI Z2-132	human	female carriage	Finland	ND	III	17	17	Lyhs et al. 2015	✔	✔	✔	✔	✔	✔
MRI Z2-137	human	UTI	Finland	ND	Ib	8	8	Lyhs et al. 2015	✔	✔		✔	✔	✔
MRI Z2-197	bovine	mastitis	UK	ND	ND	67	67	Prof. Ruth N. Zadoks,	✔	✔				
MRI Z2-198	bovine	mastitis	UK	ND	ND	67	67	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	✔
MRI Z2-200	bovine	mastitis	UK	ND	ND	67	420	Prof. Ruth N. Zadoks,	✔	✔				
MRI Z2-202	bovine	mastitis	UK	ND	ND	67	67	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	✔

Specimen ID	Host species	Source	Country of origin	Farm	Serotype	CC	ST	Reference	Experiments					
									Identification of bacteriocin producing strains	Spectrum of bacteriocin	*Galleria* challenge	Biolog assay	Hyaluronidase activity assay	*hyl*B gene
MRI Z2-187	human	invasive (sepsis or meningitis)	Sweden	ND	Ib	8	8	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z2-189	human	invasive (sepsis or meningitis)	Sweden	ND	Ib	8	8	Lyhs et al. 2015	✔	✔		✔	✔	✔
MRI Z2-058	bovine	mastitis	Finland	ND	Ib	10	10	Lyhs et al. 2015	✔	✔		✔	✔	
MRI Z1-890	bovine	mastitis	Denmark	ND	ND	196	196	Prof. Ruth N. Zadoks,	✔	✔		✔	✔	
STIR CD 01	Mullet	brain	Kuwait	A	Ia	7	7	Delannoy et al., 2013	✔	✔		✔	✔	
STIR CD 02	Mullet	brain	Kuwait	A	Ia	7	7	Delannoy et al., 2013	✔	✔		✔	✔	✔
STIR CD 03	Mullet	brain	Kuwait	A	Ia	7	7	Delannoy et al., 2013	✔	✔		✔	✔	
STIR CD 04	Mullet	brain	Kuwait	A	Ia	7	7	Delannoy et al., 2013	✔	✔		✔	✔	
STIR CD 05	Mullet	brain	Kuwait	A	Ia	7	7	Delannoy et al., 2013	✔	✔		✔	✔	
STIR CD 07	Tilapia	heart	Honduras	B	Ib	552	260	Delannoy et al., 2013	✔					
STIR CD 09	Tilapia	kidney	Columbia	C	Ib	552	260	Delannoy et al., 2013	✔					
STIR CD 10	Tilapia	kidney	Columbia	C	Ib	552	260	Delannoy et al., 2013	✔					
STIR CD 11	Tilapia	kidney	Columbia	C	Ib	552	260	Delannoy et al., 2013	✔					
STIR CD 12	Tilapia	kidney	Columbia	C	Ib	552	260	Delannoy et al., 2013	✔					

Specimen ID	Host species	Source	Country of origin	Farm	Serotype	CC	ST	Reference	Experiments					
									Identification of bacteriocin producing strains	Spectrum of bacteriocin	*Galleria* challenge	Biolog assay	Hyaluronidase activity assay	*hyl*B gene
STIR CD 13	Tilapia	eye	Costa Rica	D	Ib	552	260	Delannoy et al., 2013	✔					
STIR CD 14	Tilapia	NA	Vietnam	E	III	283	491	Delannoy et al., 2013	✔	✔		✔	✔	✔
STIR CD 17	Tilapia	heart	Honduras	B	Ib	552	260	Delannoy et al., 2013	✔			✔		
STIR CD 18	Tilapia	spleen	Honduras	B	Ib	552	260	Delannoy et al., 2013	✔			✔		
STIR CD 19	Tilapia	spleen	Honduras	B	Ib	552	260	Delannoy et al., 2013	✔			✔		
STIR CD 21	Tilapia	liver	Thailand	F	Ia	7	7	Delannoy et al., 2013	✔	✔		✔	✔	
STIR CD 22	Tilapia	liver	Thailand	G	Ia	7	7	Delannoy et al., 2013	✔	✔		✔	✔	
STIR CD 23	Tilapia	kidney	Thailand	H	Ia	7	7	Delannoy et al., 2013	✔	✔		✔	✔	
STIR CD 24	Tilapia	kidney	Thailand	I	Ia	7	7	Delannoy et al., 2013	✔	✔		✔	✔	
STIR CD 25	Tilapia	kidney	Thailand	J	III	283	283	Delannoy et al., 2013	✔	✔	✔	✔	✔	✔
STIR CD 26	Tilapia	kidney	Thailand	K	Ia	7	500	Delannoy et al., 2013	✔	✔		✔	✔	✔
STIR CD 27	Tilapia	kidney	Thailand	L	Ia	7	7	Delannoy et al., 2013	✔	✔		✔	✔	
STIR CD 28	Tilapia	kidney	Thailand	M	Ia	7	500	Delannoy et al., 2013	✔	✔		✔	✔	
MRI Z2-366	Tilapia	ND	Vietnam	MHH1	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔	✔	✔	✔	

Specimen ID	Host species	Source	Country of origin	Farm	Serotype	CC	ST	Reference	Experiments					
									Identification of bacteriocin producing strains	Spectrum of bacteriocin	*Galleria* challenge	Biolog assay	Hyaluronidase activity assay	*hyl*B gene
MRI Z2-367	Tilapia	ND	Vietnam	MHH1	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-368	Tilapia	ND	Vietnam	MHH1	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-369	Tilapia	ND	Vietnam	MHH1	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-370	Tilapia	ND	Vietnam	MHH1	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-371	Tilapia	ND	Vietnam	MHH1	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-372	Tilapia	ND	Vietnam	MHH1	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-373	Tilapia	ND	Vietnam	MHH1	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-374	Tilapia	ND	Vietnam	MHH1	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-375	Tilapia	ND	Vietnam	MHH2	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-376	Tilapia	ND	Vietnam	MHH2	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-377	Tilapia	ND	Vietnam	MHH2	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-378	Tilapia	ND	Vietnam	MHH2	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-379	Tilapia	ND	Vietnam	MHH3	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	✔
MRI Z2-380	Tilapia	ND	Vietnam	MHH3	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-381	Tilapia	ND	Vietnam	MHH4	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	

Specimen ID	Host species	Source	Country of origin	Farm	Serotype	CC	ST	Reference	Experiments					
									Identification of bacteriocin producing strains	Spectrum of bacteriocin	*Galleria* challenge	Biolog assay	Hyaluronidase activity assay	*hyl*B gene
MRI Z2-382	Tilapia	ND	Vietnam	MHH4	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-383	Tilapia	ND	Vietnam	MHH4	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-384	Tilapia	ND	Vietnam	MHH4	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	✔
MRI Z2-385	Tilapia	ND	Vietnam	MHH4	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-386	Tilapia	ND	Vietnam	MHH4	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-387	Tilapia	ND	Vietnam	MHH4	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-388	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-389	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-390	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	✔
MRI Z2-391	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-392	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔		✔	✔	
MRI Z2-393	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔			✔	
MRI Z2-394	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔				
MRI Z2-395	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔				
MRI Z2-396	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔				

Specimen ID	Host species	Source	Country of origin	Farm	Serotype	CC	ST	Reference	Experiments					
									Identification of bacteriocin producing strains	Spectrum of bacteriocin	*Galleria* challenge	Biolog assay	Hyaluronidase activity assay	*hyl*B gene
MRI Z2-397	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔				
MRI Z2-398	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔				
MRI Z2-399	Tilapia	ND	Vietnam	CT	III	283	283	Prof. Ruth N. Zadoks, unpublished	✔	✔	✔			

ND, not determined

Table A- 2 Other bacterial species used in evaluation of the spectrum of GBS bacteriocin.

Bacterial species	Specimen ID	Isolated from	Characteristics	Source or reference
Streptococcus dysgalactiae subsp. *dysgalactiae*	QMP Z3-611	Bovine mastitis	ND	Prof.Ruth N. Zadoks (University of Glasgow, UK)
Streptococcus dysgalactiae subsp. *dysgalactiae*	QMP Z3-820	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-184	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSLS3-189	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-243	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-257	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-368	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-376	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-470	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-477	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-516	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL Z3-088	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-204	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-210	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-215	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-290	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-294	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-317	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-409	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-420	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-455	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-472	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-476	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-478	Bovine mastitis	ND	Prof.Ruth N. Zadoks

Bacterial species	Specimen ID	Isolated from	Characteristics	Source or reference
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-482	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-521	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-522	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-539	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-540	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	FSL S3-541	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	QMP Z3-488	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	QMP Z3-580	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococcus dysgalactiae subsp. *dysgalactiae*	QMP Z3-854	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococus canis	FSL Z3-046	Bovine mastitis	ND	Richards et al., 2012
Streptococus canis	FSL Z3-048	Bovine mastitis	ND	Richards et al., 2012
Streptococus canis	FSL Z3-058	Bovine mastitis	ND	Richards et al., 2012
Streptococus canis	FSL Z3-116	Bovine mastitis	ND	Richards et al., 2012
Streptococus canis	FSL Z3-117	Bovine mastitis	ND	Richards et al., 2012
Streptococus canis	FSL Z3-121	Bovine mastitis	ND	Richards et al., 2012
Streptococus canis	FSL Z3-156	Bovine mastitis	ND	Richards et al., 2012
Streptococus canis	FSL Z3-158	Bovine mastitis	ND	Richards et al., 2012
Streptococus canis	FSL Z3-159	Bovine mastitis	ND	Richards et al., 2012
Streptococus uberis	FSL Z1-036	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococus uberis	FSL Z1-63	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococus uberis	FSL Z1-100	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococus uberis	FSL Z1-124	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococus uberis	FSL Z3-343	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococus uberis	FSL Z3-366	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococus uberis	QMP Z3-523	Bovine mastitis	ND	Prof.Ruth N. Zadoks

Bacterial species	Specimen ID	Isolated from	Characteristics	Source or reference
Streptococus uberis	QMP Z3-524	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococus uberis	QMP Z3-527	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Streptococus uberis	QMP Z3-567	Bovine mastitis	ND	Prof.Ruth N. Zadoks
Enterococcus faecalis	17M701947B	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecalis	17M212227S	ND	vancomycin sensitive	Prof.Daniel Walker
Enterococcus faecalis	17M659576E	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecalis	18M400091D	ND	vancomycin sensitive	Prof.Daniel Walker
Enterococcus faecalis	18M400202Y	ND	vancomycin sensitive	Prof.Daniel Walker
Enterococcus faecalis	18M203048B	ND	vancomycin sensitive	Prof.Daniel Walker
Enterococcus faecalis	18M400202Y	ND	vancomycin sensitive	Prof.Daniel Walker
Enterococcus faecium	17M691899C	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecium	17M690836D	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecium	17M689442T	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecium	17M709979D	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecium	17M712841V	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecium	17M652735R	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecium	17M643747J	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecium	17M661474S	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecium	18M606770J	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecium	18M607481L	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecium	102757	ND	vancomycin resistant	Prof.Daniel Walker
Enterococcus faecium	777245	ND	vancomycin resistant	Prof.Daniel Walker
Lactobacillus jensenii	LMG 06414	human, vaginal discharge	ND	BCCM, Gent, Belgium
Lactobacillus gasseri	LMG 13134	vaginal tract	ND	BCCM, Gent, Belgium
Lactobacillus crispatus	LMG 11440	pregnant woman, vagina	ND	BCCM, Gent, Belgium

ND, not determined

Table A- 3 Indicator strains used in determination of bacteriocin production.

Specimen ID	Host species	Source	Country of origin	Serotype	CC	ST
MRI Z2-093	human	UTI	Finland	III	17	17
MRI Z2-081	human	skin and soft tissue infection	Finland	Ia	23	23
MRI Z2-115	human	skin and soft tissue infection	Finland	VI	1	1
MRI Z1-710	bovine	mastitis	Denmark	NA	314	314
MRI Z1-050	bovine	mastitis	Denmark	Ia	103	103
MRI Z1-851	bovine	mastitis	Denmark	NA	196	196
MRI Z2-044	bovine	mastitis	Finland	Ia	103	103
MRI Z2-198	bovine	mastitis	UK	NA	67	67
MRI Z2-007	bovine	mastitis	Finland	NA	103	103
MRI Z1-872	bovine	mastitis	Denmark	NA	314	314
MRI Z1-363	bovine	mastitis	Denmark	III	17	17
MRI Z2-001	bovine	mastitis	Finland	III	632	632
STIR CD 01	Mullet	brain	Kuwait	Ia	7	7
STIR CD 07	Tilapia	heart	Honduras	Ib	552	260
STIR CD 09	Tilapia	kidney	Columbia	Ib	552	260
STIR CD 13	Tilapia	eye	Costa Rica	Ib	552	260
STIR CD 17	Tilapia	heart	Honduras	Ib	552	260
STIR CD 21	Tilapia	liver	Thailand	Ia	7	7
STIR CD 23	Tilapia	kidney	Thailand	Ia	7	7
MRI Z2-384	Tilapia	ND	Vietnam	III	283	283
MRI Z2-358	Tilapia	ND	Vietnam	III	283	283
MRI Z2-391	Tilapia	ND	Vietnam	III	283	283
MRI Z2-392	Tilapia	ND	Vietnam	III	283	283
MRI Z2-399	Tilapia	ND	Vietnam	III	283	283

Appendix ii

Antimicrobial susceptibility of *Streptococcus agalactiae* (section 3.3.4, 3.3.5, 4.3.3 and 4.3.4)

Table A- 4 Antimicrobial susceptibility of *Streptococcus agalactiae* isolates based on zone diameter (mm) as measured by the disc diffusion method and interpreted based on criteria for human isolates defined by the Clinical and Laboratory Standard Institute (CLSI).

Specimen ID	Host species	Penicillin G	Ampicillin	Erythromycin	Clindamycin	Tetracycline	Ceftriaxone
MRI Z1-050	bovine	31	31	26	24	13 (R)[a]	29
MRI Z1-158	bovine	ND	ND	ND	ND	ND	ND
MRI Z1-354	bovine	30	31	25	23	13 (R)	27
MRI Z1-363	bovine	28	27	24	21	25	27
MRI Z1-586	bovine	30	28	15 (R)	6 (R)	12 (R)	29
MRI Z1-597	bovine	29	28	25	22	15 (R)	29
MRI Z1-600	bovine	ND	ND	ND	ND	ND	ND
MRI Z1-707	bovine	29	29	28	25	12 (R)	32
MRI Z1-710	bovine	31	30	26	25	14 (R)	30
MRI Z1-715	bovine	31	30	29	28	28	32
MRI Z1-717	bovine	ND	ND	ND	ND	ND	ND
MRI Z1-719	bovine	30	31	24	21	24	30
MRI Z1-803	bovine	31	31	6 (R)	6 (R)	13 (R)	32
MRI Z1-808	bovine	ND	ND	ND	ND	ND	ND
MRI Z1-811	bovine	30	31	26	23	17 (R)	31
MRI Z1-822	bovine	33	37	27	26	27	35
MRI Z1-851	bovine	ND	ND	ND	ND	ND	ND
MRI Z1-858	bovine	ND	ND	ND	ND	ND	ND
MRI Z1-872	bovine	ND	ND	ND	ND	ND	ND
MRI Z2-001	bovine	30	31	26	24	11 (R)	30
MRI Z2-002	bovine	ND	ND	ND	ND	ND	ND
MRI Z2-005	bovine	33	32	27	24	11 (R)	33
MRI Z2-007	bovine	ND	ND	ND	ND	ND	ND
MRI Z2-041	bovine	31	29	23	20	12 (R)	30
MRI Z2-044	bovine	29	30	24	21	11 (R)	30
MRI Z2-045	bovine	28	28	24	21	12 (R)	26
MRI Z2-053	bovine	29	30	24	21	13 (R)	29
MRI Z2-060	bovine	ND	ND	ND	ND	ND	ND
MRI Z2-062	bovine	29	30	24	22	11 (R)	29
MRI Z2-065	bovine	29	29	25	22	11 (R)	30
MRI Z2-077	human	28	28	23	20	12 (R)	27
MRI Z2-081	human	28	30	24	21	15 (R)	28
MRI Z2-082	human	29	28	24	20	12 (R)	28
MRI Z2-089	human	29	30	25	22	12 (R)	29
MRI Z2-093	human	29	29	25	22	12 (R)	29
MRI Z2-098	human	30	27	25	22	13 (R)	30

Specimen ID	Host species	Penicillin G	Ampicillin	Erythromycin	Clindamycin	Tetracycline	Ceftriaxone
MRI Z2-101	human	30	30	14 (R)	11 (R)	16 (R)	29
MRI Z2-102	human	29	30	13 (R)	12 (R)	23	29
MRI Z2-103	human	ND	ND	ND	ND	ND	ND
MRI Z2-106	human	ND	ND	ND	ND	ND	ND
MRI Z2-115	human	25	27	22	20	24	25
MRI Z2-120	human	ND	ND	ND	ND	ND	ND
MRI Z2-121	human	ND	ND	ND	ND	ND	ND
MRI Z2-126	human	29	29	26	22	13 (R)	28
MRI Z2-130	human	28	29	25	21	26	29
MRI Z2-132	human	27	26	24	23	13 (R)	28
MRI Z2-137	human	29	27	27	22	12 (R)	29
MRI Z2-197	bovine	ND	ND	ND	ND	ND	ND
MRI Z2-198	bovine	31	31	27	25	27	31
MRI Z2-200	bovine	ND	ND	ND	ND	ND	ND
MRI Z2-202	bovine	34	34	27	23	27	33
MRI Z2-187	human	27	27	23	12 (R)	13 (R)	27
MRI Z2-189	human	24	27	15 (R)	19	23	27
MRI Z2-058	bovine	27	29	23	20	24	27
MRI Z1-890	bovine	26	27	23	21	24	28
STIR CD 01	Mullet	27	24	26	22	24	26
STIR CD 02	Mullet	26	**23**	24	21	23	27
STIR CD 03	Mullet	26	24	25	21	24	25
STIR CD 04	Mullet	26	24	24	21	23	27
STIR CD 05	Mullet	25	**23**	24	22	24	25
STIR CD 07	Tilapia	33	33	31	29	26	34
STIR CD 09	Tilapia	35	35	34	30	32	37
STIR CD 10	Tilapia	32	33	28	28	28	33
STIR CD 11	Tilapia	33	33	31	29	27	33
STIR CD 12	Tilapia	34	34	31	28	32	33
STIR CD 13	Tilapia	35	35	31	28	30	34
STIR CD 14	Tilapia	28	27	26	22	12 (R)	26
STIR CD 17	Tilapia	35	33	32	27	29	33
STIR CD 18	Tilapia	34	34	30	28	26	33
STIR CD 19	Tilapia	33	32	31	27	26	33
STIR CD 21	Tilapia	25	25	24	22	23	27
STIR CD 22	Tilapia	24	24	24	21	23	23
STIR CD 23	Tilapia	26	26	24	22	24	26
STIR CD 24	Tilapia	26	26	25	21	25	26
STIR CD 25	Tilapia	29	28	27	24	11 (R)	28
STIR CD 26	Tilapia	26	26	24	22	23	25
STIR CD 27	Tilapia	25	25	25	21	24	25
STIR CD 28	Tilapia	26	26	24	22	23	25
MRI Z2-366	Tilapia	27	27	24	22	12 (R)	26
MRI Z2-367	Tilapia	27	27	25	20	11 (R)	26
MRI Z2-368	Tilapia	26	27	26	23	12 (R)	26
MRI Z2-369	Tilapia	26	27	25	22	11 (R)	26

Specimen ID	Host species	Penicillin G	Ampicillin	Erythromycin	Clindamycin	Tetracycline	Ceftriaxone
MRI Z2-370	Tilapia	27	27	24	23	11 (R)	27
MRI Z2-371	Tilapia	28	26	26	22	12 (R)	26
MRI Z2-372	Tilapia	28	27	25	22	12 (R)	25
MRI Z2-373	Tilapia	28	28	24	22	11 (R)	26
MRI Z2-374	Tilapia	28	27	23	21	11 (R)	26
MRI Z2-375	Tilapia	27	26	26	22	11 (R)	27
MRI Z2-376	Tilapia	28	26	25	21	12 (R)	27
MRI Z2-377	Tilapia	26	26	24	22	12 (R)	26
MRI Z2-378	Tilapia	28	27	24	23	12 (R)	27
MRI Z2-379	Tilapia	26	25	24	20	11 (R)	25
MRI Z2-380	Tilapia	27	27	26	22	11 (R)	27
MRI Z2-381	Tilapia	27	27	26	22	12 (R)	26
MRI Z2-382	Tilapia	28	29	27	23	12 (R)	28
MRI Z2-383	Tilapia	27	26	25	23	12 (R)	27
MRI Z2-384	Tilapia	27	28	25	22	12 (R)	27
MRI Z2-385	Tilapia	26	27	26	22	12 (R)	28
MRI Z2-386	Tilapia	28	30	26	22	12 (R)	30
MRI Z2-387	Tilapia	27	26	26	23	11 (R)	26
MRI Z2-388	Tilapia	27	26	25	22	11 (R)	27
MRI Z2-389	Tilapia	29	28	27	23	12 (R)	28
MRI Z2-390	Tilapia	27	27	24	21	12 (R)	25
MRI Z2-391	Tilapia	27	26	24	23	11 (R)	26
MRI Z2-392	Tilapia	28	28	24	21	11 (R)	26
MRI Z2-393	Tilapia	27	27	25	22	11 (R)	27
MRI Z2-394	Tilapia	26	27	24	21	12 (R)	28
MRI Z2-395	Tilapia	26	27	26	22	12 (R)	25
MRI Z2-396	Tilapia	26	26	25	22	11 (R)	26
MRI Z2-397	Tilapia	26	26	24	21	12 (R)	26
MRI Z2-398	Tilapia	26	25	24	21	12 (R)	24
MRI Z2-399	Tilapia	27	25	25	22	12 (R)	25

[a](R) means resistance. For interpretative criteria, see section 2.2.2,Table 2-1.
ND, not determined

Appendix iii

The DNA sequence of the agalacticin A gene used for plasmid construction (section 2.4.1)

AGGAGGTAAAACATATGGACACCTACGTTCGCCCGATTGACAATGGCCGCATCACGACTGGTTT
TAATGGTTATCCGGGTCATTGCGGCGTTGATTACGCAGTACCAACCGGCACGATTATTCGCGCG
GTGGCAGACGGCACGGTCAAGTTTGCGGGTGCAGGCGCGAACTTCTCTTGGATGACCGATCTGG
CTGGTAACTGTGTTATGATCCAACATGCCGATGGCATGCATAGCGGTTACGCGCACATGTCCCG
TGTTGTGGCGCGTACCGGTGAAAAAGTCAAACAGGGCGACATTATTGGTTATGTGGGTGCAACC
GGTATGGCGACGGGTCCGCACTTGCACTTTGAGTTCCTGCCGGCTAACCCGAATTTTCAGAACG
GTTTCCACGGCCGTATCAATCCGACGAGCCTGATCGCGAATGTTGCTACCTTTAGCGGTAAGAC
TCAGGCGAGCGCGCCTAGCATCAAGCCGCTGCAGTCGGCCCCGGTGCAGAACCAGAGCAGCAA
ACTGAAAGTGTATCGTGTCGACGAACTGCAAAAAGTGAACGGTGTCTGGCTGGTTAAGAACAA
TACCTTGACGCCGACCGGTTTCGACTGGAACGATAACGGCATTCCGGCGAGCGAGATCGATGA
AGTCGACGCAAACGGTAACCTGACCGCCGATCAAGTGCTGCAAAAGGGCGGCTACTTCATTTTC
AATCCGAAAACCCTGAAAACGGTCGAGAAGCCAATCCAGGGTACGGCAGGCCTGACCTGGGCC
AAGACCCGTTTCGCGAATGGTAGCAGCGTTTGGCTGCGTGTTGATAATTCCCAAGAACTGCTGT
ACAAAGAGCACCACCACCACCACCACTGA

The protein sequence of agalacticin (section 2.4.4)

MDTYVRPIDNGRITTGFNGYPGHCGVDYAVPTGTIIRAVADGTVKFAGAGANFSWMTDLAGNCVM
IQHADGMHSGYAHMSRVVARTGEKVKQGDIIGYVGATGMATGPHLHFEFLPANPNFQNGFHGRIN
PTSLIANVATFSGKTQASAPSIKPLQSAPVQNQSSKLKVYRVDELQKVNGVWLVKNNTLTPTGFDW
NDNGIPASEIDEVDANGNLTADQVLQKGGYFIFNPKTLKTVEKPIQGTAGLTWAKTRFANGSSVWL
RVDNSQELLYKEHHHHHH

Appendix iv

Use of agalacticin A in combination with broad spectrum antibiotics (section 4.3.4)

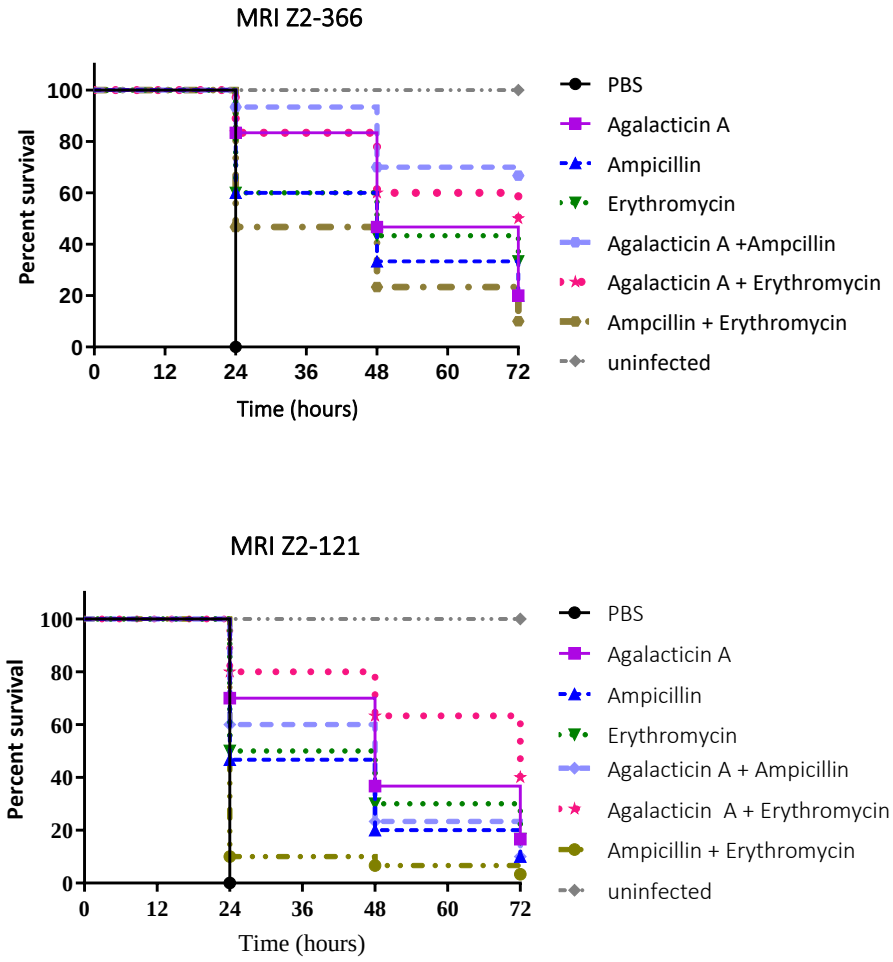


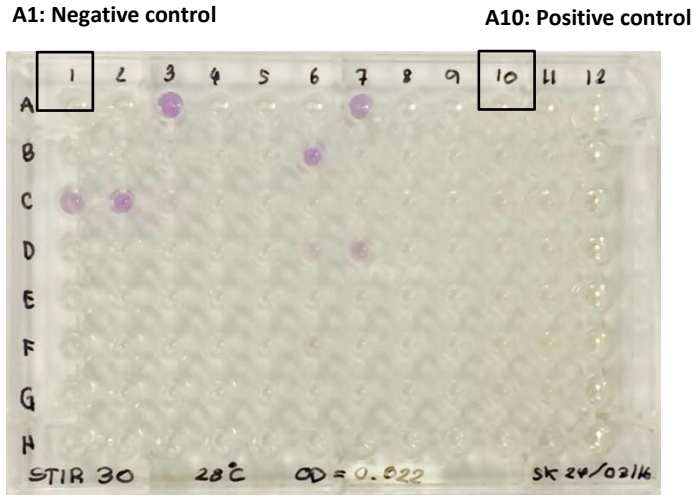
Figure A-1 Survival of *Galleria mellonella* larvae in a Comparison of single agent and combinations of agalacticin or antibiotic for treatment of group B *Streptococcus* infection in section 4.3.4.

Hypervirulent strains ST283 (MRI Z2-366) and ST17 (MRI Z2-121) were used to challenge 10 larvae per treatment group. A single agent and combined agents were administered 2 h post challenge. Three experiments were performed for each isolate. Survival was observed at 24, 48 and 72 h post challenge. Treating with a single agent, MRI Z2-366 challenged groups have median survival at 48 h for all single agents but in MRI Z2-121 were variable as 48 h of agalacticin A, 24 h of ampicillin and 36 h of erythromycin. The combined agents of agalacticin A to antibiotic provided longer median survival as 72 h for MRI Z2-366 and 48 h for MRI Z2-121 treated with agalacticin A + ampicillin and at 72 h for agalacticin A + erythromycin treatment in both strains.

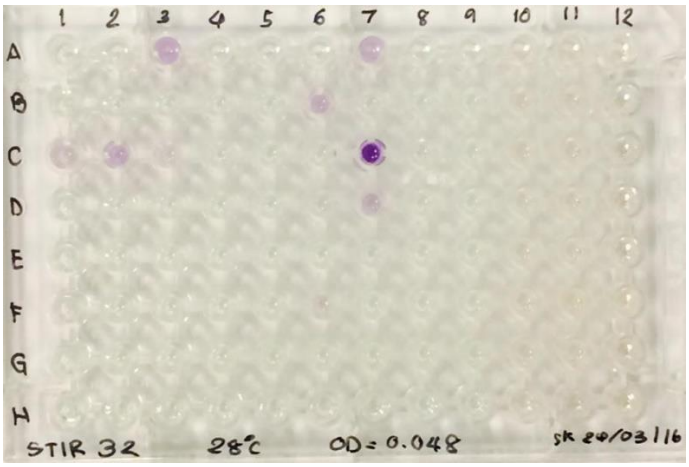
Appendix v

Preliminary testing of CC552 on Biolog GEN III (Chapter 5)

CC552 was excluded from the study on carbon utilization using Biolog GEN III. Preliminary tests using STIR-CD-30 and STIR-CD-32 grown at 28°C for 48 h failed to detect growth of those fish-specific strains in the positive control well. Because locus 3 is also present in CC283 and CC7 (Delannoy et al., 2016), exclusion of CC552 does not jeopardize the detection of locus 3-dependent phenotypes.



STIR- CD-30



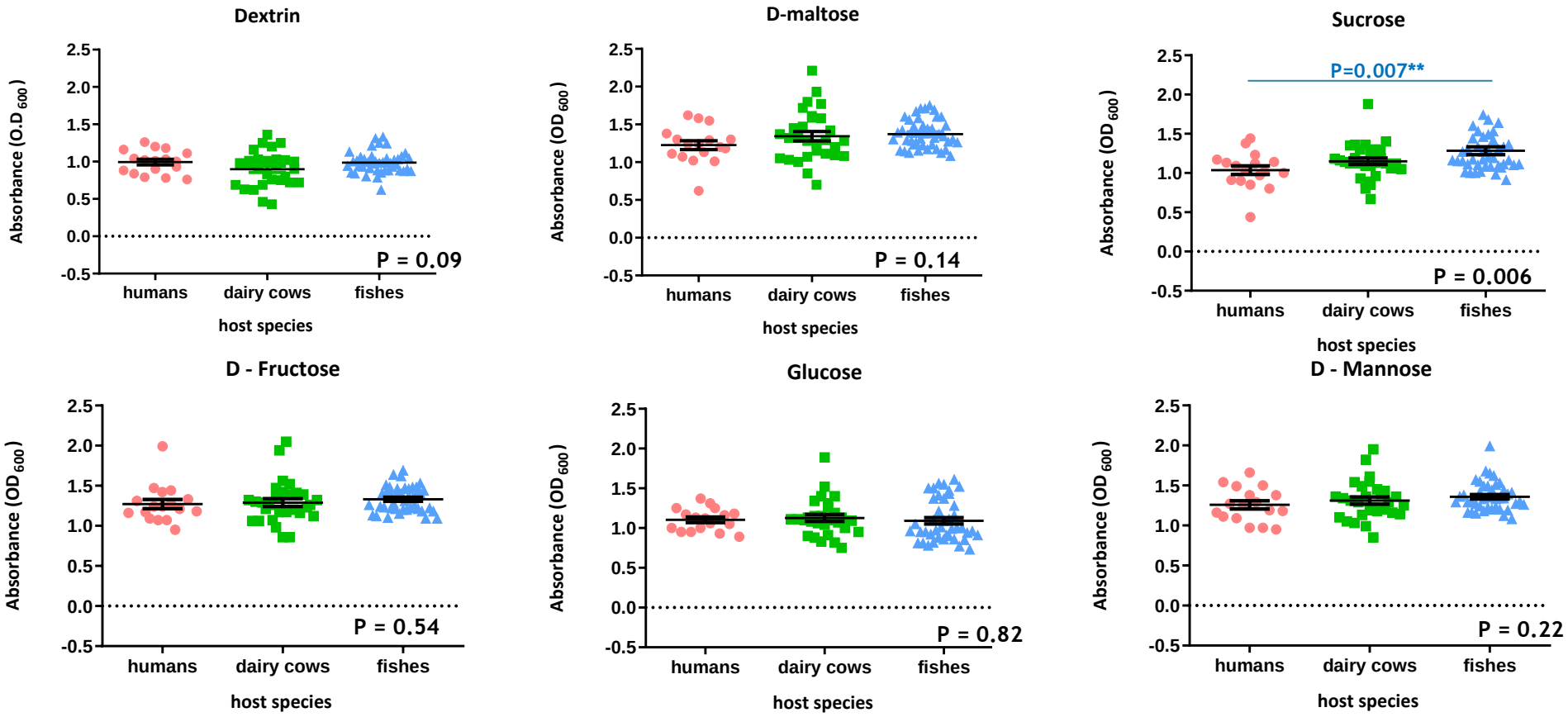
STIR- CD-32

Detecting carbon utilization of poikilothermic group B *Streptococcus* (GBS) on Biolog GEN III

Tetrazolium redox dye (purple) indicated the GBS metabolic activity in the presence of various carbon sources or chemical tests; A1, negative control and A10, positive control. Testing of a *Streptococcus agalactiae* isolate from clonal complex 552 on Biolog GEN III system showed failure to grow in the positive control well after 48 hrs at 28°C.

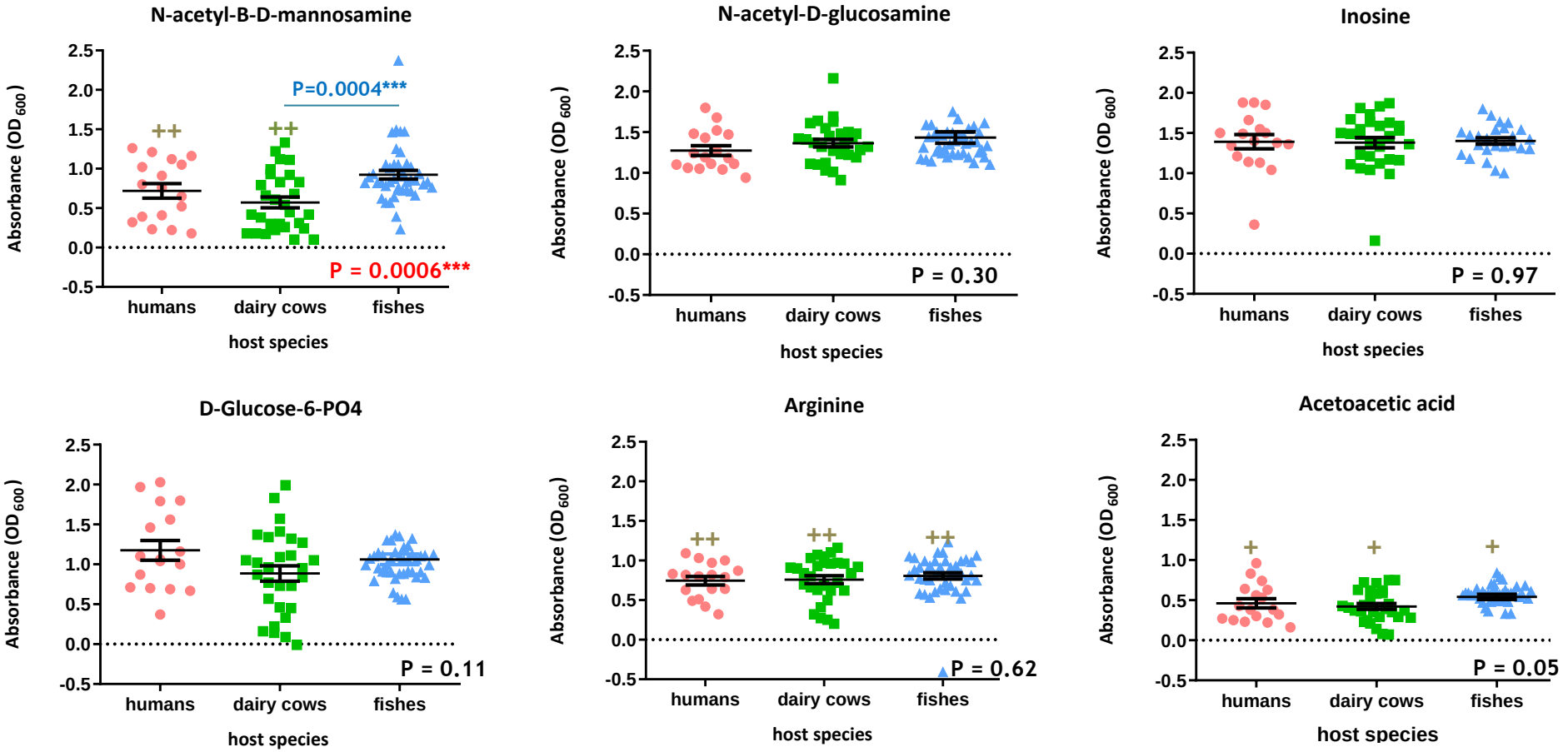
Appendix vi

Streptococcus agalactiae's metabolic profiling using Biolog GENIII system: high utilization (section 5.3.1)



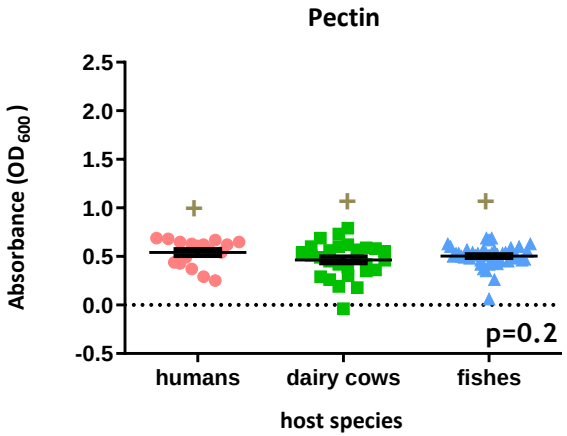
Numbers present p-value using One Way ANOVA in comparison of three host species. P value above plots are from multicomparison between two host species.

S. agalactiae's metabolic profiling using Biolog GENIII system: high to moderate utilization (section 5.3.1)

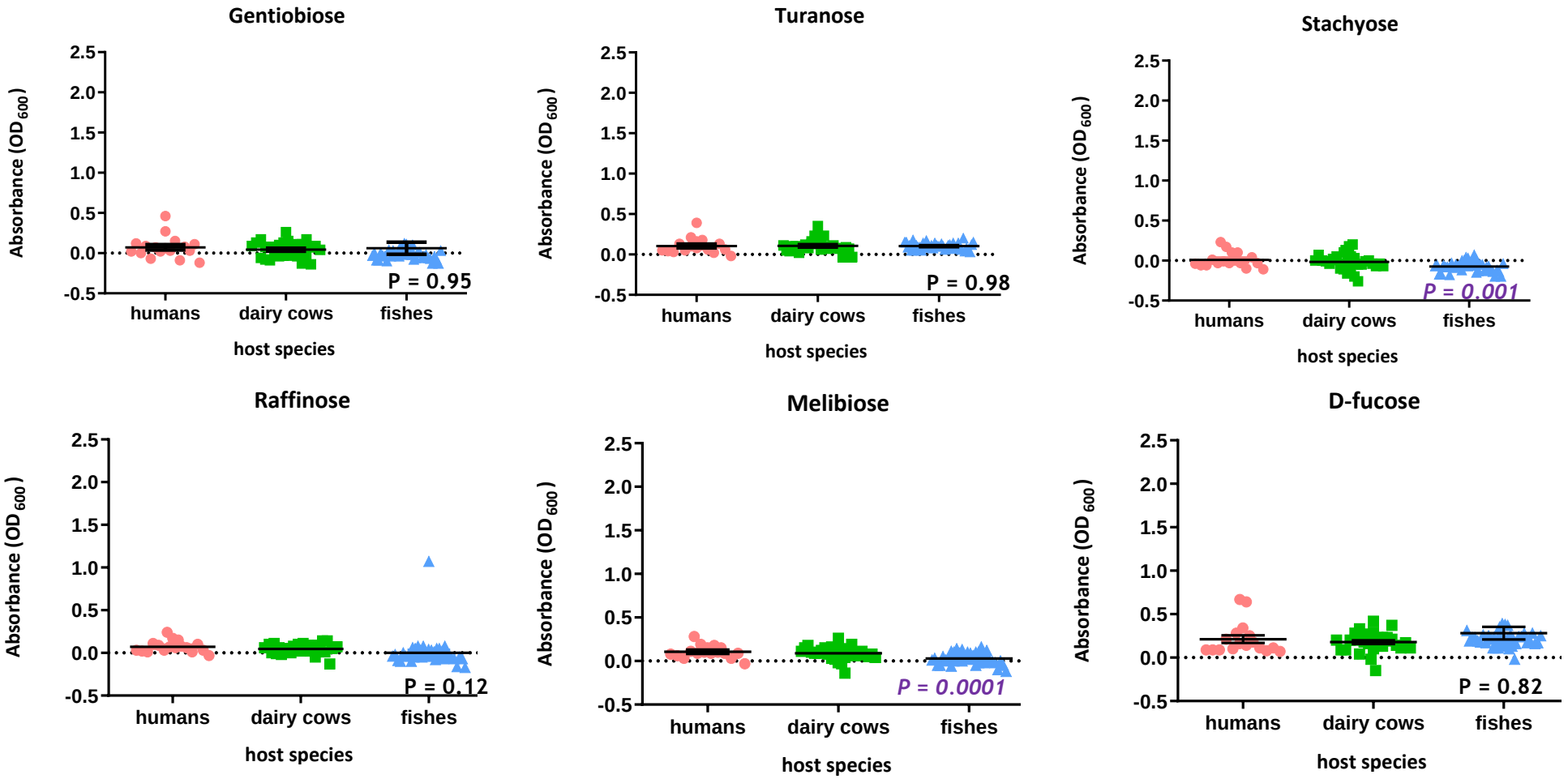


These compounds are high median utilization but many individual isolates show low use. Median utilisation is moderate for N-acetyl-B-D-manosamine and arginine and is low for acetoacetic acid and pectin pectin. ++ means moderate utilization (median between 0.5 and 1.0) and + means low utilization (median around or below 0.5).

S. agalactiae's metabolic profiling using Biolog GENIII system: high to moderate utilization (continued)

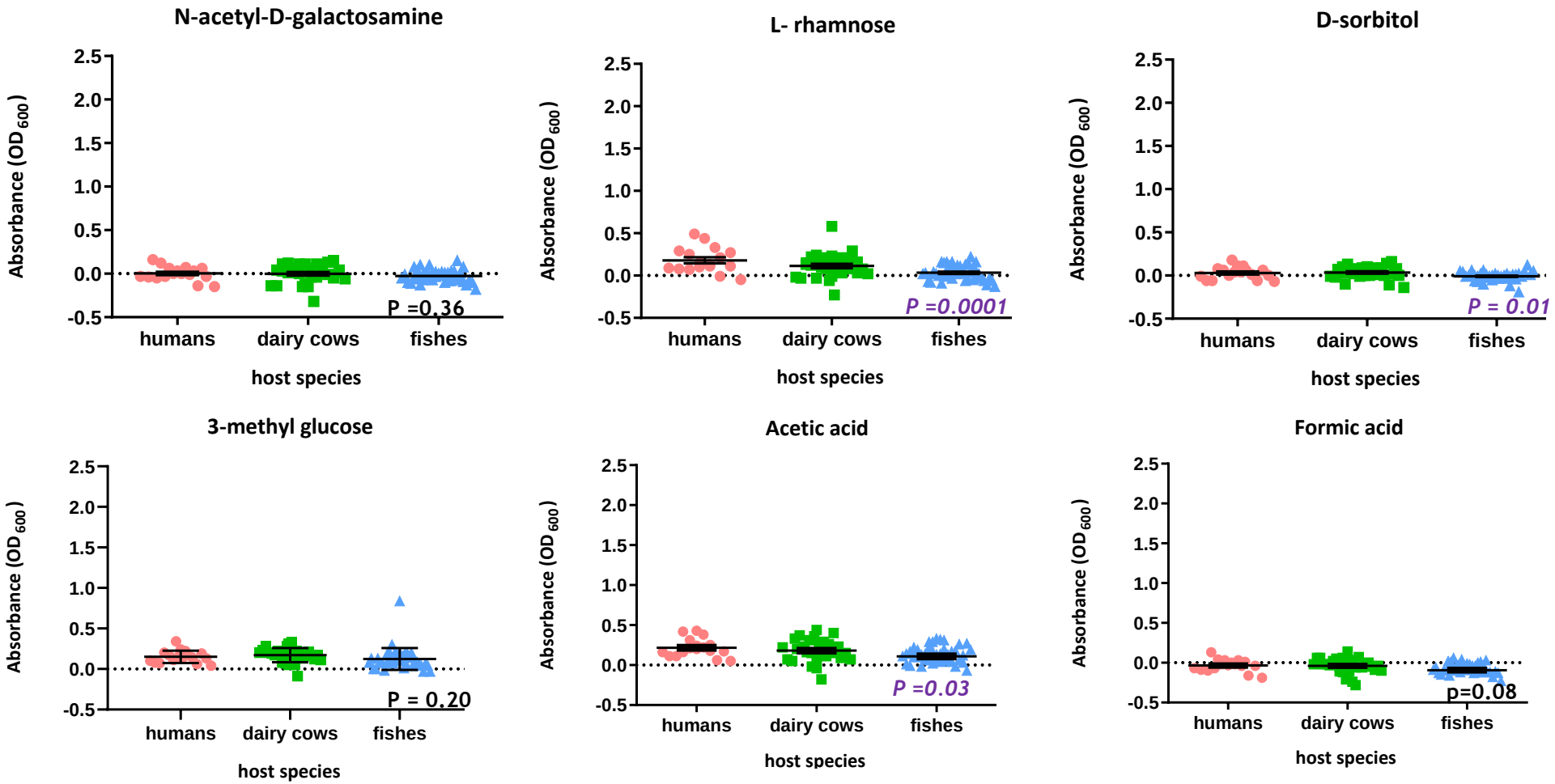


S. agalactiae's metabolic profiling using Biolog GENIII system: low utilization (section 5.3.1)



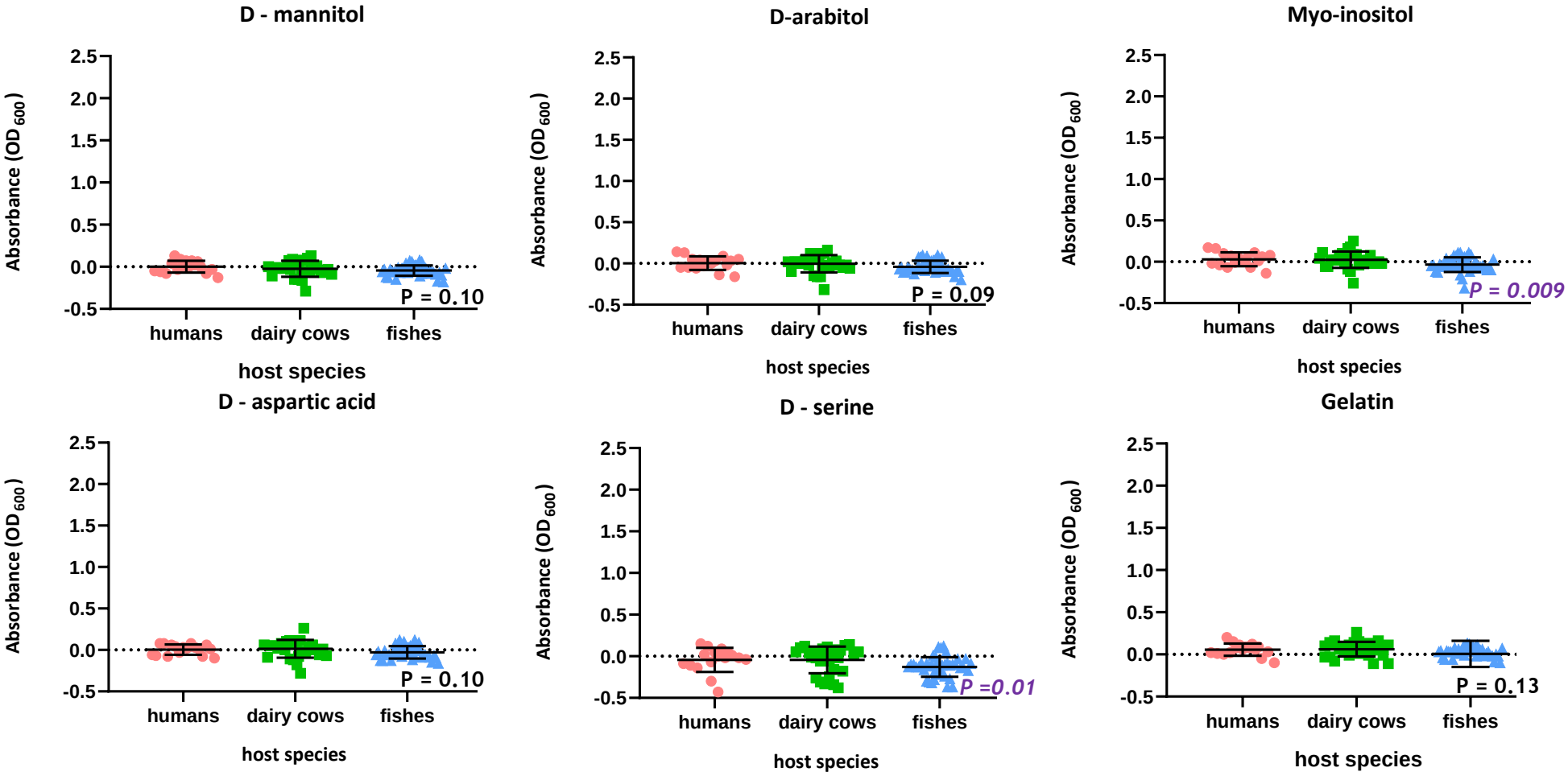
The italic P-value (purple) means that statistical significance does not necessarily mean biological significance due to all of them being non-users.

S. agalactiae's metabolic profiling using Biolog GENIII system: low utilization (continued)



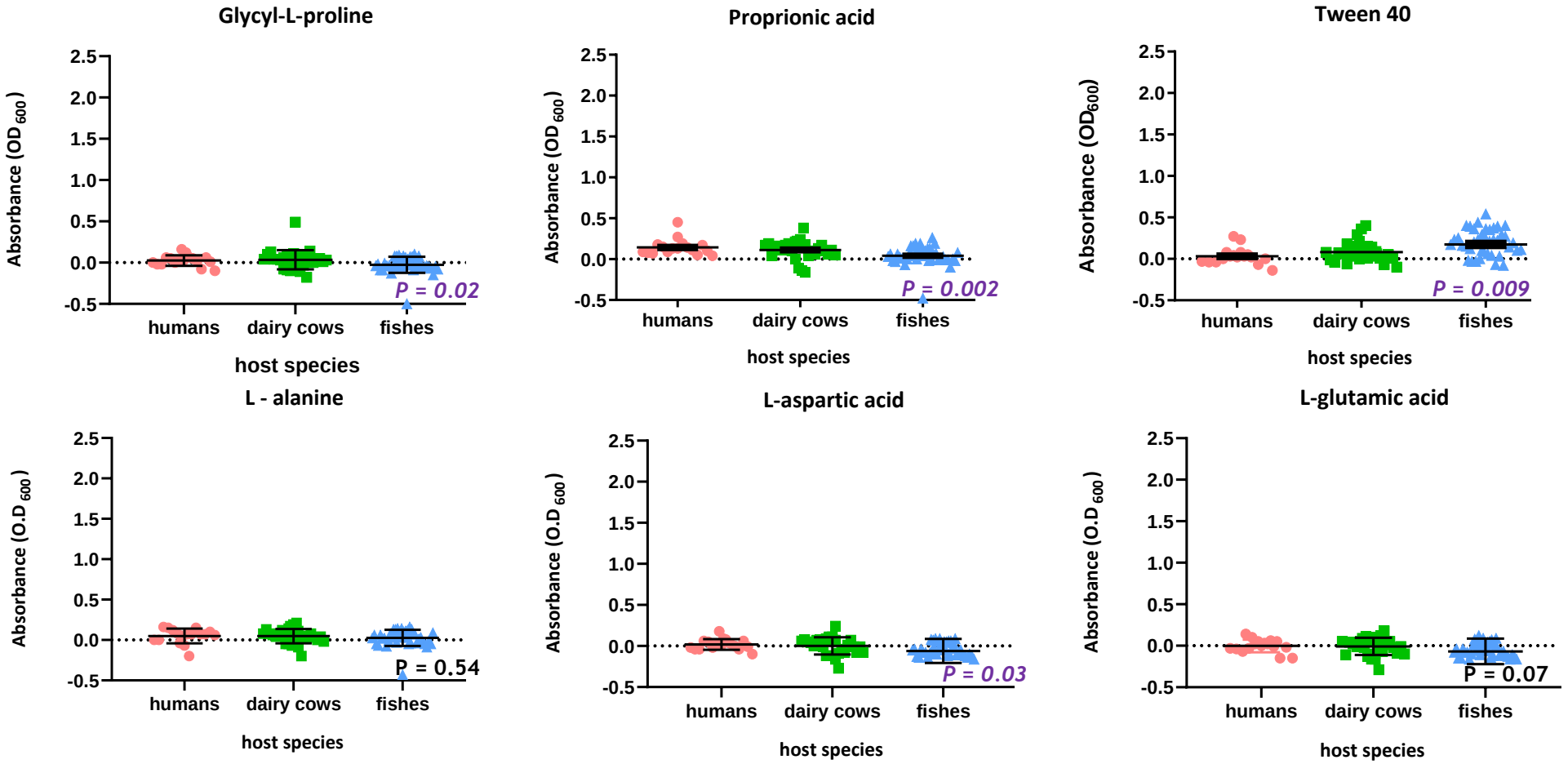
The italic P-value (purple) means that statistical significance does not necessarily mean biological significance due to all of them being non-users.

S. agalactiae's metabolic profiling using Biolog GENIII system: low utilization (continued)



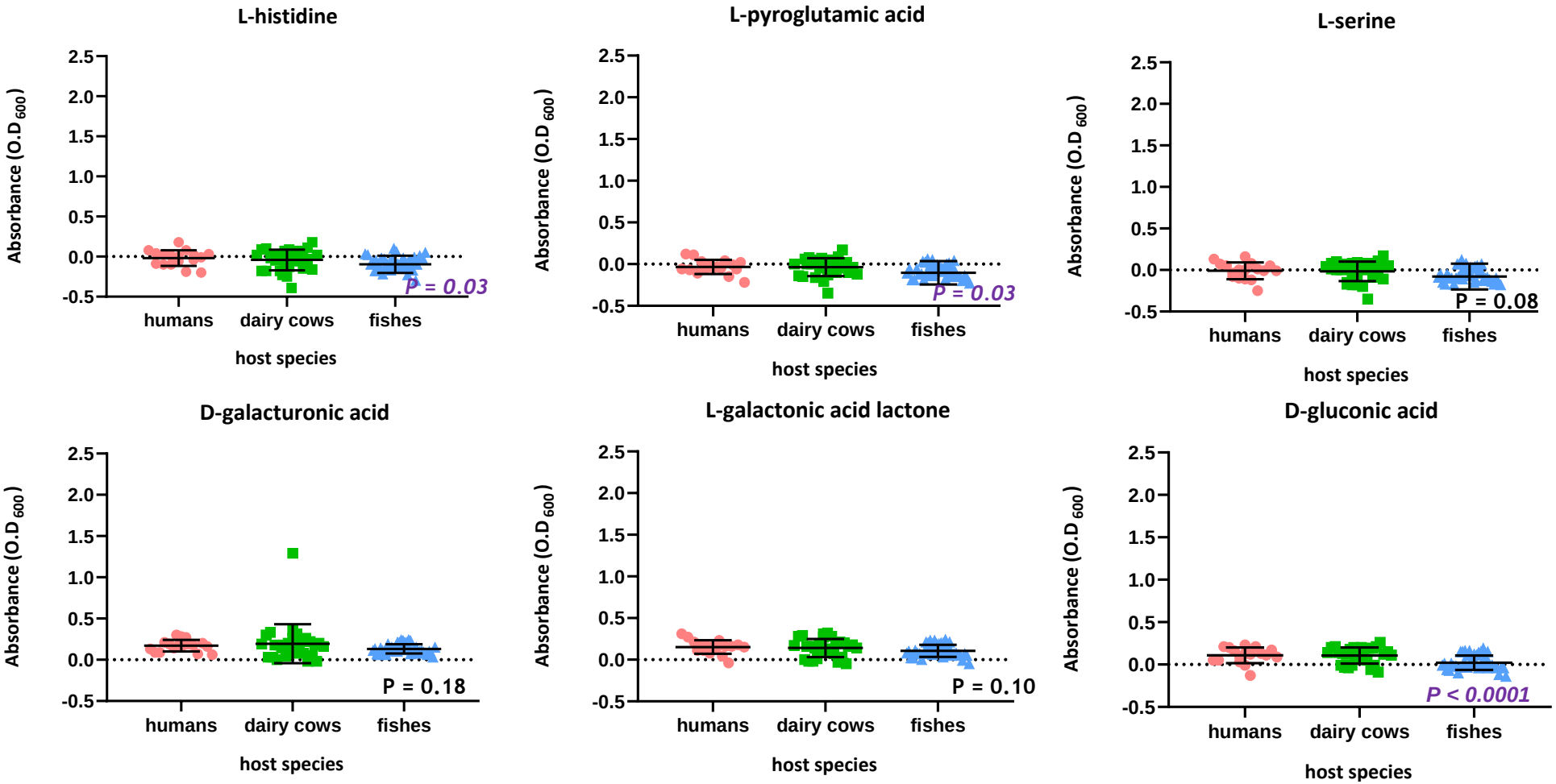
The italic P-value (purple) means that statistical significance does not necessarily mean biological significance due to all of them being non-users.

S. agalactiae's metabolic profiling using Biolog GENIII system: low utilization (continued)



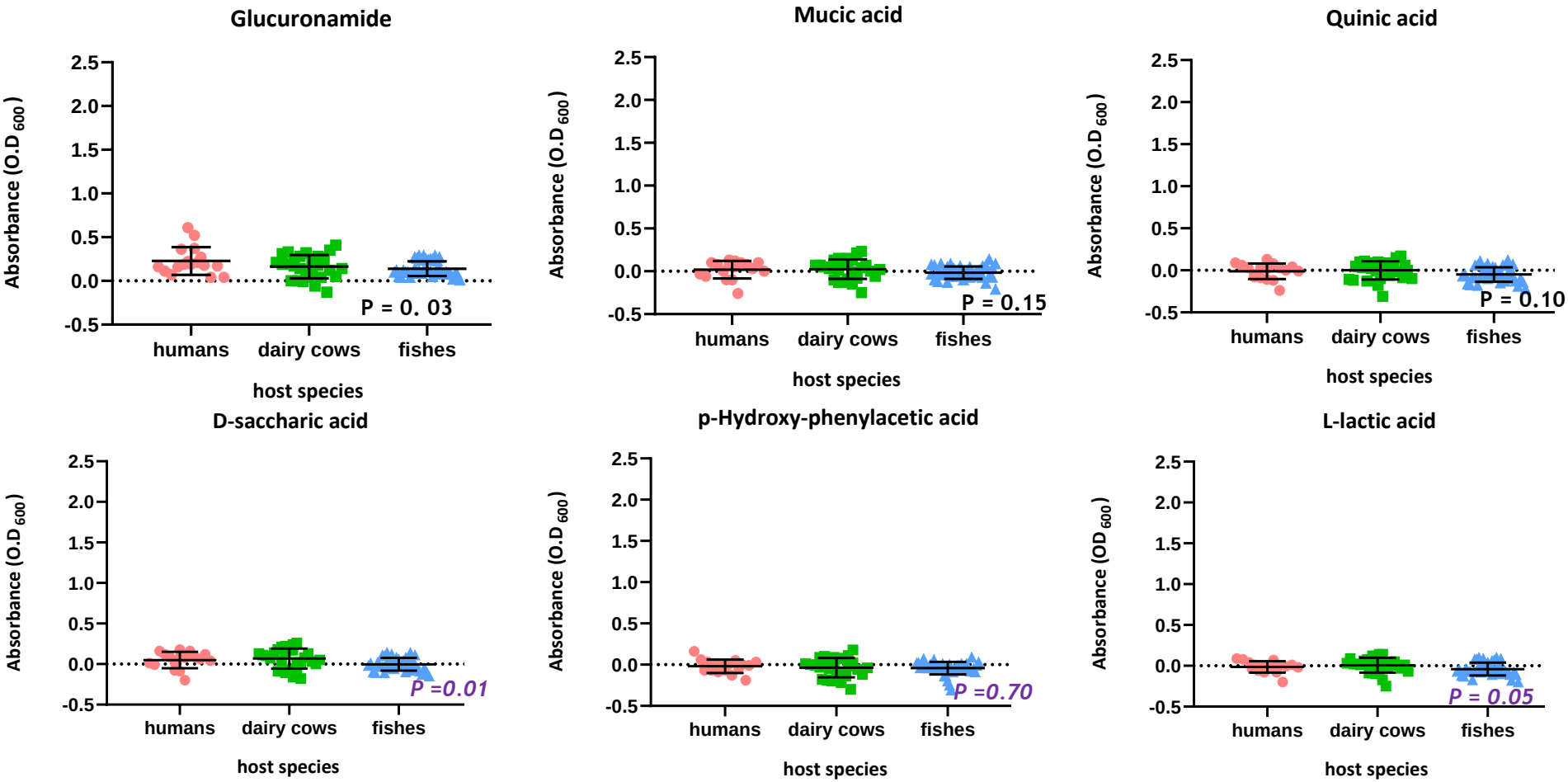
The italic P-value (purple) means that statistical significance does not necessarily mean biological significance due to all of them being non-users.

S. agalactiae's metabolic profiling using Biolog GENIII system: low utilization (continued)



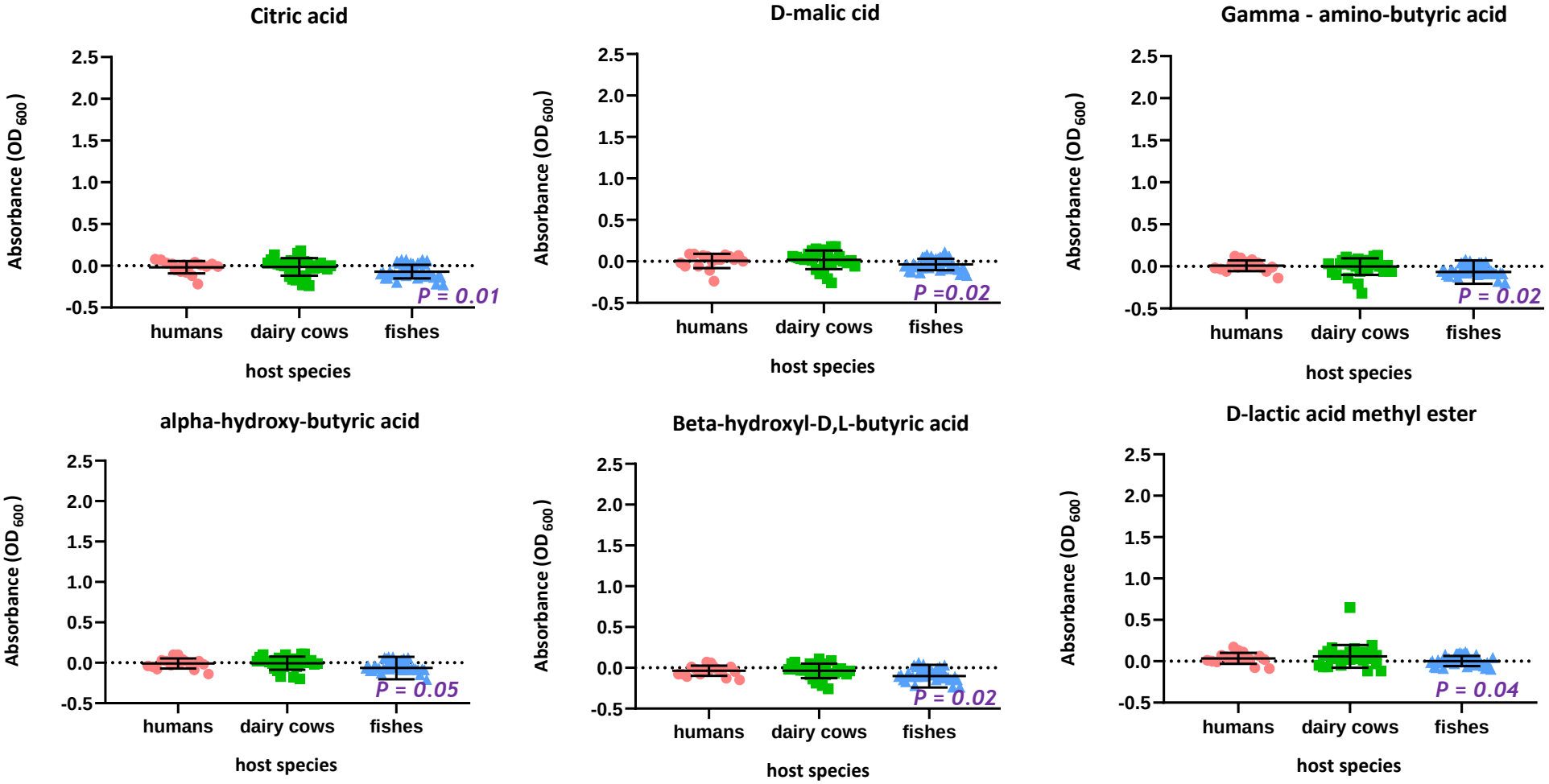
The italic P-value (purple) means that statistical significance does not necessarily mean biological significance due to all of them being non-users.

S. agalactiae's metabolic profiling using Biolog GENIII system: low utilization (continued)



The italic P-value (purple) means that statistical significance does not necessarily mean biological significance due to all of them being non-users.

S. agalactiae's metabolic profiling using Biolog GENIII system: low utilization (continued)



The italic P-value (purple) means that statistical significance does not necessarily mean biological significance due to all of them being non-users.

Appendix vii

Hyaluronidase activity assay and salicin utilization (section 6.3.3)

Table A- 5 Ability of Group B *Streptococcus* in salicin utilization and hyaluronidase activity

sample ID	host	serotype	CC[a]	ST[b]	salicin (OD_{600})	Hyaluronidase test (average diameter; mm)[d]
MRI Z1-808	Bovine	ND[c]	1	1	1.27	16.33
MRI Z2-062	Bovine	V	1	1	1.25	0.00
MRI Z1-707	Bovine	ND	1	1	1.12	15.00
MRI Z2-115	human	VI	1	1	2.14	6.00
MRI Z2-089	human	II	1	1	1.53	0.00
MRI Z2-077	human	V	1	462	1.19	16.67
MRI Z1-354	bovine	Ia	7	7	1.76	16.67
MRI Z1-586	bovine	ND	7	7	1.53	0.00
STIR-CD-02	fish	Ia	7	7	2.01	15.33
STIR-CD-04	fish	Ia	7	7	1.77	15.33
STIR-CD-24	fish	Ia	7	7	1.76	10.00
STIR-CD-01	fish	Ia	7	7	1.75	14.33
STIR-CD-05	fish	Ia	7	7	1.67	14.67
STIR-CD-03	fish	Ia	7	7	1.65	16.33
STIR-CD-21	fish	Ia	7	7	1.64	14.00
STIR-CD-27	fish	Ia	7	7	1.61	13.67
STIR-CD-28	fish	Ia	7	500	1.61	13.33
STIR-CD-23	fish	Ia	7	7	1.58	15.33
STIR-CD-22	fish	Ia	7	7	1.48	15.00
STIR-CD-26	fish	Ia	7	500	1.46	16.33
MRI Z2-045	bovine	Ib	8	8	1.42	12.67
MRI Z2-041	bovine	Ib	8	8	1.40	14.00
MRI Z2-053	bovine	Ib	8	8	1.19	14.00
MRI Z2-082	human	Ib	8	8	1.81	14.00
MRI Z2-137	human	Ib	8	8	1.52	13.00
MRI Z2-189	human	Ib	8	8	1.42	12.33
MRI Z2-187	human	Ib	8	8	1.21	13.33
MRI Z1-363	bovine	III	17	17	0.38	14.33
MRI Z2-121	human	III	17	17	0.25	16.33
MRI Z2-132	human	III	17	17	0.00	17.33
MRI Z2-093	human	III	17	17	-0.02	15.00
MRI Z1-597	bovine	ND	19	19	1.73	0.00
MRI Z1-811	bovine	ND	19	19	1.37	0.00
MRI Z2-101	human	III	19	19	1.59	0.00
MRI Z2-102	human	III	19	19	1.32	0.00
MRI Z1-158	bovine	Ia	23	23	1.72	16.67
MRI Z1-600	bovine	ND	23	23	1.58	15.00
MRI Z1-719	bovine	ND	23	23	1.26	9.67

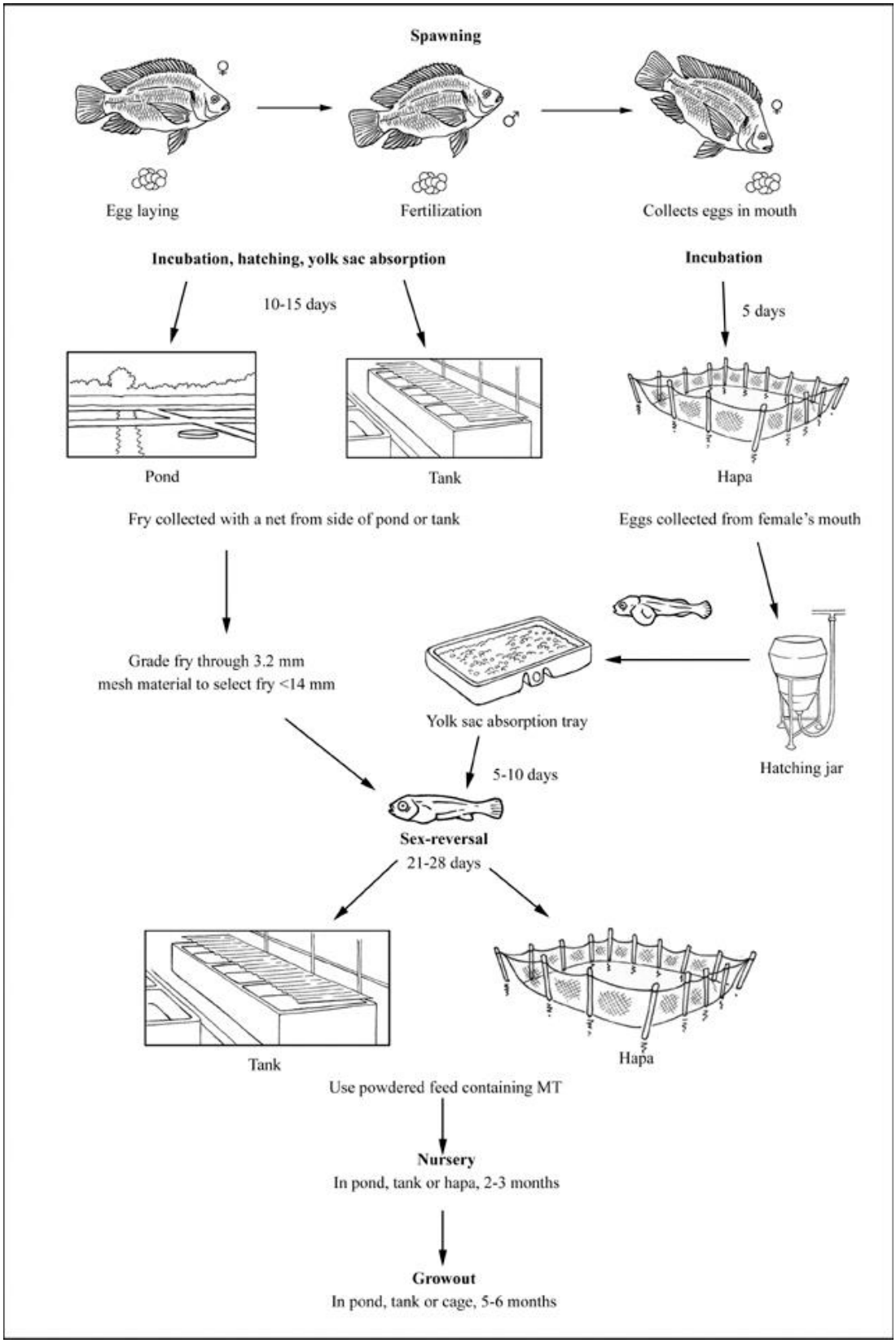
sample ID	host	serotype	CC[a]	ST[b]	salicin (OD_{600})	Hyaluronidase test (average diameter; mm)[d]
MRI Z1-822	bovine	ND	23	23	0.26	6.33
MRI Z2-103	human	Ia	23	23	1.77	15.33
MRI Z2-081	human	Ia	23	23	1.76	15.67
MRI Z2-126	human	Ia	23	23	1.47	15.67
MRI Z2-202	bovine	ND	67	67	1.03	13.33
MRI Z2-198	bovine	ND	67	67	0.20	13.00
MRI Z1-858	bovine	ND	103	314	1.88	14.67
MRI Z1-710	bovine	ND	103	314	1.84	12.67
MRI Z1-803	bovine	ND	103	314	1.55	15.00
MRI Z1-715	bovine	ND	103	103	1.50	14.33
MRI Z2-007	bovine	ND	103	103	1.32	16.67
MRI Z2-044	bovine	Ia	103	103	1.31	15.33
MRI Z1-050	bovine	Ia	103	103	0.12	13.00
MRI Z1-890	bovine	ND	196	196	1.74	18.00
MRI Z2-065	bovine	IV	196	196	1.36	16.33
MRI Z2-098	human	IV	196	196	1.36	12.67
MRI Z2-130	human	IV	196	196	1.25	15.00
MRI Z2-001	bovine	III	632	632	1.30	9.67
MRI Z2-005	bovine	ND	632	632	1.07	9.33
MRI Z2-384	fish	III	283	283	0.93	17.33
STIR-CD-25	fish	III	283	283	0.26	15.33
STIR-CD-14	fish	Ia	283	491	0.21	16.67
MRI Z2-380	fish	III	283	283	0.16	18.00
MRI Z2-392	fish	III	283	283	0.15	18.33
MRI Z2-385	fish	III	283	283	0.14	18.67
MRI Z2-391	fish	III	283	283	0.13	18.00
MRI Z2-382	fish	III	283	283	0.13	18.00
MRI Z2-387	fish	III	283	283	0.12	17.33
MRI Z2-386	fish	III	283	283	0.12	18.00
MRI Z2-390	fish	III	283	283	0.11	19.00
MRI Z2-369	fish	III	283	283	0.11	17.67
MRI Z2-378	fish	III	283	283	0.11	17.67
MRI Z2-367	fish	III	283	283	0.10	17.00
MRI Z2-377	fish	III	283	283	0.08	17.67
MRI Z2-375	fish	III	283	283	0.08	18.33
MRI Z2-383	fish	III	283	283	0.07	17.67
MRI Z2-389	fish	III	283	283	0.07	18.33
MRI Z2-374	fish	III	283	283	0.07	17.67
MRI Z2-393	fish	III	283	283	0.07	18.33
MRI Z2-372	fish	III	283	283	0.07	18.33
MRI Z2-388	fish	III	283	283	0.06	17.33
MRI Z2-371	fish	III	283	283	0.06	17.33
MRI Z2-366	fish	III	283	283	0.06	18.00
MRI Z2-370	fish	III	283	283	0.06	17.67
MRI Z2-376	fish	III	283	283	0.05	17.67

sample ID	host	serotype	CC[a]	ST[b]	salicin (OD_{600})	Hyaluronidase test (average diameter; mm)[d]
MRI Z2-373	fish	III	283	283	0.05	18.33
MRI Z2-368	fish	III	283	283	0.05	16.67
MRI Z2-381	fish	III	283	283	0.02	16.33
MRI Z2-379	fish	III	283	283	-0.02	16.67

[a] CC, clonal complex
[b] ST, sequence type
[c] ND, not determine
[d] The zone diameter from experiments in triplicate was measured.

Appendix viii

Tilapia production cycle (section 7.2)



FAO 2005-2019. Cultured Aquatic Species Information Programme. *Oreochromis niloticus*. Figure by Rakocy, J. E. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated 18 February 2005. [Cited 29 April 2019].