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The signal-based microbial spoilage on New Zealand export lamb

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Declaration

Unless otherwise acknowledged the work presented in this thesis is my own. No part has been submitted for another degree in any other institute of learning.

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Abstract

Lamb is one of New Zealand's primary exports; however, the spoilage of lamb causes considerable financial loss. Psychrotolerant, anaerobically growing spoilage bacteria are responsible for the spoilage of fresh chilled vacuum-packaged lamb exported from New Zealand. These spoilage bacteria interact with through a system called "quorum sensing". Different types of quorum sensing signals are produced by spoilage bacteria in response to the bacterial cell population. Through this cell-to-cell interaction, the expression of certain genes is regulated, followed by changes in bacterial activity. The main objective of this research was to determine the influence of quorum sensing signals produced by those psychrotolerant, anaerobically growing spoilage bacteria on the spoilage of New Zealand lamb.

Quorum sensing of psychrotolerant, anaerobically growing spoilage bacteria was studied using *in vitro* and *ex vivo* methods. Two types of *in vitro* quorum sensing signals (Type-I and Type-II) were identified for New Zealand reference type strains *Hafnia alvei* and *Serratia liquefaciens*, and Type-II quorum sensing signal was found from a lamb isolate *Carnobacterium divergens*. These two types of signals were also discovered in spoiled chilled vacuum-packed lamb. After random EZ-Tn5 transposon mutagenesis to *luxI/R*-type genes and *luxS* genes, which are responsible for Type-I and Type-II signals respectively in *H. alvei*, *S. liquefaciens*, and *C. divergens*, wild type and mutant strains were compared. Type-I quorum sensing signaling molecules influenced the expression of *lipB* that regulates the secretion of hydrolytic enzymes produced by

H. alvei and *S. liquefaciens*. These enzymes are believed to contribute to lamb spoilage. Cinnamaldehyde added to fresh vacuum-packed New Zealand lamb as an inhibitor of quorum sensing enabled an extension of the shelf-life of lamb by 2-8 days, through deactivating the Type-I quorum sensing system. Inhibition of quorum sensing has the potential in lamb preservation.

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Abbreviations

AB	autoinducer bioassay
ABC	ATP-binding cassette
AHL	*N*-acyl-homoserine lactone
AI-2	autoinducer Type II
AI-3	autoinducer Type III
AIP	autoinducing peptides
ANOVA	analysis of variance
ATCC	American Type Culture Collection
BCG	bromocresol green
BLAST	basic logical alignment search tool
bp	base pair
C4-HSL	*N*-butyryl-homoserine lactone
C6-HSL	*N*-hexanoyl-homoserine lactone
3-oxo-C6-HSL	*N*-(3-oxohexanoyl) homoserine lactone
C8-HSL	*N*-octanoyl-homoserine lactone
C10-HSL	*N*-decanoyl-L-homoserine lactone
cAMP	cyclic adenosine monophosphate
CBA	Columbia Blood Agar
cDNA	complementary deoxyribonucleic acid
CFME	cell-free meat extract
DFD	dark, firm and dry
CFU	colony forming units
cps	counts of photons per second
DNA	deoxyribonucleic acid
dNTP	deoxy-ribonucleoside triphosphate
DPD	4, 5-dihydroxy-2, 3-pentanedione
ESI-MS-MS	electrospray ionization tandem mass spectrometry

ESR	Institute of Environmental Science and Research
FAO	Food and Agriculture Organization
$FMNH_2$	1, 5-dihydroriboflavin 5'-(dihydrogen phosphate)
Folin's reagent	sodium 1, 2-naphthoquinone-4-sulfonate
g	acceleration due to gravity
LAB	lactic acid bacteria
LB	Luria broth
LCMS/MS	liquid chromatography-tandem mass spectrometry
MC	meat coupon
$MgSO_4$	magnesium sulfate
MIA	Meat Industry Association
MJ	meat juice
MRM	multiple reaction monitoring
Na_2CO_3	sodium carbonate
NaCl	sodium chloride
NaOH	sodium hydroxide
NCBI	National Centre for Biotechnology Information
NCTC	National Collection of Type Cultures
NZRM	New Zealand Reference Culture Collection
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
ppm	parts per million
PQS	*Pseudomonas* quinolone signal
PSE	pale, soft and exudative
QS	quorum sensing
QSI	quorum sensing inhibitor
R-THMF	(2R, 4SL)-2-methyl-2, 3, 3, 4-tetrahydroxytetrahydrofuran
RA	relative abundance

RLU	relative light units
RNA	ribonucleic acid
rpm	revolutions per minute
RT-qPCR	quantitative reverse transcription PCR
RTE	ready to eat
SAM	S-adenosyl methionine
SOB	super optimal broth
TCA	trichloroacetic acid
TLC	thin-layer chromatography
UV	ultraviolet
VP	vacuum-packaged
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Nucleotides

A	adenine
C	cytidine
G	guanosine
T	thymidine

Chapter One: General Introduction and Project Objectives

1.1 Introduction

Meat is a perishable food that is rich in nutrients such as protein, fat, carbohydrate and minerals to provide a balanced source of nourishment for humankind (Heinz and Hautzinger, 2009; Doulgeraki et al., 2012). These nutrients are also used by some microorganisms that degrade meat resulting in physical and chemical spoilage (Gram et al., 2002; Heijnen et al., 2004). Every year, meat products are rejected by the consumer because of poor quality, largely due to the activities of microorganisms (Nychas et al., 2008). These microorganisms could be from animal sources (such as hides, feeds, feces, etc.) and/or the abattoir and can contaminate meat during handling, transportation, distribution, and storage. Hence, to maintain the quality of meat, it is important to control the growth of microorganisms (FAO, 1991).

The growth of microorganisms is influenced by intrinsic factors (the initial microbial load, physical and chemical properties of meat, such as the pH (5.4-6.6), water activity ($a_{w,}$ > 0.99) and extrinsic factors such as storage temperature and oxygen availability (Dave and Ghaly, 2011; Bruckner et al., 2012). Understanding the types of microbiota that grow on meat is important to determine the likely effect on spoilage or food safety (Kumar and Anand, 1998). Bacteria responsible for spoilage produce proteolytic, saccharolytic and lipolytic enzymes (Braun et al., 1999a; Loureiro, 2000a), causing slime formation (only occurs aerobically), changes in flavour, odour, tenderness, texture and juiciness of meat (Casaburi et al., 2015; Ercolini et al., 2006).

Nowadays, meat and meat products are exported over long distances from local sources to international supermarkets (Nychas et al., 2008). A premium price is achieved, only if the meat is sold as “fresh” in distant markets; however, ready-to-eat meat products, which have been processed to inhibit pathogens are popular as well. A high degree of meat quality and safety is required to provide fresh products with a long shelf-life. One of the most popular methods to preserve fresh meat quality is to vacuum-pack large “primal” cuts, in which oxygen is obstructed preventing the growth of aerobic spoilage bacteria e.g. *Pseudomonas*, and fungi (Gill, 1989).

Temperature also plays a pivotal role in controlling spoilage. Frozen meat, for example, is less susceptible to spoilage with a longer shelf-life but after prolonged storage, the color and palatability of frozen meat may be altered and not desirable. Studies suggest that a chill temperature of -1.5 °C is the recommended storage temperature for chilled products delivered worldwide (Jeremiah and Gibson, 2001). At this temperature, the growth of anaerobic bacteria is reduced and the storage lifetime of meat is conservatively estimated to be around 70 days (James and James, 2002). However, if the transit is extended, spoilage is still possible at -1.5 °C (Psaraftis and Kontovas, 2013).

All meat is susceptible to microbial spoilage. To date, there are studies extensively on beef spoilage, but little information on lamb (Mills et al., 2014). There is a relatively large proportion of lamb or products traded internationally from New Zealand. According to the annual report of the meat industry association (MIA) of New Zealand, in the year of 2018, 92% of local sheep meat is exported to more than 120 countries and brings \$3.4 billion to the country (Figure 1.1). By understanding lamb spoilage, the quality of New Zealand exported lamb can be better controlled.

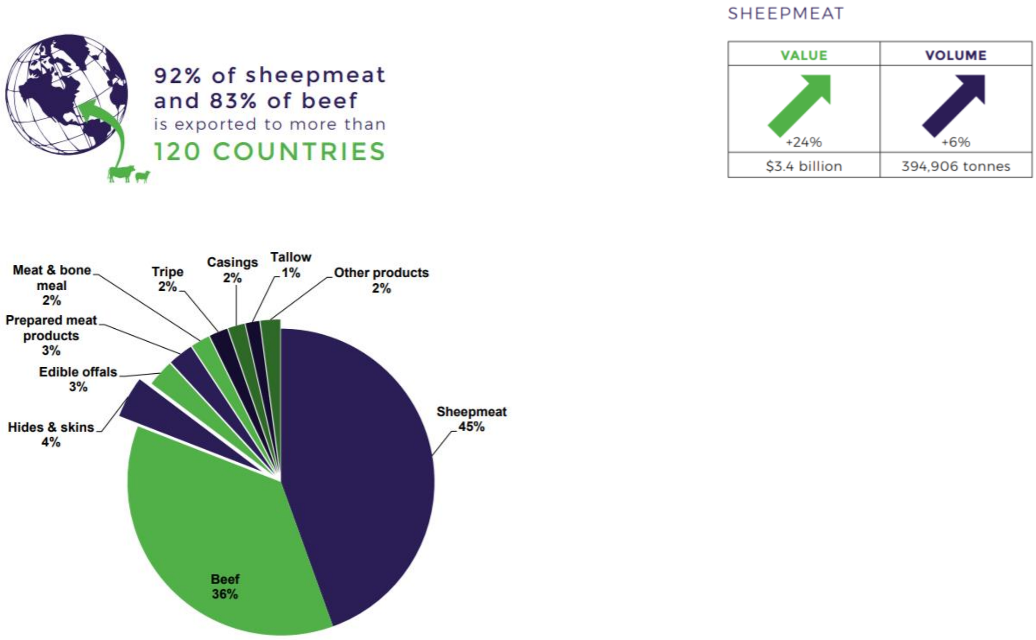


Figure 1.1. The sheepmeat (lamb) exported from New Zealand in 2018, extracted from the MIA 2018 annual report https://www.mia.co.nz/.

Chilled vacuum-packaged (VP) meats undergo a mild fermentation with naturally occurring microbiota dominated by the growth of lactic acid bacteria (LAB), most often *Carnobacterium* spp., (up to 50% *C. divergens* and up to 26% *C. maltaromaticum* of the LAB isolates obtained) (Leisner et al., 2007). When the microbiota reaches a certain level during storage and transport, e.g. 10^3 colony-forming units (CFU)/cm^2 for *Brochothrix* spp., or 10^8 CFU/cm^2 for lactic acid bacteria and psychrophilic *Enterobacteriaceae*, this results in an early end to shelf-life (Gribble et al., 2014).

In comparison to other meat, lamb has higher pH and fat, which enables bacteria with a higher spoilage potential to grow (Mills et al., 2014). Furthermore, muscle metabolites in lamb are in a different concentration (Gill and Newton, 1978), a few commercial promotion also suggest that lamb has higher amount of protein and nutrients such as Vitamin K and D than other meat, which makes the microbiota of lamb spoilage different and worth understanding.

The synergistic or antagonistic effects between bacteria is another important factor that affects bacterial growth (Bruckner et al., 2012). Interactions among microorganisms influence the selection of microbiota and regulate their physiological activities (Bruhn et al., 2004). Microorganisms communicate via signaling compounds to regulate gene expression in a coordinated manner (Gram et al., 2002). This communication system is known as “quorum sensing” (QS) or “autoinduction”, and the signaling compounds are known as “autoinducers” (Davies et al., 1998). QS systems have been extensively reviewed in relation to bacterial pathogenicity, bioluminescence, sporulation, antibiotic production, biofilm formation, and virulence (Ng and Bassler, 2009; Novick and Geisinger, 2008; Smith et al., 2004; Williams and Cámara, 2009). Since microbial activity is a cause of spoilage (Nychas et al., 2009), the QS systems of spoilage bacteria may also be involved in spoilage of food, such as meat.

Two of the common autoinducers found in spoilage bacteria are Type-I, acyl-N-homoserine lactones (AHLs) and Type II, furanosyl borate diester (AI-2). AHLs have only been found in Gram-negative species. AHL molecules comprise a universal homoserine lactone ring with a fatty acid acyl chain. These vary in the length and composition of the acyl side chain (Kumari et al., 2006). Short-chain (with 4-8 carbons) AHL molecules diffuse in and out of cells by passive transport and long-chained (over 10 carbons) molecules diffuse via active transport mechanisms e.g. efflux and influx (Skandamis and Nychas, 2012a). AI-2 is derived from the recycling of S-adenosyl-homocysteine to homocysteine, so it is found universally in almost all types of microorganisms. However, it is still controversial whether AI-2 is synthesized to signal (Diggle et al., 2007). These two QS signaling molecules have the ability to contribute to food spoilage. Riedel et al., 2001 found that AHLs regulated the expression of the *lipB* gene and mediated the secretion of hydrolytic enzymes such as metalloprotease, protease, and lipase in *Serratia liquefaciens* MG1. Blana, 2010 showed that AI-2 affected the growth of *S. liquefaciens*. Microbial and enzymatic activity further leads to spoilage.

It has been suggested that disrupting the QS circuit of spoilage bacteria might play an important role in preventing food spoilage (Bai and Rai, 2011). Quorum sensing inhibitors (QSIs) have been developed to interfere with the communication among microorganisms (Rasmussen and Givskov, 2006a) and in this study, QSIs are investigated as potential inhibitors of meat spoilage. A few bacteria from both terrestrial and marine environment are discovered to produce enzymes which inactivates, degrades or modifies QS molecules (Chu et al., 2014; Rehman and Leiknes, 2018), but the topic whether or how to apply this kind of quorum quenching bacteria to food industry will continue to be hot and worth exploring.

1.2 Project questions and objectives

The major research questions in this project were:

- Can autoinducers be detected in spoiled VP chilled lamb meat (hereinafter called lamb)?
- Is there a correlation between signaling molecules associated with spoiled VP chilled lamb and those produced by spoilage bacteria?
- What mechanisms are influenced by QS molecules and associated with meat spoilage?
- What quenching system or quenching compounds can be utilized to delay spoilage and extend the shelf-life of VP chilled lamb?

In this regard, the study aimed to discuss the involvement of QS systems in lamb spoilage, especially during storage in chilled vacuum-packaging, and to examine the potential for QSIs in controlling spoilage of lamb.

Chapter Two: Literature Review

2.1 Spoilage of meat

2.1.1 Pre-slaughter cause of meat deterioration

Meat may show signs of deterioration even before slaughter. When animals are exposed to pre-slaughter stress, the glycogen inside muscles is broken down via an anaerobic glycolytic pathway and ultimately alters the meat pH by producing lactic acid (Miller, 2002). When animals are exposed to long-term stress, meat pH increases, resulting in dark, firm and dry (DFD) meat, whilst short-term stress lowers the pH of muscle and leads to pale, soft and exudative (PSE) meat (Chambers et al., 2001; Miller, 2002). DFD meat has an increased water holding capacity and is more sensitive to spoilage, whereas PSE meat has less water holding capacity to spoil the tissue (Scheffler and Gerrard, 2007).

After slaughtering, microbial and enzymatic activities are the leading causes of meat spoilage during processing and storage.

2.1.2 Microbial spoilage

In the meat industry, microorganisms are present ubiquitously from the animal source to the abattoir, and eventually to meat products. The composition of the initial microbiota in meat and meat products depends on factors such as (a) animal age, (b) husbandry practices, (c) handling and hygiene of slaughtering, (d) preservation during processing, packaging, distribution, and (e) storage (Cerveny et al., 2009).

There are a variety of natural microorganisms found in meat and meat products, including *Pseudomonas*, *Escherichia*, *Lactobacillus*, *Salmonella*, *Bacillus*, *Micrococcus*, *Streptococcus*, *Sarcina*, *Clostridium* (Arnaut-Rollier et al., 1999; Lin et al., 2004; Nychas and Tassou, 1997), *Candida* spp., *Cryptococcus* spp., *Rhodotorula* spp., *Cladosporium*, *Penicillium*, *Geotrichum*, *Mucor* and *Sporotrichum* (Garcia-Lopez et al., 1998). Some of them may be pathogens (Jay et al., 2005).

Various compounds are produced from microbial metabolism and they have different effects on meat. For example, compounds like fatty acids, ketones, and alcohols lead to fruity odors in meat, whereas, sulfides such as hydrogen sulfide, methylsulfide and dimethylsulphide produce putrid and sulfurous odors (Dainty, 1996). Other compounds like methylamine, dimethylamine, trimethylamine, diamines, cadaverine, and putrescine have also been detected during bacterial spoilage and studied as indicators of meat spoilage (Garcia-Lopez et al., 1998; Jay et al., 2005).

2.1.3 Enzymatic spoilage

The enzymatic deterioration of meat is a natural process resulting from cell autolysis, as well as microbial activity during the slaughter, leading to meat spoilage (Tauro et al., 1986). Enzymatic reactions include proteolysis and lipid hydrolysis of protein and fats respectively, in the muscle tissues which further break down to simpler compounds, affecting the meat quality.

Endogenous proteolytic enzymes calpains, cathepsins and aminopeptidases digest the z-line proteins (titin, nebulin, desmin, and α-actinin) of the myofibril in meat, and result in post mortem breakdown of polypeptides (Huss, 1995; O'Halloran et al., 1997; Toldrá and Flores, 2000). At low temperatures, these proteolytic enzymes are active, and the microbial strains with high proteolytic enzymatic activity potentially increase the biogenic amine formation, which leads to meat deterioration (Biji et al., 2016; Kuwahara and Osako, 2003).

The enzymatic hydrolysis of fats is termed lipolysis. During lipolysis, the glycerides in meat are digested and form glycerols and free fatty acids. These free fatty acids lead to off-flavor development (FAO, 1986; Huis in 't Veld, 1996). Lipases, phospholipase, and esterases are the lipolytic enzymes found in meat of which phospholipase A1 and phospholipase A2 are the main enzymes (Toldrá, 2006). They may be endogenous (in animal blood) or from secreted by psychrotrophic microorganisms (Ghaly et al., 2010).

2.2 Microbial spoilage in stored vacuum-packed lamb

2.2.1 Vacuum-packaging for chilled raw meat

After slaughtering, meat is preserved at a chilled temperature (-1.5 °C – 5 °C). At this range of temperature, the growth of most microbes is slowed during storage, and meat spoilage is retarded (Lambert et al., 1991). Vacuum-packaging is a method that removes air from the package before sealing (Robertson, 2013), and is widely used to preserve and transport meat or meat products from local to overseas market (MIA annual report 2018). Under an oxygen-deficient environment, the shelf-life of the chilled meat can extend within the pack by slowing the microbial growth. However, vacuum-packaged (VP) meats (Figure 2.1) are not often found in retail display as when the meat or meat products are preserved in an oxygen-depleted atmosphere, the color of the meat turns purple due to deoxymyoglobin (Gill and Gill, 2005) and be rejected by customers.

Figure 2.1. Examples of vacuum-packed meats. Left: normal vacuum-packed meat; Right: purplish vacuum-packed meat due to deoxymyoglobin.

2.2.2 Growth of different microbes on vacuum-packed chilled lamb

Chemical and physical parameters such as temperature, pH, water activity and atmospheric conditions influence the growth and activity of microorganisms. These growth conditions select for microbiota on meat and meat products (Bell, 2001). Packaging and storage strategies aim to extend shelf-life when VP lamb is stored at chilled temperatures and with good temperature control, lamb cuts should have a storage life of 10-12 weeks (James and James, 2002; Kiermeier et al., 2013). Although the shelf-life of VP chilled lamb is greatly extended using appropriate measures, the likelihood of spoilage is always there.

When the meat is placed into bags with limited oxygen permeability and a vacuum is applied before sealing (Kropf, 2004), the growth of aerobes would be restricted within the pack (Gill, 1989). However, the growth of anaerobic microorganisms e.g. lactic acid bacteria (LAB), predominated by *Carnobacterium* spp. and *Leuconostoc* spp. (Jones et al., 2008), has been shown to have some effects. Specific spoilage microorganisms *Brochothrix* spp., (e.g. *B. thermosphacta* and *B. campestris*), *Shewanella putrefaciens* and psychrophilic (microorganisms that are able to grow and replicate at low temperatures, -20 °C to 10 °C) *Clostridium* spp. (e.g. *C. estertheticum*, and *C. gasigenes*) are found on chilled VP lamb (Mills et al., 2014; Pennacchia et al., 2011; Seelye and Yearbury, 1979). Other psychrotrophic *Enterobacteriaceae* spp. notably, *Hafnia alvei*, *Serratia* spp., *Rahnella aquatilis*, and *Yersinia enterocolitica* also grow on VP lamb stored at chilled temperatures (Brightwell et al., 2007; Borch et al., 1996; Gill, 2004). These aforementioned psychrotrophic bacteria cause spoilage such as a sweet odor and greening by LAB (Mol et al., 1971); a cheesy odor caused by *B. thermosphacta* and a sulfurous odor caused by *Enterobacteriaceae* (Walker, 2003) in VP meats. Carbon dioxide (CO_2) is also produced as the end product of tissue and microbial respiration in VP meat products and causes a blown pack spoilage by *Clostridium* spp. (Perdue, 2009).

Lactic acid bacteria (LAB) are highly competitive in a modified atmosphere (Kameník, 2013). Psychrotrophic LAB, such as *Carnobacterium divergens*, *C. maltaromaticum*, *Lactobacillus sakei*, *L. curvatus*, *L. fuchuensis*, *Weissella* spp., and *Leuconostoc* spp., grow to high numbers in vacuum-packed meat stored at chilled temperatures (Jones et al., 2008; Kameník, 2013; Mills et al., 2014). Heterofermentative species, e.g. *Carnobacterium* and *Leuconostoc* spp., produce not only lactic acid but also spoilage-related compounds including sulfides, ethanol and butyric acid. They are usually the two major bacteria at the end of shelf-life (Jones, 2004).

Psychrotrophic *Brochothrix* spp. (mainly *B. thermosphacta*) have a higher spoilage potential than LAB and are important agents of meat spoilage. They are facultative anaerobes and widely distributed in the environment. *B. thermosphacta* is closely related to the genera *Lactobacillus* and *Listeria,* and found on beef, lamb, fish and cured meats (Gribble and Brightwell, 2013; Stackebrandt and Jones, 2006) and may become a major contaminating microorganism on fresh and processed meat due to its ability to grow on chilled meat and tolerance to high salt concentrations. It is often associated with the early spoilage of red meats (Gill et al., 1988; McClure et al., 1993). *B. thermosphacta* is able to proliferate at low pH (5.5 to 5.6) aerobically and become the dominant bacterial species in the presence of oxygen. However, in oxygen-depleted conditions, such as VP packages, a slightly higher pH, over 5.8 is required for growth at chilled temperatures, along with a small amount of oxygen (Grau, 1980; Newton et al., 1978).

In a chilled vacuum packaged environment, psychrotrophic *Enterobacteriaceae* groups commonly occur in lower numbers on meat compared to those of LAB, although these bacteria are more likely to cause spoilage at "abuse temperatures", over 5 °C (Borch et al., 1996; Brightwell et al., 2007; Pennacchia et al., 2011). These meat-associated *Enterobacteriaceae* metabolize amino acids to malodorous volatile compounds, like diamines and sulfuric compounds, leading to spoilage of lamb and beef products (Baylis, 2006; Bell, 2001; Newton et al., 1978). In VP chilled meat, spoilage by *Enterobacteriaceae* is typically associated with sulfurous odors and potential greening of the surface and/or drip through the development of sulfmyoglobin (Bell, 2001; Fox, 1966).

Lamb has a higher pH compared to other meat. Compare to the pH values of other meat 5.3-5.8 (Honikel, 2004), the pH of many lamb cuts is over 5.8, favoring the growth of bacteria such as *Brochothrix* spp., and *Enterobacteriaceae* with a high spoilage potential. In lamb, *Brochothrix* spp., *B. thermosphacta* and *B. campestris* were found not to be entirely inhibited in VP packages (Shaw et al., 1980; Talon et al., 1988) and could be a cause of meat spoilage at chilled temperatures (Gribble and Brightwell, 2013). *Enterobacteriaceae* were reported to grow and potentially spoil VP packaged lambs at pH values between 5.5 and 6.5 when stored at chilled temperatures −1.5 to 7 °C (Gribble and Brightwell, 2013). However, LAB will still have a growth rate advantage and become predominant at chilled storage temperatures (Gill et al., 1988). Furthermore in lamb, the glucose concentration is three times higher than in other meat e.g. beef (Bell, 2001; Gill and Newton, 1978), and this will potentially influence the amount and type of microbial growth

S. putrefaciens is associated with high pH meat spoilage, producing hydrogen sulfide and greening, and has been reported to cause spoilage of chilled beef (Gill and Newton, 1978), but Kiermeier et al., 2013 stated that *S. putrefaciens* did not seem to cause spoilage of chilled lamb in any of the culture or culture-independent studies.

2.3 Quorum sensing in vacuum-packed lamb

2.3.1 The discovery and general overview of bacterial quorum sensing

In the 1970s, a density-dependent cell-to-cell communication was recognized via luciferase enzymes in the marine bacterium *Vibrio fischeri* (Nealson et al., 1970). The phenomenon was referred to as “autoinduction”; however, it was ascribed to some unknown factors at that time. In 1973, bioluminescence production, catalyzed by luciferase was found to be attributed to the oxidation of reduced flavin phosphate ($FMNH_2$) and reacted with long-chain fatty aldehydes for light emission (Hastings et al., 1973). This reaction is as follows:

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + h\nu \text{ (490 nm)}$$

Eberhard et al., in 1981 identified a small diffusible molecule, known as *N*-(3-oxohexanoyl) homoserine lactone to induce the production of luciferase in *V. fischeri*. This molecule belongs to a group of signaling molecules called *N*-acyl homoserine lactones (AHLs). The discovery of AHLs indicated the ability of a bacterial cell or a population of bacterial cells to respond to the presence of signaling molecules in the extracellular environment. The response could provide a population-wide change in cellular behavior. The *lux* operon was deemed to be responsible for luciferase production in *V. fischeri* (Figure 2.2). Genes *luxI* and *luxR* encode for synthesis and reception of AHL molecules while genes *luxA* and *luxB* encode for two subunits of luciferase. For bioluminescence production, the fatty acid reductase complex is coded by *lux*CDE. Among these genes, *luxC* encodes for acyl-reductase, *luxD* encodes for acyl-transferase, and *luxE* encodes for acyl-protein synthase. The reduced Flavin required for light emission is supplied by *luxG* and *luxH* genes. These two downstream genes are not involved in the general regulation or expression of the *lux* system. However, they may be required for the survival and/or symbiosis of the bacteria in the marine environment. (Meighen, 1991).

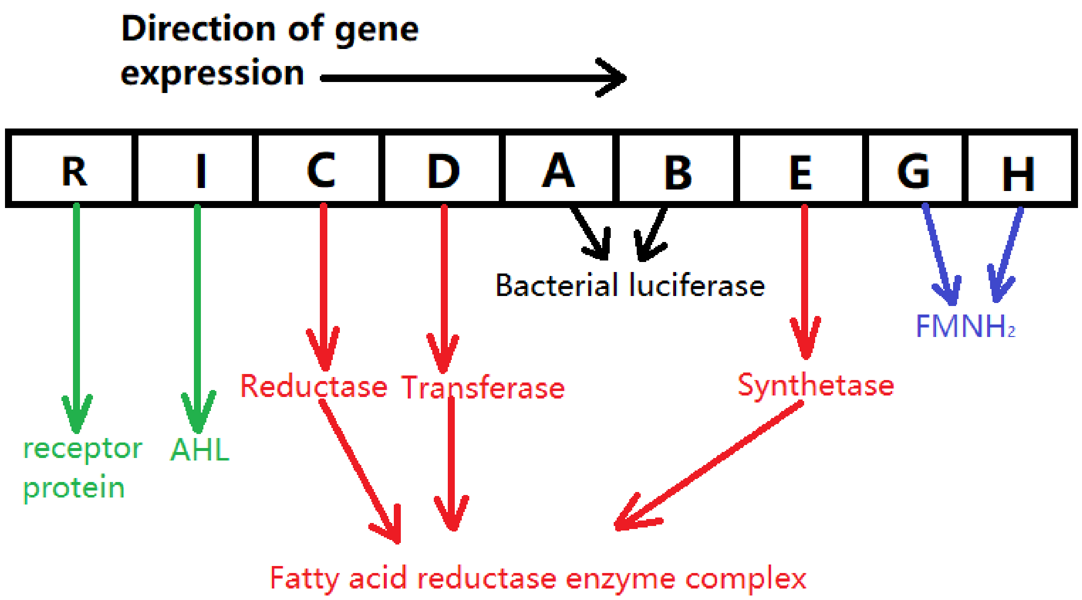


Figure 2.2. The arrangement of the *lux* operon in *V. fischeri*.

There are two general QS mechanisms bacteria use to detect and respond to QS signaling molecules and to regulate gene expression (Bai and Rai, 2011). One depends on the production of AHLs, which are synthesized by the AHL synthase LuxI homologs in Gram-negative bacteria (Schaefer et al., 1996). Acyl chains are derived from acyl carrier proteins (ACPs) from the common fatty acid biosynthesis pathway and the homoserine lactone (HSL) moiety is derived from S-adenosylmethionine (SAM) (Val and Cronan, 1998; Parsek et al., 1999). Short-chain AHLs passively diffuse across cell membranes and accumulate in the cytoplasm whilst long-chain AHLs require active efflux mechanisms, such as the efflux pump MexABOprM in *Pseudomonas aeruginosa* for exportation (Pearson et al., 1999; Moore et al., 2014). When the cell population increases, the accumulation of AHL signaling molecules reaches a threshold level and is recognized by the receptor proteins from other bacteria. These receptor proteins belong to the LuxR family and also act as transcriptional regulators. When the receptor proteins bind with AHL signaling molecules, the complexes are then involved in the expression control of QS regulons (Schuster et al., 2004). In this type of QS mechanism, the AHL signal can be decayed by AHL-degrading enzymes (Huang et al., 2003) and affect the AHL-dependent gene expression (Zhang et al., 2004).

Another QS mechanism is mediated by a membrane-associated 2-component response regulatory system. This system is present in both Gram-positive and Gram-negative bacteria (Waters and Bassler, 2005). In this mechanism, QS signaling molecules e.g. autoinducing peptides (AIPs) produced by Gram-positive bacteria, are transported by an ABC transporter and accumulate in the intercellular environment. Once the accumulated molecules reach a threshold level, these signaling molecules are detected and transferred by a sensor. A cognate receptor is then activated to modulate the expression of the QS regulon through regulatory

RNAs and intracellular transcription factors (Novick, 2003). To date, no signal decay has been reported for this type of QS mechanism.

To date, the response of QS has been found extensively in the development of genetic competence for exogenous DNA, biofilm formation, production of antimicrobial peptides or exotoxins, sporulation, transfer of conjugative plasmids, nitrogen fixation, symbiosis and regulation of virulence factor synthesis (Hoang et al., 2004; Podbielski and Kreikemeyer, 2004; Smith et al., 2004; Williams, 2007; Bai and Rai, 2011).

2.3.2 Quorum-Sensing Signals

In general terms, there are two groups of signaling molecules involved in bacterial QS systems: peptide derivatives exploited by Gram-positive bacteria and fatty acid derivatives utilized by Gram-negative bacteria (Bai and Rai, 2011). Furthermore, the QS molecules are divided into categories most commonly found in cell-to-cell signaling systems. In this research study, only two categories of autoinducer-1 (AI-1, also known as AHL) and AI-2 were considered and studied. AI-1 molecules are primarily involved in intraspecies communication (Smith et al., 2004), and only found in Gram-negative cells (Sturme, 2002). AI-2 is found in both Gram-positive and Gram-negative bacteria, and is utilized for interspecies communication (Schauder et al., 2001). These two signaling molecules are chemically distinct with different gene modulations.

Autoinducer-1: AHL mediated quorum sensing

The AI-1 system is based on the LuxI/LuxR system, as described in Section 2.3.1. The signaling molecules with different acyl chains and substitutions (Figure 2.3) are synthesized by LuxI and its homologs (Schaefer et al., 1996). When the amount of AHL production is high enough, signaling molecules diffuse into cells and are recognized and bound by LuxR and its homologs (Figure 2.4), the signals are then regulated to trigger the target gene expression through this LuxI/R system.

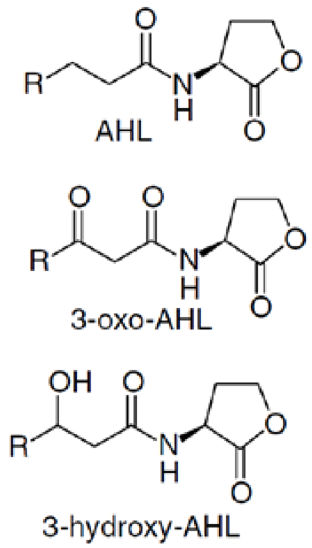


Figure 2.3. General chemical structures of AHL signaling molecules. The acyl fatty acid side chains of AHLs can be saturated or unsaturated, ranging from 4 to 18 carbons in length. Hydroxyl- or oxo- substitutions may be found on the third carbon.

So far, LuxI/LuxR and its homologous systems have only been identified in Gram-negative bacteria, and synthesize specific AHL signaling molecules. In *P. aeruginosa*, LasI/LasR system, homologous to LuxI/LuxR, has been shown to produce extracellular enzymes such as elastase, which plays an important role in the pathogenicity of *P. aeruginosa* and promoting biofilm formation (Yu et al., 2014). The homologous system RhlI/RhlR also modulates enzyme production, coordinate biosurfactant synthesis, biofilm formation and bacterial survival in the environment through AHLs (Wang et al., 2014). The expression of the virulence factors in *P. aeruginosa* is controlled through these signaling mechanisms. In addition, one AHL-dependent QS system, LuxI/R-type synthase and regulatory proteins were found in *P. fluorescens* for its environmental adaption and physiology in food spoilage (Tang et al., 2019). Studies have shown that some bacteria e.g. *Salmonella* Typhimurium and *E. coli*, do not have a LuxI homolog to synthesize AHL signaling molecules but possess a LuxR homolog known as SdiA (Ahmer et al., 1998; Kanamaru et al., 2000). The exact role of SdiA in pathogenesis is still unclear, but the transcribed protein allows the modulation of gene expression in response to the presence of AHL signaling molecules, produced by other bacteria (Michael et al., 2001). It is also possible that "LuxR orphans" may enable bacteria to gain an advantage by using exogenous AHLs to regulate their gene expression (Subramoni and Venturi, 2009), given an example that in non-AHL-producing bacteria *E. coli*, homologs of orphan LuxR can detect 3-oxo-C10-HSL (Lee et al., 2006). In other bacteria, such as *Y. enterocolitica*, the LuxR homolog YenR activates the expression of YenS, which inhibits the expression of the LuxI homolog YenI and impedes the production of AHL signaling molecules. This YenR/YenI/YenS system has been shown to control the swimming and swarming motility of the bacteria (Atkinson et al., 2006; Tsai and Winans, 2011a).

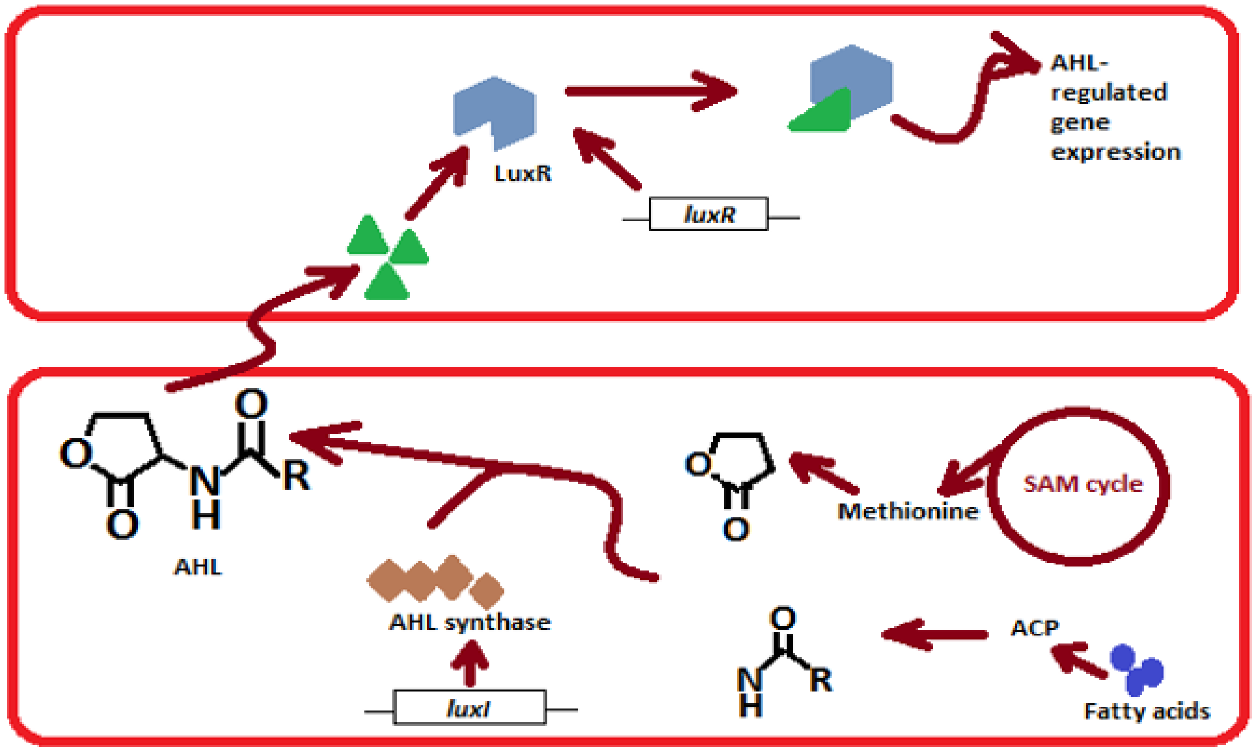


Figure 2.4. The schematic diagram of the LuxI/R regulatory system in Gram-negative bacteria.

LuxI/R is not the only type of protein to facilitate AHL-mediated QS. During the study of bioluminescence in a marine bacterium, *Vibrio harveyi*, an AHL synthase protein termed LuxM was discovered to synthesize 3-OH-C4-HSL (Bassler et al., 1993; Cao and Meighen, 1989). 3-OH-C4-HSL is detected and regulated by a protein termed LuxN (Freeman et al., 2000). When AHL is at low concentration, LuxN acts as a kinase and transfers phosphate moiety to a cytoplasmic phosphotransferase protein LuxU and then onto the σ54 dependent activator LuxO. Phosphorylation of LuxO reduces the bioluminescence in *V. harveyi* (Freeman et al., 2000). When the AHL concentration is high, i.e. at high cell density, LuxN interacts with AHL as a phosphatase, resulting in the loss of phosphate from the LuxU/O complex and repression of bioluminescence is forfeited (Cámara et al., 2002). In many other marine *Vibrio* spp., LuxM/N-type QS systems are found alongside LuxI/R-type QS systems (Milton et al., 2001).

Autoinducer-2: a universal signaling language?

AI-2 is an extracellular signaling molecule that is synthesized and detected by the QS system in both Gram-negative and Gram-positive bacteria (Cao and Meighen, 1989). It is synthesized by synthase protein LuxS from S-ribosylhomocysteine, a by-product of the SAM-e cycle metabolism, and converted into 4,5-dihydroxy-2,3-pentanedione (DPD), the precursor compound of the AI-2 signaling molecule (Figure 2.5) (Schauder et al., 2001).

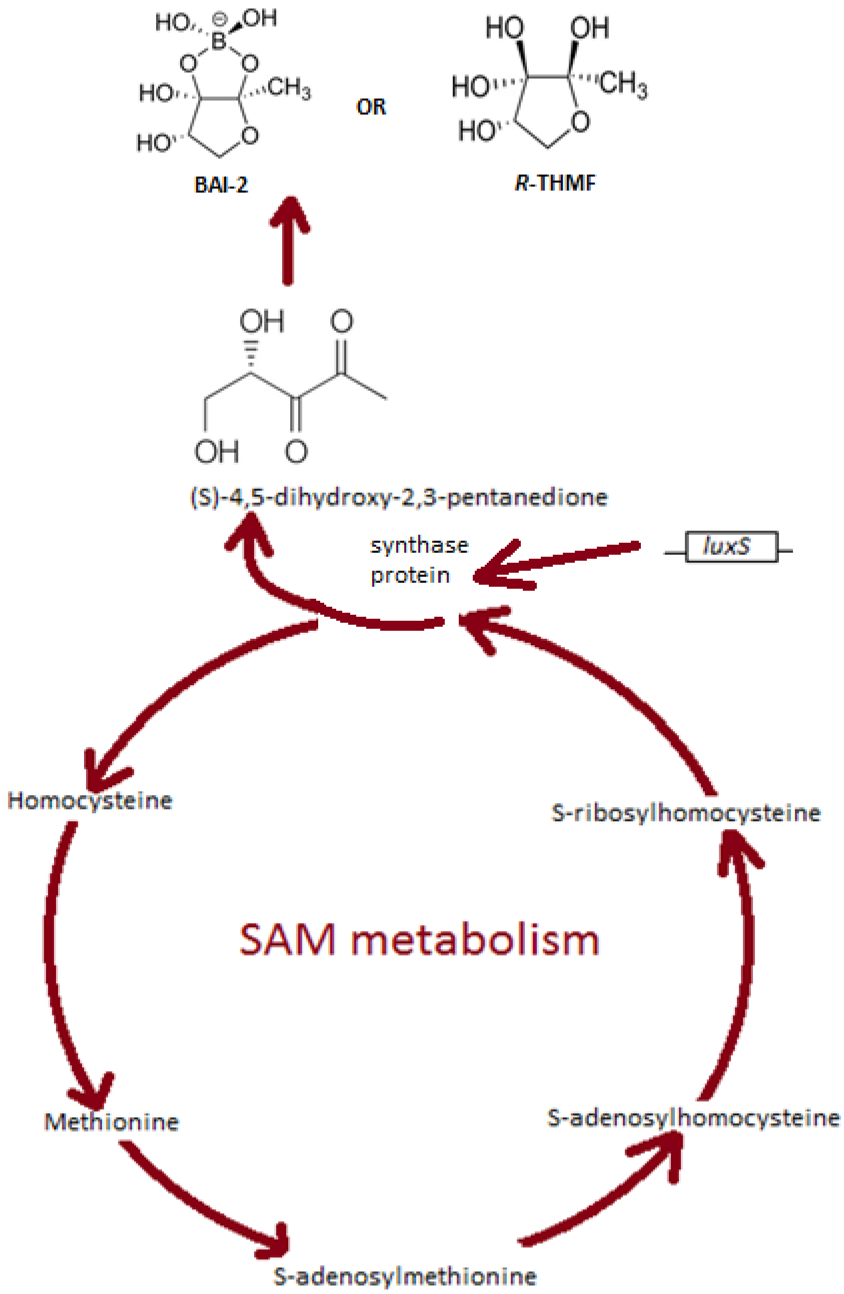


Figure 2.5. The schematic diagram of the synthesis of AI-2 signaling molecules. Modified from Schauder et al., 2001.

When the AI-2 signal is released into the cell's environment, it is detected by a bacterium by two separate mechanisms. The LuxP/LuxQ cascade has so far only been identified in *Vibrio* species. In general, *Vibrio* spp., e.g. *V. harveyi* detects furanosyl borate diester, a BAI-2 form of the AI-2 signal, by binding with an autoinducer-specific binding protein, LuxP. This AI-2/LuxP complex then interacts with LuxQ, a sensor kinase, and initiates luciferase production and bioluminescence (DeKeersmaecker and Vanderleyden, 2003a). A different Lsr mechanism, especially in *S*. Typhimurium, is used for AI-2 detection. When the AI-2 signal is released by the accumulation of bacteria, it is recognized by LsrB. This protein co-crystalizes with the furanone form of AI-2, (2R,4SL)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (R-THMF). The Lsr ABC transporter then transports the AI-2 signal into the cell, followed by phosphorylation by LsrK. The phosphorylated AI-2 then interacts with LsrR to relieve transcription repression of the Lsr cascade (Taga et al., 2001). AI-2 signaling is also regulated by glucose and cyclic adenosine monophosphate (cAMP) via the *lsr* operon. When glucose is present, there is almost no *lsr* operon expression. However, when glucose is depleted, cAMP is needed to stimulate the expression of *lsr*, while it is repressed without the inducer, the phosphorylated form of AI-2. When AI-2 accumulates, and more AI-2 is transported via LsrABC, phosphorylated via LsrK, the *lsr* transcription is de-repressed, which results in more AI-2 uptake (Wang et al., 2005).

Since the LuxS/AI-2 systems have been reported in a broad range of Gram-positive and Gram-negative bacteria, AI-2 is regarded as a universal signal for cross-species interaction (Xavier and Bassler, 2003). However, as the AI-2 production happens mainly in SAM-e cycle metabolism, it is still contestable whether it is always being produced for microbial interaction. For example, Holmes et al., 2009 did not found any evidence for the role of AI-2 in cell-to-cell communication in *C. jejuni* strain NCTC 11168.

2.3.3 QS signals in vacuum-packaged chilled food spoilage

QS signaling molecules, especially AHLs, have been detected in food spoilage systems like milk, meat and vegetables (Bruhn et al., 2004; Gram et al., 2002; Liu et al., 2006; Pinto et al., 2007). To date, there have been several relevant studies developed to investigate the role of QS in meat spoilage (Table 2.1). They covered beef, poultry and pork studies but not in lamb.

Psychrotrophic pseudomonads are responsible for the spoilage of meat and meat products stored aerobically at chilled temperatures. QS molecules have been found in the spoilage process of fresh meat stored aerobically at refrigeration temperatures (Jay et al., 2003). When cell-free meat extract from fresh meat was added to *P. fluorescens* and *Serratia marcescens* from fresh meat, there was an increase in the metabolic activity of both the tested strains (Nychas et al., 2007). This increase was attributed to the presence of QS signaling molecules in cell-free meat extract. Liu et al. in 2006 found that a variety of AHL signaling molecules, like C4-HSL, 3-oxo-C6-HSL, C6-HSL, C8-HSL, and C12-HSL, were detected in ground beef and chicken, stored aerobically at chilled temperatures. These signals were associated with the growth of *Pseudomonadaceae* and *Enterobacteriaceae* (Blana, 2010).

Table 2.1. Studies of QS signals in meat spoilage.

Source of QS	QS signaling molecules	References
Isolated *Enterobacteriaceae* strains isolated from VP chilled beef	C6-3-oxo-HSL, C6-HSL	(Gram et al., 2002)
Isolated *Enterobacteriaceae* strains and meat extracts from VP chilled beef	C6-3-oxo-HSL, C6-HSL	(Bruhn et al., 2004)
Beef, chicken, and turkey products	Borated AI-2	(Lu et al., 2004a)
Aerobically chilled stored ground beef	C4-HSL, C6-3-oxo-HSL, and undefined AHLs	(Liu et al., 2006)
Isolated *Yersinia enterocolitica* strains from fresh beef and pork extracts	C6-3-oxo-HSL, C6-HSL	(Medina-Martínez et al., 2006)
Isolated *E. coli* O157: H7 from ground beef extracts	Borated AI-2	(Soni et al., 2008[b])
Minced pork extracts stored aerobically at 5 and 20°C	Short-, medium-, and long-side-chain AHLs, AI-2	(Nychas et al., 2009)
Isolated *Pseudomonas fragi* from fresh and spoiled meat	Borated AI-2	(Ferrocino et al., 2009)
Isolated *E. coli* O157: H7 from beef and pork broths	AI-2	(Silagyi et al., 2009)
Isolated lactic acid bacteria from minced beef packaged under modified atmospheres	AI-2	(Blana et al., 2011)

Because of the inhibition of sensing AI-2 molecules by fatty acids (Soni et al., 2008[a]), a low level of AI-2 activity was found in spoiled beef steak, chicken breast, and turkey patties even though the bacterial density was high (Lu et al., 2004a). On the other hand, the activities of BAI-2, as well as AHLs, were higher in the cell-free meat extract from spoiled minced pork meat stored at refrigerated temperature (5 °C) than at 20 °C (Ammor et al., 2008). Such a difference was suggested to be linked to the presence of some psychrotolerant spoilage bacteria.

Li et al., 2019 found that AI-2/LuxS regulation significantly increased the antimicrobial activity of *Lactobacillus plantarum* AB-1 *in vitro* and on refrigerated shrimp. It gives an idea that *Lacotobacillus* spp. could use AI-2 signaling molecules for biopreservation of food. However, in the cold vacuum packaged environment, spoilage in fish was found to be enhanced by the interaction between *Enterobacteriaceae* and lactic acid bacteria, usually *Carnobacterium* spp., and/or *Lactobacillus* spp. (Jørgensen et al., 2000), which indicated that the AI-2 signaling between *Enterobacteriaceae* and lactic acid bacteria could not activate the biopreservation of *Lactobacillus* spp., or the spoilage behavior of *Enterobacteriaceae* might dominate. *H. alvei* and *Serratia* spp. have been identified as the predominant *Enterobacteriaceae* in vacuum-packaged meat (Ravn et al., 2003). To date, the *lux*I/R-type genes have been found to affect the AHL signaling molecules (mainly 3-oxo-C6-HSL) and QS regulatory mechanisms in *H. alvei* and *Serratia* spp. (X. Li et al., 2019; Takayama and Kato, 2016; Tao et al., 2008). AHL-modulated proteolytic activity has been reported in a cold-smoked salmon isolate *S. proteamaculans* B5a strain (Christensen et al., 2003) and found to be involved in food spoilage. A LuxI homolog SprI in *Serratia plymouthica* RVH1 isolated from processing raw vegetables was found to modulate the production of C4-HSL, C6-HSL, and 3-oxo-C6-HSL. Inactivation of SprI notably reduced the production of C4-HSL and C6-HSL, and completely lost the production of 3-oxo-C6-HSL. This SprI-dependent quorum sensing was also found to be involved in the production of extracellular enzymes such as protease, chitinase, and nuclease, as well as 2,3-butanediol fermentation (Van Houdt et al., 2006). The production of these exoenzymes was reduced in the *sprI* mutant and was restored when external C6-HSL or 3-oxo-C6-HSL was added (Van Houdt et al., 2007).

These findings provide evidence that bacterial spoilage of meat is influenced by QS-regulated hydrolysis and AHL-based QS systems are involved. The presence of AHL signaling molecules in vacuum-packaged chilled meat and meat products indicates that the existing preservation techniques might be insufficient. To counteract the effect of QS systems, quorum sensing inhibitors or quenchers can be exploited and developed as novel preservatives in the meat industry.

2.4 Quorum quenching

The principle of quorum quenching is to degrade or disrupt quorum sensing signals (Alagarasan and Aswathy, 2017). Possible strategies include inhibitor compounds that inactivate signaling synthase, compounds that mimic signaling molecules and block the signal receptor, compounds that affect signaling molecules, bacterial mutants that generate false sensing, and compounds that confuse bacteria indicating that the cell density is too high and cease bacterial growth and/or metabolic activity (Figure 2.6) (Chan et al., 2011, 2009; Skandamis and Nychas, 2012b).

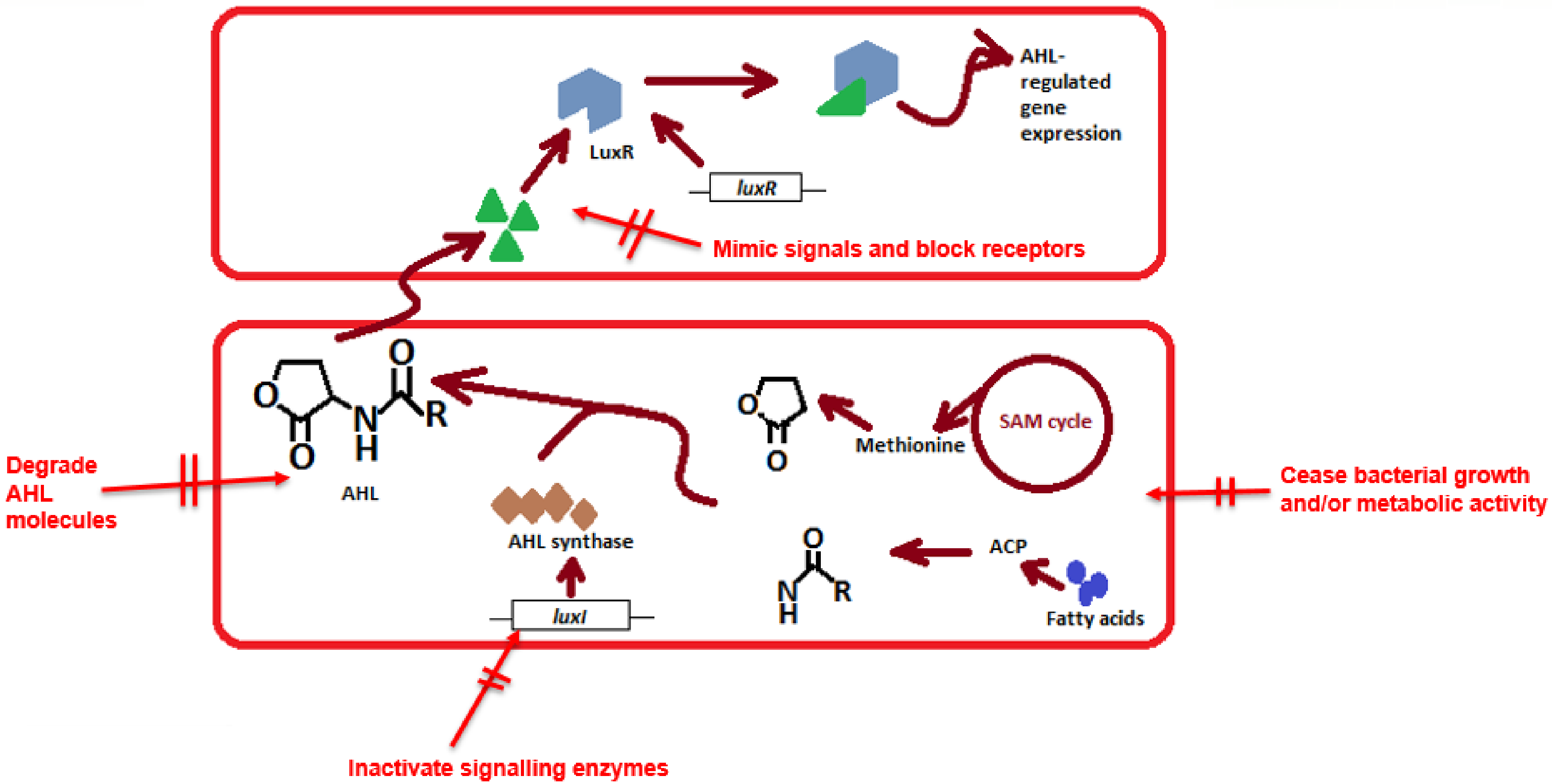


Figure 2.6. The schematic diagram of quorum quenching system/strategies inhibitors use to control quorum sensing.

Closantel and triclosan are known inhibitors of QS synthesis. Closantel inhibits the histamine kinase sensor while triclosan blocks the ACP reductase and intervenes in the synthesis of AHLs (Basavaraju et al., 2016; Zhang and Dong, 2004).

A promising group of QS inhibitors that mimic AHL molecules are the halogenated furanones extracted from an Australian red alga, *Delisea pulchra*. These furanones prevent receptors from binding a substrate or decrease the concentration of receptors in the cell (Basavaraju et al., 2016). They also act on AHL-dependant transcriptional activity. The expression of AHL-regulated virulence factors in *P. aeruginosa* and *S. liquefaciens* has been reported to be reduced by the inhibitors (Givskov et al., 1996). A flavor lactone γ -caprolactone was also recently found to mimic AHL signals and stimulate transcription of the Rhodococcal qsd operon, which is involved in quorum quenching activities (Chane et al., 2019).

In particular, enzymes that are capable of degrading the N-acyl homoserine lactone (AHL) molecules used by numerous bacteria (Michael et al., 2019; Chan et al., 2010). Application of these AHL-degrading bacteria has been exploited in aquacultures and agriculture, to limit the spread of pathogenic bacteria and dissociate biofilm formation (Galloway et al., 2012; Grandclément et al., 2016). Enzymatic reactions of quorum quenching are commonly classified into four roles: 1) the lactonases that open the homoserine lactone ring (Uroz et al., 2008); 2) the amidases (or acylases) that cleave AHLs and release free fatty acid and homoserine lactone (Lin et al., 2003); 3) the reductases that convert 3-oxo-substituted AHL to their cognate 3-hydroxyl-substituted

AHL (Bijtenhoorn et al., 2011) and 4) cytochrome oxidases that oxidizes the acyl chain (Chowdhary et al., 2007). However, this classification of quorum quenching remains tentative as the evidence of those quorum quenching enzymes which have been identified at the molecular and biochemical levels is limited (Grandclément et al., 2016). Gualpa et al., 2019 discovered *in silico* and *in vitro* that *Azospirillum brasilense* Az39 is capable of degrading AHLs by hypothetical enzymes, and some penicillin G (PENG)-resistant bacteria from penicillin-contaminated river sediments were isolated, showing quorum quenching activity towards AHL signaling molecules (Hiroyuki et al., 2019). But to date, there is no bacterium with quorum quenching activity can be directly applied to food industry for meat preservation.

Natural products are important in modern drug development (Veeresham, 2012). Plants used in traditional medicine, dietary phytochemical extracts from fruits, herbs, and spice extracts have potential as a source of new biologically active QSI compounds from natural sources (Bai and Rai, 2011; Vattem et al., 2007). For example, a previous study reported that there is a minimum of 3 different QS inhibitors present in garlic extract, one of which was identified as an acyclic disulphur compound and exerted a strong antagonistic effect on LuxR-based QS (Rasmussen et al., 2005). The natural furocoumarins extracted from grapefruit juice can act as potent AI-1 and AI-2 inhibitors of *Salmonella enterica, E.coli* and *P. aeruginosa* (Girennavar et al., 2008). Also, with a similar structure to furanone, sodium ascorbate (Vitamin C) has been reported to have anti-QS activity by targeting the Las system of *Pseudomonas* spp., and subsequently inhibiting the rhamnolipid and signal production, thus reducing virulence factor production (El-Mowafy et al., 2014). The antimicrobial potential of these plant extracts has been widely used in the food industry, whereas their QS inhibition ability remains to be evaluated as alternative preservatives in food spoilage. Natural QS inhibitors with low level of toxicity are considered as an attractive option for food preservation, paving the way for the development and application to improve food quality and safety.

However, the naturally occurring QS inhibitors also have deficiencies, such as low levels of production and associated toxicity. Synthetic QSIs can overcome these limitations. Synthetic QSI target the biosynthetic pathway leading to signal production (e.g. inhibiting fatty acids biosynthesis, SAM biosynthesis, etc.), changes in signal's chain length and the AHL ring moiety, and analogs of the signaling molecules to block the receptor (Kalia, 2013). A variety of synthetic QSIs have been shown to be efficient in regulating bacterial activity, like cell-to-cell communication and biofilm formation (Geske et al., 2005; Lönn-Stensrud et al., 2007; Janssens et al., 2008; Rasch et al., 2007). However, even although the application of synthetic biotechnology promotes the development of the food industry (Li et al., 2019), there is still an increasing concern of consumers on the safety of synthetic food additives, leading to a high enthusiasm in natural inhibitors in food industry.

QS inhibitors do not kill microorganisms directly thus they are usually used together with biocidal agents (Bhardwaj et al., 2013), most of which are synthetic chemicals and usually not an option for food. QSI appears to be effective *in vitro* and various animal models (Kalia, 2013), thus it may have potential in the food industry. However, the occurrence of resistance to QSIs has been noticed (Maeda et al., 2012). Most current QSI research is based on type strains with adequate control experiments including toxicity tests and QS inhibition selector systems to obtain more reliable compounds that are true QS inhibitors (Defoirdt et al., 2013). In my study, not only reference type strains but also isolated strains from lamb were targeted for QSI investigation.

2.5 Conclusion

A number of psychrotrophic anaerobically growing spoilage bacteria have been identified in lamb. Due to their ability to grow and reproduce in oxygen limiting conditions at low temperatures, they are associated with the spoilage in fresh, chilled, vacuum-packed lamb. QS signals play an essential role in interaction amongst those spoilage bacteria to cause spoilage. Hydrolytic enzymes produced by spoilage bacteria are transported by an ABC transporter system that is triggered by AHL signals. When these enzymes become exoenzymes, they cause food deterioration. AHLs, as well as their involvement in diary and seafood spoilage, have been well studied. To the best of our knowledge, limited research has been carried out prior to the present study, investigating the involvement and mechanism of QS signals in lamb spoilage. Spoilage can be controlled when AHL signals are inhibited by QSIs.

Chapter Three: General Materials and Methods

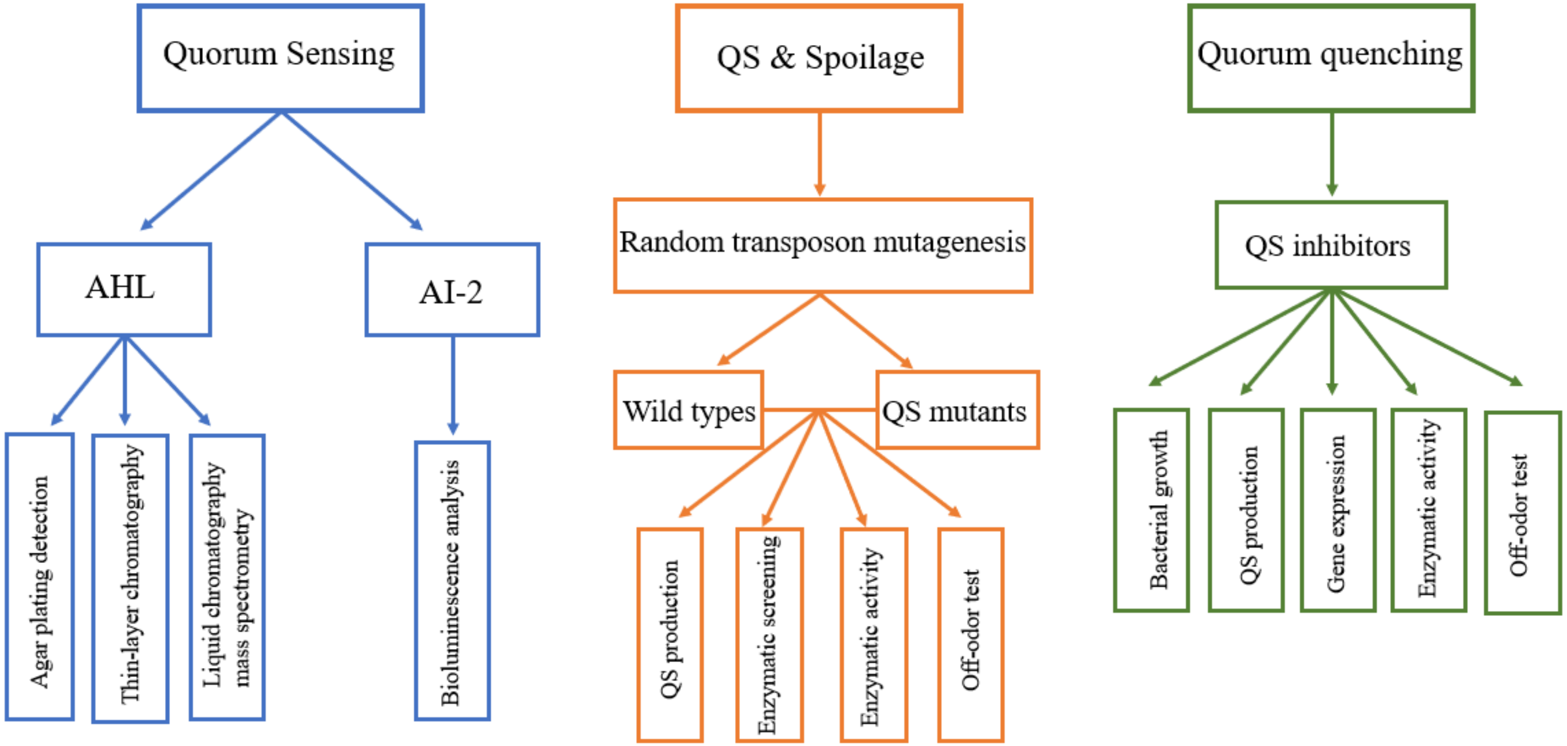
3.1 Research Framework

This research project was divided into three parts (Figures 3.1).

In the first part, a selection of common psychrotrophic anaerobically growing spoilage bacteria were tested to detect quorum sensing (QS) signaling molecules. *N*-Acyl - homoserine lactone (AHL) molecules were primarily screened using biosensors on agar plates, and when AHLs produced by specific bacteria were detected, molecules were further differentiated by thin-layer chromatography (TLC). In addition, liquid chromatography-tandem mass spectrometry (LCMS/MS) was used to characterize AHL signaling molecules produced by spoilage bacteria. AI-2 production by spoilage bacteria was detected by a bioluminescence assay using biosensors. Presence and characterization of QS signaling molecules in spoiled chilled vacuum-packaged (VP) lamb shoulder were carried out using the same methods.

In the second part, bacteria producing QS signaling molecules were selected, and random transposon mutagenesis was carried out to develop QS-related gene knockdown mutants of those bacteria. The wild type and mutant strains of each species were compared for QS molecule production and hydrolytic enzymatic activity.

In the third and final part, a group of QS inhibitors were selected for quorum quenching investigation. Their influences on the growth of spoilage bacteria, QS molecule production, and spoilage were studied. At the end of the study, the efficiency of one of the potential QS inhibitors (QSI), was analyzed to increase the shelf-life of an anaerobically stored lamb



Figures 3.1. The general workflow of this research project.

3.2 Bacterial strains

Three biosensor strains; *Agrobacterium tumefaciens* NTL4/pZLR4 (ATCC® BAA-2240), *Vibrio harveyi* MM32 (ATCC® BAA-1121), and *V. harveyi* BB170 (ATCC® BAA-1117) were obtained from the American Type Culture Collection (ATCC). *Chromobacterium violaceum* CV026 (NCTC 13278) was obtained from the National Collection of Type Cultures (NCTC) of Public Health England (Table 3.1). These biosensors were used in this study to detect QS signals by giving phenotypic color change. They were revived on specific agar plates and incubated at 30 °C aerobically overnight. *A. tumefaciens* NTL4/pZLR4 and *C. violaceum* CV026 were cultivated on Luria–Bertani agar (Fort Richard Laboratories, New Zealand) with 50 µg/mL gentamicin (Sigma Aldrich, USA), and 25 µg/mL kanamycin (Sigma Aldrich), respectively. Colonies were inoculated into respective broth media with antibiotics and incubated at 30 °C for overnight with agitation (160 rev min^{-1}), using a Labnet Orbit 1000 shaker (Total Lab Systems Ltd, New Zealand). Marine agar (Fort Richard Laboratories) was used for the proliferation of *V. harveyi* MM32 followed by inoculating into 10 mL of autoinducer bioassay (AB) medium (described in Section 3.5.1) and incubation at 30 °C overnight with agitation (160 rev min^{-1}).

The bacterial strains capable of spoilage and used in this research were either New Zealand strains isolated from chilled vacuum-packaged lamb, or from New Zealand Reference Culture Collection (NZRM) obtained from the Institute of Environmental Science and Research (ESR, New Zealand). The isolates were revived on Columbia Blood Agar (CBA) containing 5% sterile sheep blood (Fort Richard Laboratories, New Zealand) and incubated at 25 °C overnight. Stock solutions were stored in -80 °C freezers until use.

Table 3.1. Bacterial strains used in this study.

Bacterial strains	Description
Brochothrix thermosphacta NZRM3320	Spoilage bacterium; obtained from ESR
Brochothrix campestris NZRM3569	Spoilage bacterium; obtained from ESR
Hafnia alvei NZRM860	Spoilage bacterium, *Enterobacteriaceae*; obtained from ESR
Hafnia alvei FAM01	Spoilage bacterium, *Enterobacteriaceae*; isolate from chilled lamb cuts
Serratia liquefaciens NZRM861	Spoilage bacterium, *Enterobacteriaceae*; obtained from ESR
Serratia liquefaciens FAM02	Spoilage bacterium, *Enterobacteriaceae*; isolate from chilled lamb cuts
Carnobacterium divergens FAM03	Spoilage bacterium, lactic acid bacterium; isolate from chilled lamb cuts
Carnobacterium maltaromaticum FAM04	Spoilage bacterium, lactic acid bacterium; isolated from chilled lamb cuts
Rahnella aquatilis NZRM3285	Spoilage bacterium, *Enterobacteriaceae*; obtained from ESR
Yersinia enterocolitica NZRM3595	Spoilage bacterium, *Enterobacteriaceae*; obtained from ESR
Klebsiella oxytoca NZRM2301	Spoilage bacterium, *Enterobacteriaceae*; obtained from ESR
Shewanella putrefaciens NZRM3404	Spoilage bacterium; obtained from ESR
C. violaceum CV026	AHL sensor strain; violacein reporter; NCTC 13278
A. tumefaciens NTL4/pZLR4	AHL sensor strain; β-galactosidase reporter; ATCC® BAA-2240
V. harveyi BB170	AI-2 producer strain; bioluminescent reporter; ATCC® BAA-1117
V. harveyi MM32	AI-2 sensor strain; bioluminescent reporter; ATCC® BAA-1121

3.3 AHL standards

N-butyryl-homoserine lactone (C4-HSL), N-pentanoyl-L-homoserine lactone (C5-HSL), *N*-hexanoyl-homoserine lactone (C6-HSL), *N*-(3-oxohexanoyl) homoserine lactone (3-oxo-C6-HSL), *N*-octanoyl-homoserine lactone (C8-HSL) and *N*-decanoyl-L-homoserine lactone (C10-HSL) were procured from Sigma Aldrich, NZ and stock solutions were prepared to 1 mM in acetonitrile (Sigma Aldrich) and stored in -20 °C freezer.

3.4 QS inhibitor candidates

A selection of AHL inhibiting compounds such as 99% Acros trans-cinnamaldehyde, 95% Acros 4-Hydroxy-2(5H)-furanone, > 95% Fisher L-ascorbic acid, 99% Acros vanillin, and >98% Acros curcumin were obtained from Thermo Fisher Scientific, New Zealand. Fresh loose garlic was obtained from a local supermarket.

3.5 Growth media

3.5.1 Autoinducer bioassay (AB) medium

This medium was specifically used to dilute the *V. harveyi* biosensor strain, for bioluminescence detection of AI-2 signaling molecules. The recipe was as per "ATCC Medium: 2746 Autoinducer Bioassay (AB) Medium".

NaCl	17.5 g

$MgSO_4$	12.3 g
Deionized water	970 mL

All ingredients were mixed in a sterile 1 L Schott glass bottle and the pH of the solution was adjusted to 7.5 with 3M NaOH. The solution was autoclaved at 121℃ for 15 min and after cooling down, the following ingredients from sterile stocks were added:

1M potassium phosphate (pH 7.0)	10 mL
0.1M L-arginine	10 mL
Casamino acids	2.0 g
Glycerol	10 mL

3.5.2 Super optimal broth (SOB)

This medium is a nutrient-rich medium for bacterial growth. It was used for growing bacterial strains before and after introducing random transposon mutagenesis.

Tryptone	20.0 g
Yeast extract	5.0 g
NaCl	0.5 g
1M KCl	2.5 mL
1M $MgCl_2$	10.0 mL
Deionized water	Make it to 1 L

All the ingredients were mixed in a sterile 1 L Schott glass bottle and sterilized by autoclaving at 121ºC for 15 min.

3.5.3 Lamb juice and the cell-free meat extract (CFME) of lamb

The lamb juice used in this study was homogenized from lamb using the method described by Brightwell et al., 2007. As mentioned in Section 3.5.3, fresh lamb loin and lamb shoulder meat was procured from CMP Rangitikei Ltd., New Zealand, meat pieces were aseptically cut and put into sterile plastic bags. Saline (0.85% NaCl) was added to the meat at a ratio of 1:1 w/w. The lamb was homogenized using a peristaltic blender (Colworth stomacher Model 400) for 5 min, normal setting at ambient temperature. 10 mL aliquots of the homogenate were dispensed into sterile tubes and stored in the cold room until further use. Lamb juice from spoiled lamb pieces that had been stored in -1.5 °C fridge for over 120 days was also obtained for the study. The CFMEs were obtained by centrifugation of the homogenate at 5000 × g using a Heraeus Multifuge X3R centrifuge (Thermo Fisher Scientific, Germany) for 10 min at 4 ℃ and the supernatants were filtered through 0.22 μm sterile filters (Thermo Fisher Scientific). The sterile CFMEs were stored at -20 °C for further use. CFME from fresh lamb was used as blank control, and CFMEs from spoiled lamb or lamb with spoilage bacterial growth were used for QS signaling molecule detection

3.5.4 Bromocresol green (BCG) agar plates

These plates were used for protease screening indicated by BCG dye.

Lamb slurry	5.0 g
Yeast extract	1.5 g
NaCl	5.0 g
Bacto™ agar	15.0 g
Casein	10.0 g
BCG	0.15 g
Deionized water	1 L

All the ingredients were mixed and heated to dissolve in a 1 L Schott glass bottle and the bottle was sterilized and autoclaved at 121°C for 15 mins. The sterile media was cooled to 52°C in a water bath and poured into sterile Petri dishes, allowed to set then stored at 4 °C until further use. The storage time of these plates was 7 days.

3.5.5 Rhodamine B agar plates

These plates were used for lipase screening, indicated by Rhodamine B under ultraviolet (UV) light.

Lamb slurry	5.0 g
Yeast extract	1.0 g
Sucrose	1.0 g
$(NH_4)_2SO_4$	1.0 g
K_2HPO_4	1.0 g

$MgSO_4.7H_2O$	0.1 g
$FeSO_4.7H_2O$	0.1 g
Bacto™ agar	15.0 g
Rhodamine B solution (0.1%, W/V)	3.0 mL
Deionized water	To 1 L

All the ingredients were mixed and heated in a 1 L glass Schott bottle. The bottle was sterilized and autoclaved at 121°C for 15 mins and cooled to 52°C in a water bath. The agar was then poured into sterile Petri dishes, allowed to set the stored at 4 °C, for up to 7 days.

All the chemicals listed above, ≥95% (HPLC) Rhodamine B, and technical grade of casein were obtained from Sigma Aldrich. The Bacto™ of agar, yeast extracts and tryptone used for media preparation were obtained from Fort Richard Laboratories.

3.6 Water

For general use and preparation of media, deionized water purified through a Millipore, Elix™ 10 water purification was used in this study. For molecular applications, distilled, DNase and RNase free water (Invitrogen Ltd., USA) was used.

3.7 Anaerobic conditions

Anaerobic cultures were prepared in anaerobic culture tubes (Chemglass Life Sciences, USA). An anaerobic chamber containing $H_2/CO_2/N_2$ (5:5:90, Don Whitley Scientific, UK) was used. The atmosphere inside the chamber was auto-monitored to ensure the chamber remained oxygen-free.

3.8 Growth of spoilage bacteria

All the spoilage bacteria listed in Table 3.1 were grown in different conditions (specified in Section 4.1.2). The cultures were centrifuged at 5000 × *g* for 10 min at 4 °C and the cell-free supernatants were obtained by filtering supernatant through 0.22 µm sterile filters (Thermo Fisher Scientific, Australia). The optical density (OD) at 590 nm was measured by a nanophotometer (Implen, Germany) and correlated with plate counts. The calibration of OD values against bacterial numbers was also conducted.

3.9 AHL detection using 96 well microplate assay

In 96 well microplates, *Agrobacterium tumefaciens* NTL4/pZLR4 was used as the biosensor strain to detect AHL signaling molecules produced by spoilage bacterial strains. 50 µL of *A. tumefaciens* NTL4/pZLR4 was grown overnight in 200 µL of Luria broth (LB) medium (supplemented with 50 µg/mL gentamicin), with 50 µL cell-free extracts (CFEs) of spoilage bacterial strains and 10 µL of X-gal (40 µg/ml) solution at 30 °C. The β-galactosidase activity was detected to infer AHL production.

3.10 Measurement of β-galactosidase activity

AHL production was quantitatively compared by β-galactosidase expression in cell-free solutions, measured by a simple absorbance assay. In the presence of AHL signaling molecules, the expression of β-galactosidase resulted in a green-blue precipitate. Briefly, the overnight mixed cultures (Section 3.9) were centrifuged at 8000 × *g* for 5 min and the supernatant was discarded. 100 µL of 20 mM KH_2PO_4 (pH 7.0) was added into the precipitate, mixed well and left for 2 min. The absorbance was measured at 635 nm by spectrophotometer (Shimadzu UV-2401PC UV-VIS, USA). The absorbance value was recorded as the induction of β-galactosidase activity.

3.11 AHL detection using an agar plating bioassay

Two biosensor strains *C. violaceum* CV026 and *A. tumefaciens* NTL4/pZLR4 were used to detect AHL signaling molecules. AHL detection was carried out using a plating bioassay (McClean et al., 1997) with slight modifications. Briefly, 10 mL of semi-solid (0.7% v/w) Bacto™ agar, sterilized by autoclaving at 121 °C for 15 min, was inoculated with 100 µL of overnight biosensor culture and poured immediately over a pre-made solid Bacto™ agar plate (1.5% v/w). For the *A. tumefaciens* NTL4/pZLR4 bioassay, 100 µL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (aslo known as X-gal, Life Technologies, USA) solution (40 µg/mL in dimethylsulfoxide, Life Technologies) was also added into the sterile semi-solid agar. Wells of diameter 6.0 mm were punched on the inoculated plate using a sterile pipette tip. 60 µL of cell-free supernatant of spoilage bacteria or the previously prepared CFME from spoiled chilled VP lamb shoulder was added to these wells. The plates were incubated at 30 °C aerobically overnight. All media were supplemented with relevant antibiotics.

3.12 AHL differentiation by Thin-layer chromatography (TLC)

Separation and characterization of AHL production was done using thin-layer chromatography according to (Shaw et al., 1997) with the modifications. Briefly, 2.5 mL of the cell-free supernatant of tested spoilage bacteria that showed AHL signal production, as well as CFME of the spoiled VP lamb shoulder were extracted with an equal amount (2.5 mL) of acidified ethyl acetate (0.1% v/v acetic acid, Merck, UK) twice. AHL molecules were extracted by this liquid-to-liquid method. The combined extracts were centrifuged and evaporated under nitrogen gas to dry. The extract residues were re-suspended in 1 mL of acidified ethyl acetate. To determine AHL profiles, 10 μL of each re-suspended solvent was loaded onto TLC silica gel plates (60 RP-18, F254S 20 aluminum sheets 20 × 20 cm, Merck, New Zealand). The chromatograms were developed in methanol: water (60:40 v/v) and air-dried. The dried TLC plates were overlaid with a thin film of sterile Bacto™ agar (0.7% w/v) containing the relevant biosensor strains and incubated at 30 °C overnight for optimal signal development.

3.13 AHL identification by liquid chromatography-tandem mass spectrometry (LCMS/MS)

AHL profiles were identified by LCMS/MS. AHL molecules were extracted from 5 mL of the cell-free supernatants of spoilage bacteria, as well as the CFME of the spoiled VP lamb shoulder, by the same amount (5 mL) of acidified ethyl acetate, as per Section 3.9. Samples were kept at 4 °C until injection, and 10 μL of each sample was injected onto an Ascentis® C18, 3 μm HPLC column, 15 cm × 2.1 mm (Sigma Aldrich) via a Thermo Electron Surveyor auto-sampler (Thermo Fisher Scientific). Separation was obtained using a gradient of 1% acetic acid in water to 1% acetic acid in acetonitrile at a flow rate of 0.3 mL/min. Detection was achieved by multiple reaction monitoring (MRM) in positive-ion mode. The LC fractions were subjected to electrospray ionization tandem mass-spectrometry (ESI-MS-MS) (Ortori et al., 2011). Initially, each parent ion was screened by MS/MS fragmentation for an ion with an m/z of 102, putative AHLs with a breakdown ion with an m/z of 102 were observed as HSL derivatives. In mass spectrometry, since the charge number for ions "z" is almost always 1, the "m/z" value is often considered to be the mass.

3.14 AI-2 detection by bioluminescence bioassay

AI-2 signal molecules were detected using a bioluminescence-based broth bioassay, utilizing the *V. harveyi* MM32 biosensor strain. The method followed Yu et al., 2013, with modifications. Briefly, the overnight grown culture of *V. harveyi* MM32 was diluted to 1:1000 with fresh AB medium (as described in Section 3.5.1) and 90 µL of dilute cell suspension was mixed with 10 µL of each of the cell-free extracts of spoilage bacteria and the spoiled lamb shoulder in a Costar 96-well black polystyrene microtitre plate (Sigma Aldrich). The microtiter plate was incubated at 30 °C in the dark and the luminescence of each sample was measured using a Wallac Victor 2 Model 1420 Multilabel photon counter (PerkinElmer Life Sciences, USA), half-hourly until the negative control exhibited an increase in luminescence. The intensity of bioluminescence was calculated in Relative Light Units (RLU), indicated by counts of photons per second (cps).

3.15 Random transposon mutagenesis

Random transposon mutagenesis was used in this study to disrupt relevant genes in the spoilage bacteria. Cultures were grown in 25 mL of SOB (Section 3.5.2) and incubated overnight at 30 °C with agitation (160 rev min^{-1}), according to Wilson and Keefe, 2001. 250 µL of the overnight culture was added to 25 mL of SOB and shaken (160 rev min^{-1}) at 30 °C until the optical density of the culture reached 0.5-0.7 at 600 nm. The culture was then placed on ice for 15 min then centrifuged at 5000 rpm for 10 min at 4 °C. The supernatants were discarded and the pellets were nitrogen-dried to evaporate the remaining liquid. To the cell pellets, 25 mL of 10% glycerol diluted in DNase-free water was added and the cells were suspended by gently pipetting up and down. The suspensions were again centrifuged at 7,500 x *g* for 10 min at 4 °C and the supernatants were discarded. Again, 25 mL of 10% glycerol was added to pellets and discarded again after centrifugation. The residual cells were mixed with the remaining glycerol solution by gently pipetting up and down. 100 µL of the final test sample was transferred to a microcentrifuge tube for electroporation or stored at -80°C until further use.

To mutagenize bacteria, 25 µL of the test sample was transferred to a cold electroporation cuvette (previously bathed in ice) and 1 µl of the EZ-Tn5 <KAN-2> Tnp Transposon (Lucigen, USA) was added (Figure 3.2). The sample was electroporated at 2.5 kV, 200 Ω and 25 µF. Cells were immediately revived by adding 975 µl of pre-warmed SOB and incubated at 30 °C for 2 h with shaking at 200 rev min^{-1}. 100 µL of the transformed cells were then plated onto pre-warmed LB plates with 50 µg/mL kanamycin and incubated overnight at 35°C to obtain mutant strains.

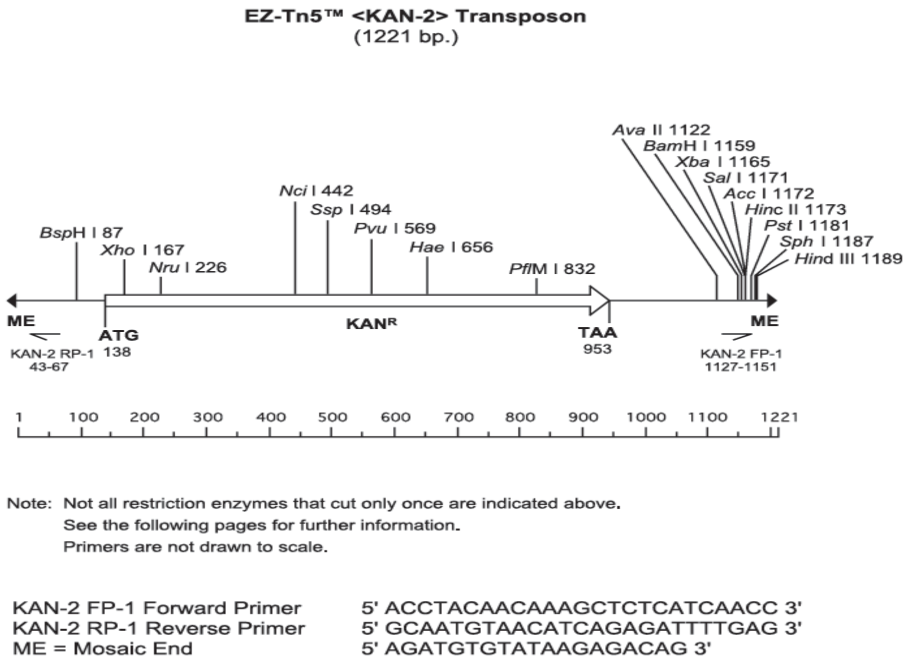


Figure 3.2. EZ-Tn5™ <KAN-2> Transposon used in this study. Retrieved from "Protocol for EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit".

3.16 DNA extraction, PCR and purification

Nucleic acid was extracted using a High Pure PCR Template Preparation Kit (Roche, Germany). The extraction was done as per Section 2.6 “Isolation of nucleic acid from bacteria or yeast” and Section 2.8 “Protocol for washing and elution” from the manufacturer’s instructions (Rosche, 2018).

The extracted DNA was amplified by PCR, in a T100™ thermocycler (BioRad, USA). Specific genes and primers will be listed in the later chapters. All the reagents and oligonucleotides used were obtained from Life Technologies Ltd, USA. The primers specific to putative quorum sensing regulatory genes were targeted and designed using the National Centre for Biotechnology Information (NCBI) with Primer3 software searched in the NCBI database. Primer3 has been widely used for primer design, in numerous high-throughput genomics applications, web services, and software packages (Untergasser et al., 2012). The primers of EZ-Tn5 transposon were provided in Lucigen EZ-Tn5™ <KAN-2> Tnp Transposome™ kit. Each 20 µL of reaction included DNase free water, 1 µmol of each primer, 1.5 mM of MgCl, 200 µmol of dNTPs, 1 unit of *Taq* DNA polymerase and 1X buffer.

The PCR products (~ 5 µL) were visualized on a 0.8% (w/v) agarose gel in 0.5 x TBE buffer (Invitrogen), stained with ethidium bromide (BioRad) under UV transillumination. 5 µL of DNA molecular weight 1kb Plus marker (Invitrogen) was loaded onto the gel to determine the size of the PCR products.

PCR products of desired size were purified from bands excised from agarose gels as per the manufacturer's instructions (Qiagen, 2010), using a QiaQuick® gel extraction kit (Bio-strategy, New Zealand). After gel extraction and purification, the PCR products were sent to Massey Genome Service, Palmerston North for sequencing.

3.17 Screening of exogenous enzymes by agar plate assays

For protease screening, 20 µL of suspensions of bacterial isolates in LB broth was loaded onto blank paper discs (Thermo Fisher Scientific) and placed on a BCG agar plate (Section 3.5.4). The plates were incubated at 30°C for 24 - 48 h to check the proteolysis by phenotypic color change.

To screen for lipolysis, 20 µL of suspensions of bacterial isolates in LB broth were loaded on blank paper discs and placed on Rhodamine B agar plates (Section 3.5.5) and incubated at 30 °C for 48 h. Lipolysis made a phenotypic color change around the loading spot and when the plates were read under UV light, the zones were indicated as orange.

3.18 Semi-quantitative measurement of enzymatic activity

Protease activity was measured using the protocol by Tsuchida et al., 1986, with a slight modification. 200 µL of the culture was mixed with 500 µL of 1% (w/v) of azocasein (Sigma Aldrich) in 50 mM phosphate buffer and incubated at 40 °C for 20 min. 1 mL of 10% (w/v) trichloroacetic acid (TCA, BioRad) was added to terminate the enzymatic reaction at room temperature for 15 min. The mixture was centrifuged at 7,500 × g for 5 mins and the supernatant was separated and mixed with 2.5 mL of 0.5M Na_2CO_3. 1 mL of Folin's reagent (sodium 1, 2-naphthoquinone-4-sulfonate, Sigma Aldrich) was added into the mixture, incubated for 30 mins at room temperature in the dark until a blue color was developed. The absorbance was then measured at 440 nm using a nanophotometer (Implen, Germany).

Lipase activity was measured by using lamb fat as the substrate. Briefly, the protocol by Shirai and Jackson, 1982 was followed with some modifications. 250 μL of the culture was mixed with 750 μL of 1% (w/v) of pre-melted lamb fat in 50 mM phosphate buffer. The mixture was then incubated in a 40 °C water bath for 20 minutes. 1 mL of 10% (w/v) TCA was added to terminate the enzymatic reaction at room temperature for 15 mins. The mixture was centrifuged at 7,500 x *g* for 5 mins and to the supernatant 2.5 mL of 0.5M Na_2CO_3 was added and mixed thoroughly. 1 mL of p-nitrophenyl butyrate (Sigma Aldrich) was added and incubated for 30 min at room temperature in the dark until a yellow color was developed. The absorbance was measured at 400 nm using an implen nanophotometer.

3.19 Gene expression studies by RT-qPCR

After the strains grew under different conditions specified in later chapters, the total RNA of the strain was extracted using High Pure RNA Isolation Kit (Roche) following the manufacturer's instructions. To assess the purity of the eluted total RNA, gel electrophoresis was carried out. If there was any band shown on the gel, indicating DNA contamination in the sample, DNase treatment was implemented to the RNA isolation. RNA integrity and quantity were measured using an Invitrogen Qubit 4 fluorometer (Thermo Fisher Scientific) according to the manufacturer's protocol.

To synthesize cDNA, for each 8 µL of reaction a standardized quantity of 5 µg RNA per sample, 1 µL of random hexamer (50 ng/µL, Invitrogen), 1 µL of annealing buffer (Invitrogen) and nuclease-free H_2O (Invitrogen) were used. After incubation for 5 min at 65 °C, the mixture was quickly cooled on ice for 1 min. 2X First-Strand Reaction Mix (10 µL, Invitrogen) and 2 µL of SuperScript III/RNase OUT Enzyme Mix (Invitrogen) were added followed by incubation for 10 min at 25 °C, and 50 min at 50 °C, then heat-inactivation of the reverse transcriptase at 85 °C for 5 min.

For qPCR amplification, an Applied Biosystems™ ViiA™ 7 Real-Time PCR system (Thermo Fisher Scientific) was used in transparent 96 well PCR plates (Thermo Fisher Scientific). Into each well 2X SYBR® Green PCR Master Mix™ (12.5 µL, Thermo Fisher Scientific), respective primer pair mix (1 µL, Table 5.1), and the respective cDNA (0.2 µL) were pipetted to 11.3 µL of nuclease-free H_2O (Life Technologies). The plates were sealed by ABsolute qPCR Plate Seals (Thermo Fisher Scientific), the cDNA was amplified in triplicate per candidate gene and 3X biological replicates were prepared. The housekeeping gene 16S rRNA was used as an internal reference. On the same qPCR plate, an initial stabilization at 50 °C for 2 min, a heat denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation for 15 s at 95 °C, annealing for

30 s at 60°C and extension for 30 s at 72°C; with a final extension for 10 min at 72°C were carried out. At the end of each qPCR reaction, SYBR®Green fluorescence was measured at 521 nm. The relative expression of the targeted genes was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

The database accession numbers of each target gene, and the sequences of each oligonucleotide used in this study are specified in Table 5.1.

3.20 Effect of QS inhibitors on bacterial growth

The growth of two *Enterobacteriaceae H. alvei* NZRM860 and *S. liquefaciens* NZRM861 with the addition of potential AHL inhibiting compounds in 5 mL of LB at 30 °C for 8 hours was compared against control cultures (without the addition of those AHL inhibitors). The bacterial growth of was measured by absorbance at 595nm. The growth of *Agrobacterium tumefaciens* NTL4/pZLR4 in 5 mL of LB at 30 °C for 8 hours with and without AHL inhibitors was also examined.

In fresh lamb juice, two *Enterobacteriaceae* isolates from lamb, *H. alvei* FAM01 and *S. liquefaciens* FAM02, were grown at 30 °C for 8 h with and without AHL inhibitors. The effect of the inhibitors on bacterial growth was measured by absorbance at 595nm..

3.21 Effect of cinnamaldehyde on anaerobically stored lamb spoilage

Cinnamaldehyde was added to anaerobically stored lamb juice (in anaerobic culture tubes) and vacuum-packed lamb coupons.

1) Lamb juices - 10 g of fresh lamb shoulder samples were removed aseptically and subsequently homogenized using a stomacher (Colworth stomacher Model 400) for 10 min, normal setting at ambient temperature.

2) Lamb coupons - fresh lamb shoulder samples were aseptically cut according to a cutting pattern model (surface area with diameter 25mm). Each lamb coupon weighed around 15 g.

10 ppm of cinnamaldehyde (in meat juice, cinnamaldehyde was dissolved in acetonitrile to 1% and finally concentrated to 0.001% in meat juice; in meat coupons, 15 µL of 1 mg/mL cinnamaldehyde in acetonitrile was spread over the surface) was added into these two types of lamb stored at different temperatures (specified in Section 6.2.6) to check its impact on lamb spoilage.

3.22 Statistical analyses

All the experiments in this study were conducted on three separate occasions with the measurements obtained in triplicate. Data represented the mean values of these repeats and the differences between the control and test samples were analyzed by one-way repeat ANOVA with $p \leq 0.05$, using MATLAB software (Shardt, 2015).

Chapter Four: Detection of Quorum Sensing Signals during Chilled Vacuum Packaged Lamb Spoilage

4.1 Introduction

In a microbial community, microorganisms communicate by producing diffusible QS signaling molecules. Two of the QS signaling molecules commonly associated with spoilage bacteria are acyl-N-homoserine lactones (AHLs) and furanosyl borate diester (AI-2).

AHLs are only produced by some Gram-negative species (Hawver et al., 2016). AI-2 is derived from the recycling of S-adenosyl-homocysteine to homocysteine. It is universal in almost all microorganisms and was considered as an interspecies communication signal (Federle, 2009).

The common method for the detection of AHL signaling molecules is screening by agar plates using biosensor strains, e.g. *Chromobacterium violaceum* CV026 (McClean et al., 1997) and *Agrobacterium tumefaciens* NTL4/pZLR4 (Cha et al., 1998). These biosensors are mutants of respective wild types and give visible color changes when they detect AHL signaling molecules. *C. violaceum* CV026 mainly detects non-substituted AHLs with short chains by expressing violacein genes producing a purple color. In comparison to *C. violaceum* CV026, *A. tumefaciens* NTL4/pZLR4 has a much wider detection range. It does not only detect AHL molecules with acyl side chains with 4 to 12 carbons but also with 18 carbons (Cha et al., 1998). Different AHL signaling molecules can be attained using thin-layer chromatography (Bruhn et al., 2004). AI-2 production can be monitored by a biosensor strain using a bioluminescence assay (Lu et al., 2004).

Since the beginning of the 21st century, QS signaling molecules, especially AHLs, have been detected in meat spoilage. Bruhn et al., in 2004 found an AHL similar to 3-oxo-C6-HSL in vacuum-packaged meat and suggested it was a major contributor to meat spoilage, while Liu et al., in 2006 detected a broad range of AHLs, in aerobically chill-stored beef. In Lu's study, *Vibrio harveyi* BB170 was used to sense the AI-2 activity in food including beef patties, chicken breast, turkey patties, frozen fish (Lu et al., 2004b).

In this part of study, the QS production in spoiled chilled VP lamb pieces and QS production from a few spoilage bacteria commonly associated with lamb spoilage were investigated. It was aimed to explore the convergence of the QS production and discuss the possibility beyond this linkage.

Studying QS in lamb under laboratory conditions is also challenging, as microbial signaling can be affected by many factors. Storage temperature, meat pH, fat level and presence of oxygen are some of the decisive factors during meat storage and preservation. The purpose of this part of study was also to examine the effect of different relevant physicochemical factors (e.g. atmosphere, temperature, pH and fat content) on QS molecule production in lamb.

4.2 Materials and Methods

4.2.1 Lamb juice

The lamb juice used in this study for bacterial growth was homogenized from fresh lamb pieces as per Section 3.4.3. Aseptic methods were used to ensure the lamb juice as sterile as possible.

4.2.2 Growth of spoilage bacteria

H. alvei NZRM860, *S. liquefaciens* NZRM861, *R. aquatilis* NZRM3285, *Y. enterocolitica* NZRM3595, *K. oxytoca* NZRM2301, *S. putrefaciens* NZRM3404, *B. thermosphacta* NZRM3320, *B. campestris* NZRM3569, C. divergens FAM03 and *C. maltaromaticum* FAM04 were grown in nutrient broth and fresh lamb juices at 25 °C for 24 h, 20 °C for 48 hours and 0 °C for 6 weeks, aerobically in universal tubes and anaerobically in anaerobic tubes. Lamb juices with different pH values were extracted from the loin (pH 5.7) and shoulder (pH 6.3). When testing the effect of fat, pH values of lamb juice were equalized to 6.3 by adding ~ 5 µL of 1M sodium hydroxide into 500 mL of lamb loin juice.

The growth was determined as per Section 3.7.

4.2.3 Detection of AHL molecules by well diffusion agar plating assay

The production of AHL signaling molecules from spoilage bacteria obtained under different conditions mentioned above, (i.e., in presence or absence of oxygen; at ambient or chilled temperature, with different levels of fat content and pH), and production in CFME of spoiled lamb (per Section 3.4.3) were analyzed using the well diffusion plate assay described in Section 3.10. A reference standard C6-HSL was used as a positive control, and the growth medium was used as a negative control.

4.2.4 Determination of AHL profiles by thin-layer chromatography (TLC)

Separation and characterization of the AHL molecules detected in spoilage bacteria and spoiled lamb was carried out using TLC using a method described in Section 3.11. C4-HSL, C6-HSL, 3-oxo-C6-HSL, and C8-HSL were applied to the TLC plates as reference AHL molecules. According to the clarity of color development on the TLC plate, some of the spoilage bacteria required a further extraction step using acidified ethyl acetate (0.1% v/v acetic acid) to minimize the effect of metabolites in lamb juices.

4.2.5 Identification and characterization of AHL molecules by liquid chromatography-tandem mass spectrometry (LCMS/MS)

Different types of AHL signaling molecules produced were identified by LCMS/MS (Section 3.12). In this method, five AHL standards, C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, and C10-HSL were tested along with the ethyl acetate extracts of *H. alvei* NZRM860, *S. liquefaciens* NZRM861, and spoiled lamb. The MS profiles of AHL molecules found in spoilage bacteria and spoiled lamb were compared with those of known reference AHL signals.

4.2.6 Bioluminescence bioassay for AI-2 detection

AI-2 signal molecules were detected using the method described in Section 3.13. Briefly, the production of AI-2 signaling molecules in spoiled lamb and from spoilage bacteria obtained under different growth conditions (Section 4.2.1) were analyzed. The impact of variables such as oxygen, temperature, fat content, and pH were investigated on levels of AI-2 production.

4.3 Results

4.3.1 Growth of spoilage bacteria in various conditions

The absorbance of each spoilage bacterium at stationary growth phase was measured at 590 nm and is shown in Tables 4.1A-E. In Table 4.1A, when spoilage bacteria were homogeneously and aerobically grown at 25 °C overnight, the bacterial growth in lamb loin meat juice (pH 5.7) was lower than the growth in nutrient broth (pH ~7), the most obvious decline was found in *S. putrefaciens*. The growth of *Carnobacterium* spp. did not show too much difference whenever they were grown in nutrient broth or lamb juice.

Table 4.1A. OD_{590nm} of spoilage bacteria grown in nutrient broth and fresh lamb loin meat juice aerobically at 25 °C for 24 hours.

Spoilage bacterial strains	nutrient broth	lamb loin juice
Brochothrix thermosphacta NZRM3320	1.55 (± 0.04)	1.36 (± 0.03)
Brochothrix campestris NZRM3569	1.52 (± 0.01)	1.38 (± 0.04)
Carnobacterium divergens FAM03	1.58 (± 0.03)	1.44 (± 0.03)
Carnobacterium maltaromaticum FAM04	1.61 (± 0.04)	1.57 (± 0.05)
Hafnia alvei NZRM860	1.77 (± 0.04)	1.34 (± 0.07)
Serratia liquefaciens NZRM861	1.71 (± 0.03)	1.26 (± 0.01)
Rahnella aquatilis NZRM3285	1.51 (± 0.04)	1.39 (± 0.01)
Yersinia enterocolitica NZRM3595	1.48 (± 0.07)	1.17 (± 0.04)
Klebsiella oxytoca NZRM2301	1.49 (± 0.01)	1.36 (± 0.06)
Shewanella putrefaciens NZRM3404	1.55 (± 0.03)	0.51 (± 0.04)

The mean ± standard deviation of triplicates was shown in the tables.

When spoilage bacteria were grown in lamb juices at 20 °C for 48 hours, the bacterial growth in the presence or absence of oxygen was compared and results were shown in

Tables 4.1B and 4.1C. In general, bacterial growth reduced when spoilage bacteria were grown anaerobically rather than aerobically. However, in lamb loin meat juice (pH 5.7), the growth of *S. putrefaciens* was very low and there was not much difference in growth when it was grown in different oxygen conditions. In lamb shoulder meat juice (pH 6.3), the growth of *S. putrefaciens* was higher in the presence of oxygen, rather than when it was grown anaerobically. *Carnobacterium* spp. did not grow differently when they were grown in either lamb juice anaerobically or aerobically. Overall, the bacterial growth in high pH lamb shoulder meat juice (6.3) was found higher than the growth in low pH lamb loin meat juice (5.7), except two *Carnobacterium* species.

Table 4.1B. OD_{590nm} of spoilage bacteria grown in lamb loin meat juice at 20 °C for 48 h.

Spoilage bacterial strains	Aerobically	Anaerobically
Brochothrix thermosphacta NZRM3320	1.33 (± 0.02)	1.16 (± 0.01)
Brochothrix campestris NZRM3569	1.38 (± 0.04)	1.08 (± 0.03)
Carnobacterium divergens FAM03	1.46 (± 0.02)	1.41 (± 0.02)
Carnobacterium maltaromaticum FAM04	1.48 (± 0.02)	1.44 (± 0.02)
Hafnia alvei NZRM860	1.48 (± 0.01)	1.34 (± 0.01)
Serratia liquefaciens NZRM861	1.37 (± 0.03)	1.26 (± 0.02)
Rahnella aquatilis NZRM3285	1.11 (± 0.03)	0.93 (± 0.01)
Yersinia enterocolitica NZRM3595	1.27 (± 0.04)	1.17 (± 0.03)
Klebsiella oxytoca NZRM2301	1.31 (± 0.02)	1.26 (± 0.01)
Shewanella putrefaciens NZRM3404	0.48 (± 0.05)	0.51 (± 0.04)

Table 4.1C. OD_{590nm} of spoilage bacteria growing in lamb shoulder meat juice at 20 °C for 48 h.

Spoilage bacterial strains	Aerobically	Anaerobically
Brochothrix thermosphacta NZRM3320	1.53 (± 0.02)	1.26 (± 0.01)
Brochothrix campestris NZRM3569	1.57 (± 0.02)	1.31 (± 0.02)
Carnobacterium divergens FAM03	1.49 (± 0.01)	1.42 (± 0.02)
Carnobacterium maltaromaticum FAM04	1.52 (± 0.03)	1.48 (± 0.04)
Hafnia alvei NZRM860	1.70 (± 0.04)	1.41 (± 0.04)
Serratia liquefaciens NZRM861	1.67 (± 0.03)	1.38 (± 0.03)
Rahnella aquatilis NZRM3285	1.21 (± 0.02)	1.02 (± 0.01)
Yersinia enterocolitica NZRM3595	1.33 (± 0.01)	1.21 (± 0.02)
Klebsiella oxytoca NZRM2301	1.68 (± 0.03)	1.44 (± 0.04)
Shewanella putrefaciens NZRM3404	1.39 (± 0.04)	1.18 (± 0.01)

When spoilage bacteria were grown in lamb juices at 0 °C for 6 weeks, the bacterial growth in the presence or absence of oxygen was shown in Tables 4.1D and 4.1E. Similar results were demonstrated, comparing to Tables 4.1B and 4.1C. Bacterial growth reduced in the absence of oxygen, and in lamb loin meat juice. The growth of *S. putrefaciens* in lamb loin meat juice was again found very low and with not much difference when it was grown either anaerobically or aerobically. However, in lamb shoulder meat juice, the growth of *S. putrefaciens* showed difference, higher in aerobic conditions than in anaerobic conditions. *Carnobacterium* spp. did not show a difference in growth when they were grown in either lamb juice anaerobically or aerobically. Overall, the bacterial growth at 0 °C for 6 weeks was found lower than the growth at 20 °C for 48 h (shown in Tables 4.1B and 4.1C), except for the psychrophile *R. aquatilis*. The growth of *R. aquatilis* was higher when it was grown at 0 °C for 6 weeks.

Table 4.1D. OD_{590nm} of spoilage bacteria growing in lamb loin meat juice at 0 °C for 6 weeks.

Spoilage bacterial strains	Aerobically	Anaerobically
Brochothrix thermosphacta NZRM3320	0.89 (± 0.02)	0.61 (± 0.02)
Brochothrix campestris NZRM3569	0.87 (± 0.04)	0.63 (± 0.03)
Carnobacterium divergens FAM03	0.76 (± 0.02)	0.74 (± 0.04)
Carnobacterium maltaromaticum FAM04	0.81 (± 0.03)	0.80 (± 0.04)
Hafnia alvei NZRM860	1.12 (± 0.04)	0.81 (± 0.03)
Serratia liquefaciens NZRM861	0.91 (± 0.03)	0.62 (± 0.03)
Rahnella aquatilis NZRM3285	1.28 (± 0.03)	1.11 (± 0.03)
Yersinia enterocolitica NZRM3595	0.73 (± 0.02)	0.61 (± 0.03)
Klebsiella oxytoca NZRM2301	0.78 (± 0.02)	0.69 (± 0.02)
Shewanella putrefaciens NZRM3404	0.28 (± 0.01)	0.25 (± 0.02)

Table 4.1E. OD_{590nm} of spoilage bacteria growing in lamb shoulder meat juice at 0 °C for 6 weeks.

Spoilage bacterial strains	Aerobically	Anaerobically
Brochothrix thermosphacta NZRM3320	0.96 (± 0.03)	0.85 (± 0.02)
Brochothrix campestris NZRM3569	1.01 (± 0.03)	0.89 (± 0.02)
Carnobacterium divergens FAM03	0.89 (± 0.02)	0.87 (± 0.02)
Carnobacterium maltaromaticum FAM04	0.84 (± 0.03)	0.85 (± 0.01)
Hafnia alvei NZRM860	1.25 (± 0.03)	0.96 (± 0.03)
Serratia liquefaciens NZRM861	1.04 (± 0.03)	0.88 (± 0.02)
Rahnella aquatilis NZRM3285	1.39 (± 0.03)	1.21 (± 0.01)
Yersinia enterocolitica NZRM3595	0.91 (± 0.02)	0.79 (± 0.02)
Klebsiella oxytoca NZRM2301	0.88 (± 0.02)	0.76 (± 0.02)
Shewanella putrefaciens NZRM3404	0.91 (± 0.03)	0.85 (± 0.03)

A representative bacterial growth in different growth media at 20 °C for 48 hours was demonstrated in Figure 4.1. The growth of most spoilage bacteria was significantly (p-value < 0.05) reduced in lamb loin meat juice, compared with growth in nutrient broth and lamb shoulder meat juice. The largest reduction in growth was seen in *S. putrefaciens* when it was cultivated in lamb loin meat juice. The growth of all spoilage bacteria was highest in nutrient broth. When pH decreased, the bacterial growth was reduced.

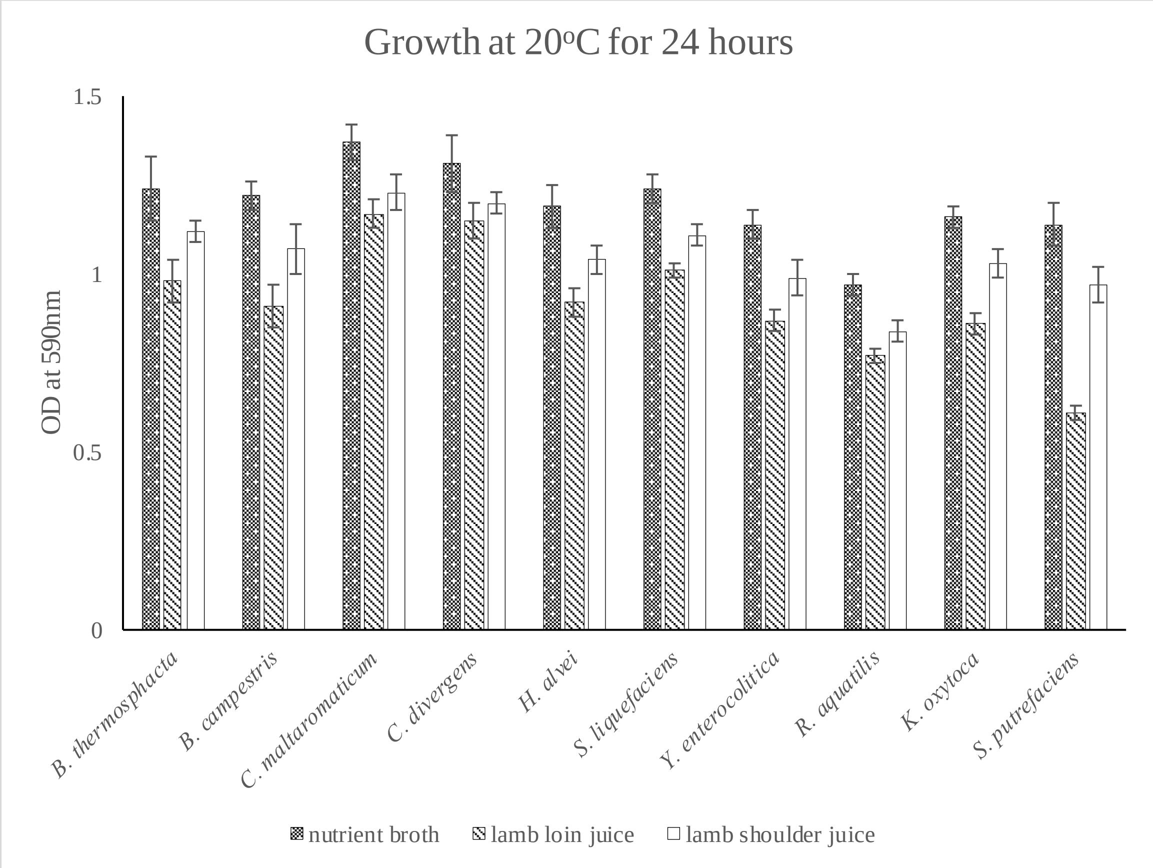


Figure 4.1. OD590nm of spoilage bacteria growing in three different substrates at 20 °C overnight. Bars show the mean of triplicates and standard deviations are indicated as error bars.

After comparing to the bacterial growth in nutrient broth, the overall growth of spoilage bacteria in lamb juices under different conditions were summarised in Table 4.2. Anaerobic condition, low pH in lamb loin meat juice and chill temperature 0 °C reduced most of bacteria, including two *Brochothrix* spp., and *Enterobacteriaceae*. However, the pH and oxygen variation did not impact the growth of *Carnobacterium* species very much. The only factor that influenced the growth of *Carnobacterium* species was temperature, their growth reduced when they were grown at 0 °C. *R. aquatilis* was the only bacterium that grew better at 0 °C than 20 °C, showing its psychrophilic feature.

S. putrefaciens did not grow well in low pH (5.7) lamb loin meat juice.

Table 4.2. Growth of spoilage bacteria in different conditions. The growth in lamb juices was compared to that in nutrient broth.

	Lamb loin meat juice aerobically at 20 °C	Lamb loin meat juice anaerobically at 20 °C	Lamb shoulder meat juice aerobically at 20 °C	Lamb shoulder meat juice anaerobically at 20 °C	Lamb loin meat juice aerobically at 0 °C	Lamb loin meat juice anaerobically at 0 °C	Lamb shoulder meat juice aerobically at 0 °C	Lamb shoulder meat juice anaerobically at 0 °C
B. thermosphacta NZRM3320	++	++	+++	++	++	+	++	+
B. campestris NZRM3569	++	++	+++	++	++	+	++	+
C. divergens FAM03	+++	+++	+++	+++	++	++	++	++
C. maltaromaticum FAM04	+++	+++	+++	+++	++	++	++	++
H. alvei NZRM860	++	++	+++	++	++	+	++	+
S. liquefaciens NZRM861	++	++	+++	++	++	+	++	+
R. aquatilis NZRM3285	++	++	+++	++	++	+	++	+
Y. enterocolitica NZRM3595	++	+	++	+	++	++	++	++
K. oxytoca NZRM2301	++	++	+++	++	++	+	++	+
S. putrefaciens NZRM3404	+	+	+++	++	+	+	++	+

+: limited growth <10^3 CFU/mL ++: moderate growth 10^3 CFU/mL – 10^5 CFU/mL +++: good growth >10^5 CFU/mL

4.3.2 AHL detection by well diffusion agar plating assay

The presence of AHL molecules was detected on well diffusion agar plates (Figure 4.2). On the plates, when AHL molecules were produced by the spoilage bacteria, a purple halo around the well was shown. It is shown that when *H. alvei*, *S. liquefaciens*, *R. aquatilis* and *Y. enterocolitica* were grown in nutrient broth overnight at 20 ºC, these bacteria produced AHL signaling molecules and was detected by the biosensor strain. A positive control C6-HSL and negative control sterile nutrient broth were shown for comparison. The other bacteria did not show any signs of AHL production. The results were reproducible when those spoilage bacteria were cultivated in lamb juice (Figure 4.3A). The biosensor strain detected the production of AHL molecules from those *Enterobacteriaceae* spp., in lamb juice. The spoiled lamb extract was also found to have AHL molecules (Figure 4.3B).

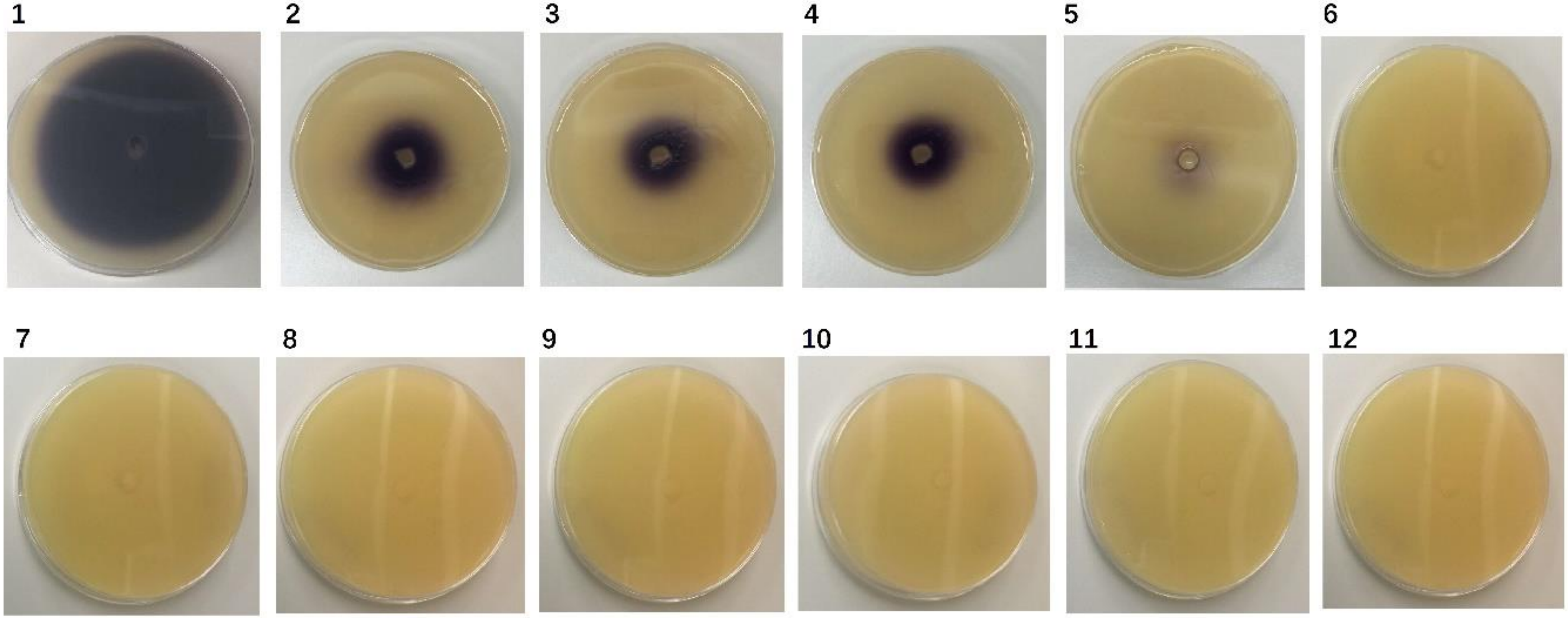


Figure 4.2. The presence of AHLs in cell-free extracts of all spoilage bacteria grown overnight in nutrient broth at 20 °C, shown by the presence of a purple zone. 1) Positive control, C6-HSL. Cell-free extracts of 2) *H. alvei*; 3) *S. liquefaciens*; 4) *R. aquatilis*; 5) *Y. enterocolitica*. 6) Negative control. Cell-free extracts of 7) *B. thermosphacta*; 8) *B. campestris*; 9) *C. maltaromaticum*; 10) *C. divergens*; 11) *K. oxytoca*; 12) *S. putrefaciens*. Note: The white stripes are the light reflection on plates.

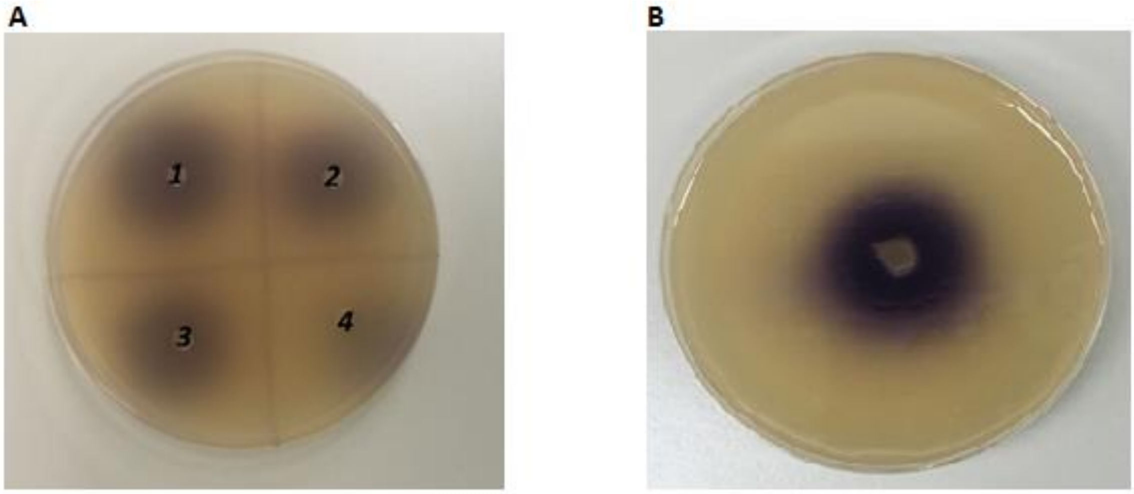


Figure 4.3. The presence of AHL molecules. A) The presence of AHLs in cell-free extracts of Enterobacteriaceae species grown overnight at 20 °C in lamb juice. Cell-free extracts of 1) *H. alvei*; 2) *S. liquefaciens*; 3) *R. aquatilis*; 4) *Y. enterocolitica*. B) The presence of AHLs in spoiled lamb extract.

The AHL production was compared when these spoilage bacteria were grown in different growth conditions. *H. alvei* was used as the representative spoilage bacterium to show the difference in AHL production (Figures 4.4 A-D). The AHL production of *H. alvei* was found to be higher when it was grown in high pH lamb shoulder juice (Figure 4.4A). However, when the pH was standardized, the AHL production was lower in the high-fat lamb shoulder (Figure 4.4B). Lower temperature (0 °C) and anaerobic conditions also significantly reduced the AHL production of *H. alvei* (Figures 4.4C & 4D).

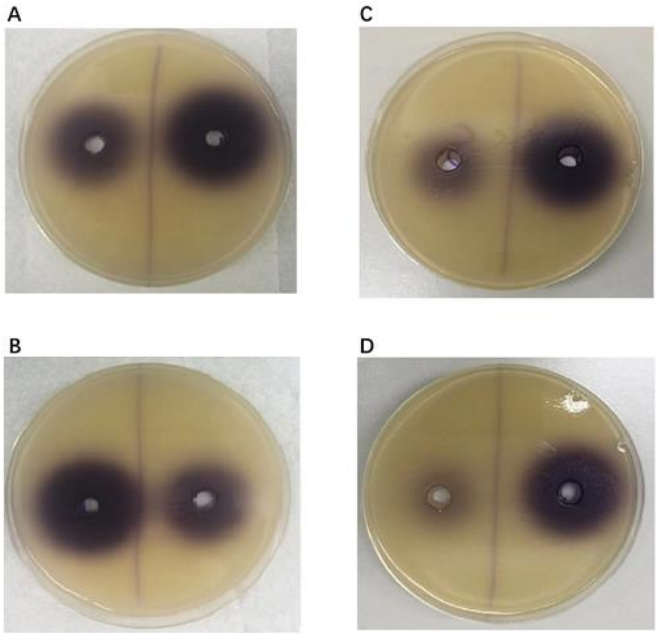


Figure 4.4. The AHL production of *H. alvei* when it was grown in different conditions. A) *H. alvei* grown overnight at 20 °C in low pH lamb loin (left) and high pH lamb shoulder (right). B) *H. alvei* grown overnight at 20 °C in two lamb juices with pH equalized. C) *H. alvei* was grown overnight anaerobically (left) and aerobically (right) at 20 °C in lamb shoulder. D) *H. alvei* was grown overnight at 0 °C (left) and 20 °C (right) in lamb shoulder.

Similar results were found in other two *Enterobacteriaceae* species *S. liquefaciens* and *R. aquatilis*, except 0 °C enhanced the AHL production of *R. aquatilis* (Figure 4.5). The AHL production of *Y. enterocolitica* was very limited and not well demonstrated.

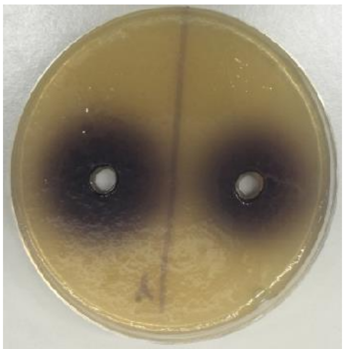


Figure 4.5. The AHL production of *R. aquatilis* when it was grown in lamb shoulder juice overnight at different temperatures. Purple zone indicated the AHL production when *R. aquatilis* was grown at 0 °C (left) and 20 °C (right).

AHL signals were also detected using *A. tumefaciens* NTL4/pZLR4, however, there was no detectable difference in the AHL production of spoilage bacteria grown in the two lamb juices (Figure 4.6).

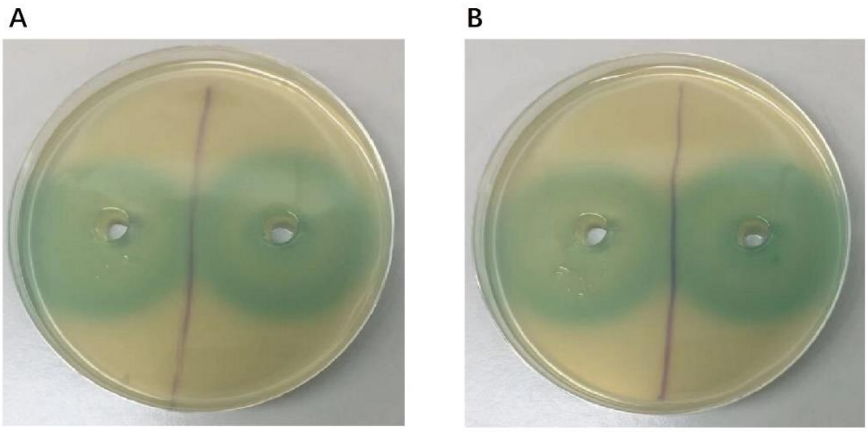


Figure 4.6. The AHL production of *H. alvei*, detected by *A. tumefaciens* NTL4/pZLR4, when it was grown in lamb with different pH values and fat contents. A) *H. alvei* was grown overnight at 20 °C in low pH lamb loin (left) and high pH lamb shoulder (right). B) *H. alvei* was grown overnight at 20 °C in lamb loin with low fat (left) and lamb shoulder with high fat (right) when pH was equalized.

4.3.3 Characterization of AHL signaling molecules by TLC

AHL signaling molecules were detected and partially identified by TLC in the cell-free supernatant extracts of Enterobacteriaceae species. When *H. alvei*, *S. liquefaciens*, *Y. enterocolitica* and *R. aquatilis* were grown in nutrient broth at 20 °C overnight, they produced AHL molecule similar to 3-oxo-C6-HSL on a TLC plate and this 3-oxo-C6-HSL-like molecule was also found in the CFME of spoiled chilled VP lamb (Figure 4.7A). When grown in fresh lamb juices at 20 °C overnight, Enterobacteriaceae species studied produced different AHL molecules (Figures 4.7B and 4.7C). *H. alvei* and *Y. enterocolitica* produced a molecule similar to 3-oxo-C6-HSL, while *S. liquefaciens* produced C4-HSL and C6-HSL-like molecules. *R. aquatilis* was found to produce a C8-HSL-like molecule in lamb shoulder juice (Figure 4.7B), while in lamb loin meat juice, it produced molecules similar to 3-oxo-C6-HSL and C8-HSL (Figure 4.7C).

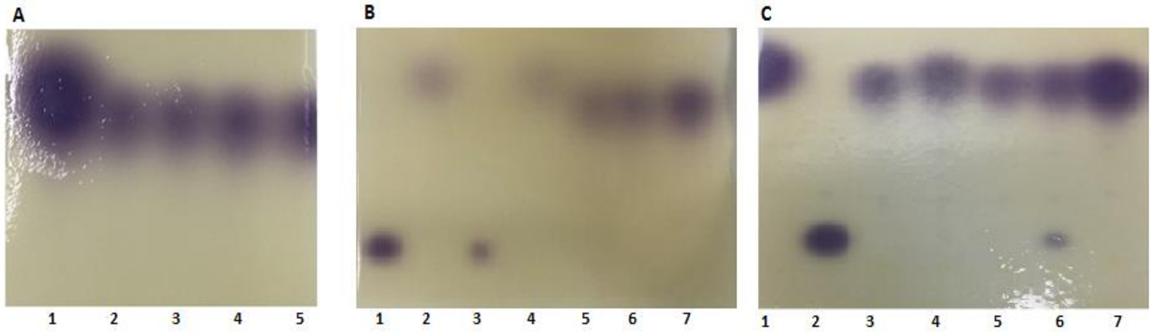


Figure 4.7. AHL profiles developed on the TLC plate. A) AHL molecules from cell-free extracts of *Enterobacteriaceae* isolates when they were grown overnight in nutrient broth at 20 °C. The cell-free extract of spoiled lamb was also investigated. 1) 3-oxo-C6-HSL. Cell-free extracts of 2) *H. alvei*; 3) *S. liquefaciens*; 4) *Y. enterocolitica*; 5) Spoiled lamb. B) AHL molecules from cell-free extracts of *Enterobacteriaceae* isolates when they were grown overnight in lamb shoulder juice at 20 °C. 1) C8-HSL; 2) C4-HSL. Cell-free extracts of 3) *R. aquatilis*; 4) *S. liquefaciens*; 5) *H. alvei*; 6) *Y. enterocolitica*. 7) 3-oxo-C6-HSL. C) AHL molecules from cell-free extracts of *Enterobacteriaceae* isolates when they were grown overnight in lamb loin juice at 20 °C. 1) C6-HSL; 2) C8-HSL. Cell-free extracts of 3) *H. alvei*; 4) *S. liquefaciens*; 5) *Y. enterocolitica*; 6) *R. aquatilis*. 7) 3-oxo-C6-HSL.

4.3.4 Identification of AHL profiles using LCMS/MS

The AHL molecules were detected and identified by LCMS/MS in cell-free supernatants of two major spoilage bacteria in Enterobacteriaceae spp., *H. alvei* and *S. liquefaciens*, as well as in the CFME of spoiled VP chilled lamb shoulder (Figure 4.8). The analyzes of all the AHL signaling molecules produced indicated the presence of the m/z 102 ion, which indicates the presence of the HSL moiety. AHL reference standards C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, and C10-HSL were also added for comparison. They migrated in the column and had m/z of 172.25, 199.08, 213.17, 228.02 and 255.25, respectively (Figure 4.8A). A wide range of AHL molecules including C4-HSL, C6-HSL, 3-oxo-C6-HSL, 3-hydroxy-C6-HSL, C10-HSL, 3-oxo-C10-HSL, C12-HSL, 3-oxo-C12-HSL, C16-HSL were found in the spoiled VP chilled lamb (Figure 4.8B). C6-HSL, 3-oxo-C6-HSL, and C12-HSL were produced by *H. alvei* (Figure 4.8C), while a wider range of AHL signaling molecules were produced by *S. liquefaciens*, ranging from C4-HSL to 3-oxo-C10-HSL, including C4-HSL, C6-HSL, 3-oxo-C6-HSL, 3-hydroxy-C6-HSL, C10-HSL and 3-oxo-C10-HSL (Figure 4.8D). The AHL molecules produced were summarised in Table 4.3. C6-HSL and 3-oxy-C6-HSL were the common AHL signaling molecules produced by these two spoilage bacteria. The relative abundance indicated that a greater amount of unsubstituted short-chain AHL molecules C4-HSL and C6-HSL had been produced or existed.

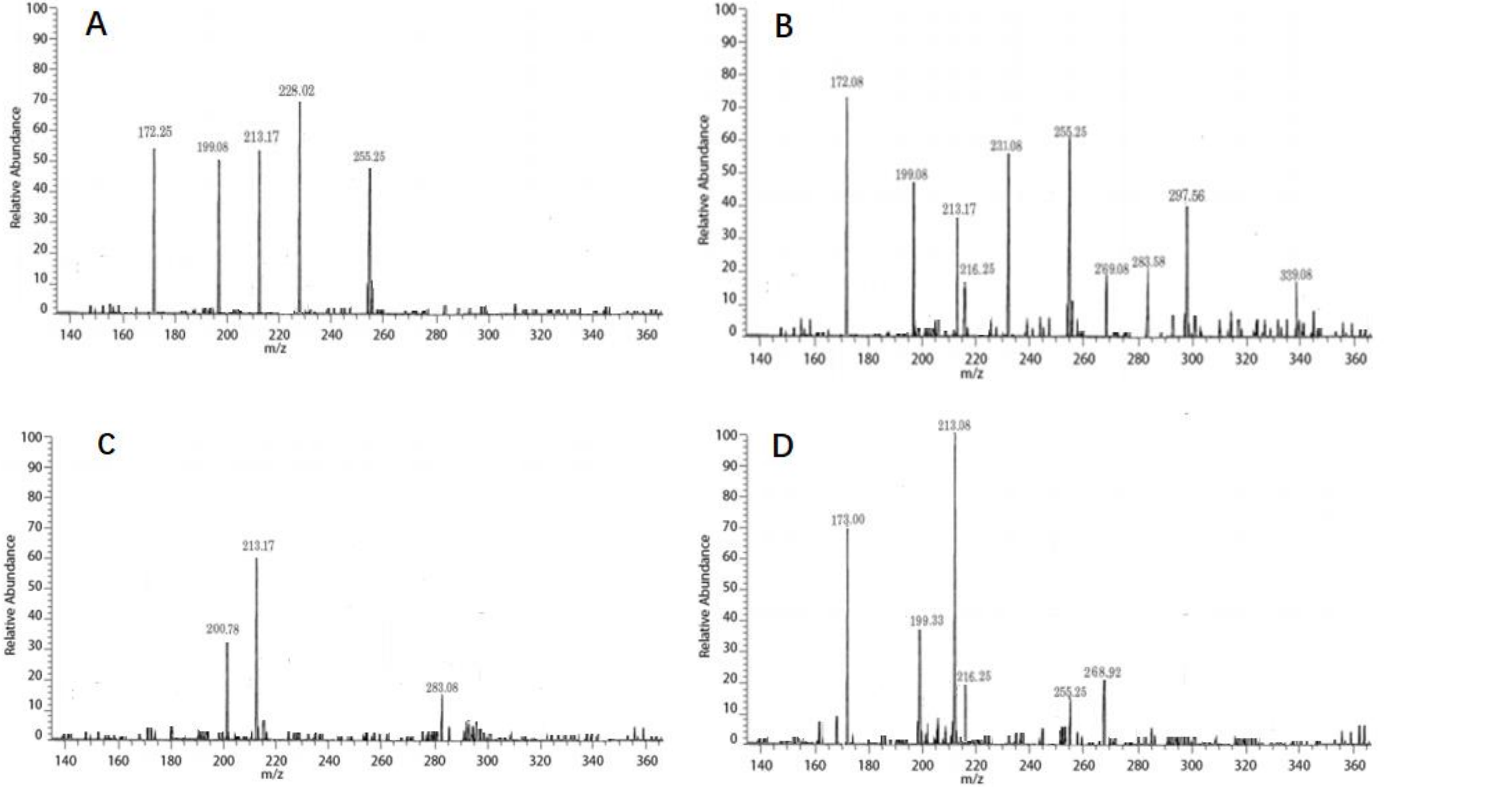


Figure 4.8. Extracted ion chromatogram of the m/z102 fragment of AHLs produced in spoiled VP chilled lamb loin, as well as in *H. alvei* and *S. liquefaciens*. The identification of the AHLs in the extract was determined using the tandem mass spectrometry (MS/MS) by comparing it with the corresponding AHL standards. (A) AHL standards C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, and C10-HSL; (B) AHL molecules in CFME of spoiled VP chilled lamb; (C) AHL molecules from *H. alvei*; (D) AHL molecules from *S. liquefaciens*.

Table 4.3. The comparison of AHLs identified in spoiled vacuum packaged lamb juice and spoilage bacteria by LCMS/MS.

AHL molecules	Molecular mass*	Spoiled VP lamb juice	*H. alvei*	*S. liquefaciens*
C4-HSL	171.89 (172.25**)	172.08	N/D	173.00
C6-HSL	199.25 (199.08**)	199.08	200.78	199.33
3-oxo-C6-HSL	213.23 (213.17**)	213.17	213.17	213.08
3- hydroxy-C6-HSL	215.25	216.25	N/D	216.25
C8-HSL	227.30 (228.02**)	N/D	N/D	N/D
C10-HSL	255.35 (255.25**)	255.25	N/D	255.25
3-oxo-C10-HSL	269.34	269.08	N/D	268.92
C12-HSL	283.41	283.58	283.08	N/D
3-oxo-C12-HSL	297.39	297.56	N/D	N/D
C16-HSL	339.5	339.08	N/D	N/D

* The molecular masses of all the AHL molecules listed in PubChem (https://pubchem.ncbi.nlm.nih.gov/).

** The m/z values of AHL standards found in this study.

N/D: not detected

4.3.5 Detection of AI-2 activity by bioluminescence assay

AI-2 signal was produced by all the spoilage bacteria when they were grown overnight at 20 °C in either nutrient broth or fresh lamb loin juice. The signal was sensed by the biosensor strain *V. harveyi* MM32, after incubation (Figure 4.9). AI-2 production was correlated with the intensity of bioluminescence measurements. The measurement was recorded at the sixth hour of incubation with biosensor strain, as the negative control Luria broth started to produce bioluminescence afterward. AI-2 production was also detected in the spoiled lamb extract. The production of AI-2 from *V. harveyi* BB170 was also sensed, by both *V. harveyi* MM32 and *V. harveyi* BB170. There was a significant drop (p-value < 0.01) in AI-2 produced by *S. putrefaciens*, ascribed to reduced growth when grown in low pH lamb loin, than in nutrient broth.

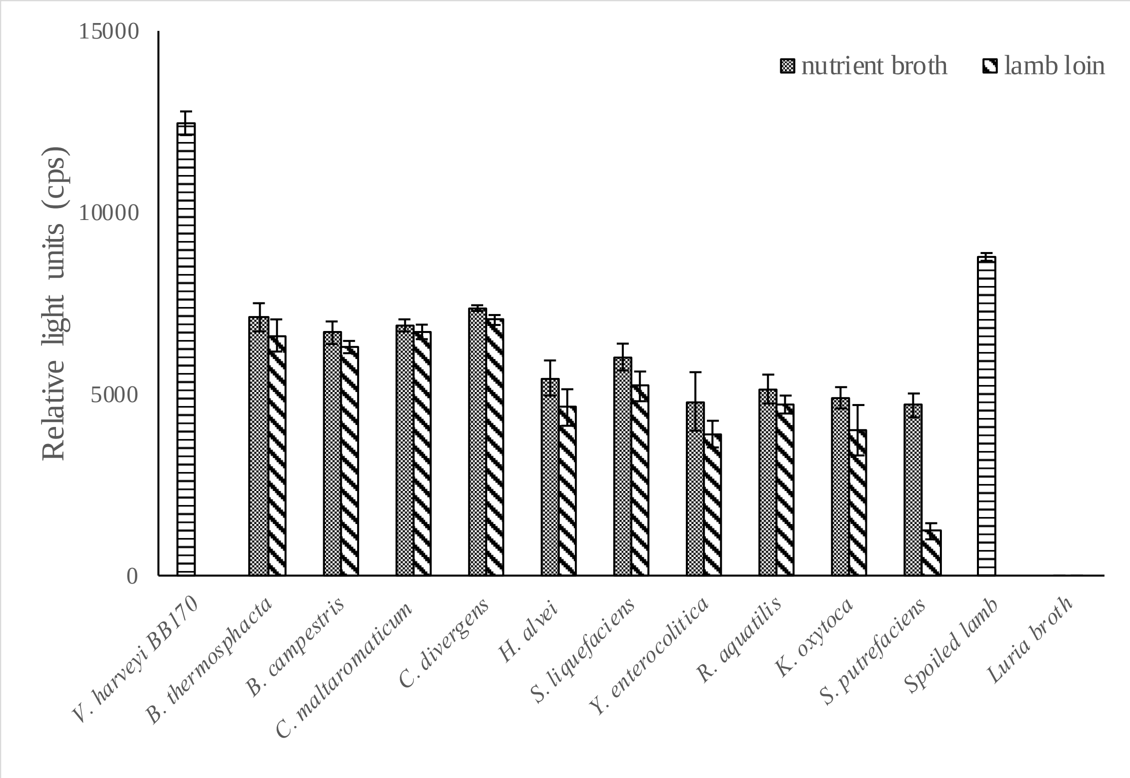


Figure 4.9. The bioluminescence production of bacteria grown overnight in nutrient broth and lamb loin juice aerobically at 20 °C, sensed by *V. harveyi* MM32. *V. harveyi* BB170 was used as a positive control. Bars show the AI-2 production in terms of relative light units. The mean and standard deviation of triplicates are indicated.

The influence of oxygen and pH values on AI-2 production was also investigated (Figures 4.10A and 4.10B). When spoilage bacteria were grown at 0 °C overnight, the AI-2 production from the cell-free extracts of those bacteria was shown lower in anaerobic conditions than in aerobic conditions, except *Carnobacterium* species (Figure 4.10A). The growth of these two bacteria was not affected by oxygen, thus there was no detectable difference in AI-2 production in those two bacteria, whether oxygen was absent or present. In low pH lamb loin meat (pH ~ 5.7), the AI-2 production of *S. putrefaciens* was significantly affected and reduced (p-value < 0.01), compared to its AI-2 production in high pH lamb shoulder juice (pH ~ 6.3). This effect was also shown in Figure 4.10B when spoilage bacteria were grown at 20 °C overnight. However, the reduction of AI-2 production from *S. putrefaciens* in lamb loin at 20 °C was not as much as the reduction at 0 °C. It may be because the influence on bacterial growth at 20 °C is not as much as the influence at 0 °C. Furthermore, when grown at 20 °C, the AI-2 production of most bacteria was found higher than it at 0 °C. The only exclusion was *R. aquatilis*, this psychrophile was found to have higher AI-2 production when it was grown at 0 °C, than 20 °C.

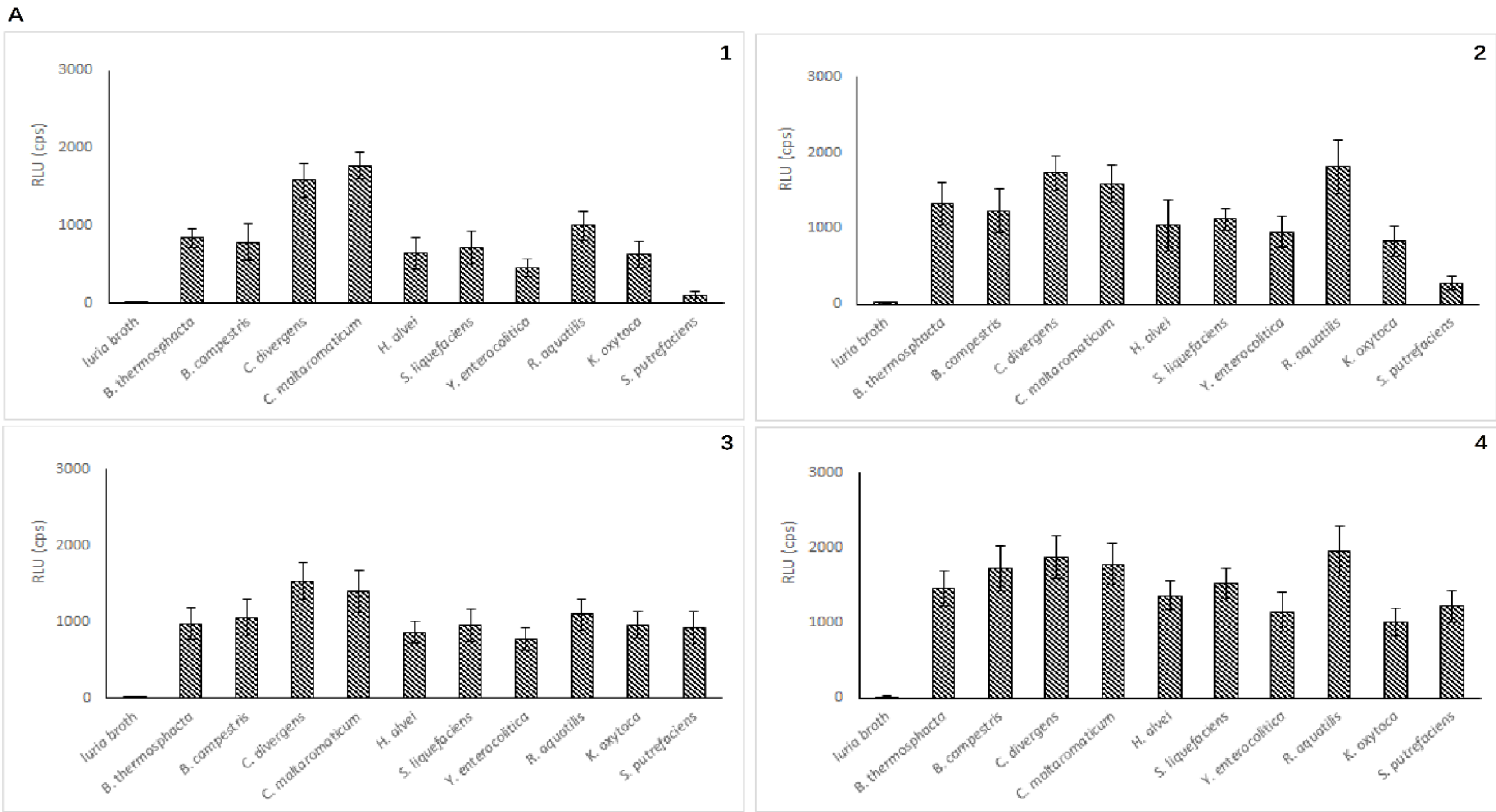


Figure 4.10A. AI-2 production in cell-free extracts of all spoilage bacteria when they were grown at 0 °C overnight, measured in relative light units. 1) In lamb loin anaerobically; 2) in lamb loin aerobically; 3) in lamb shoulder anaerobically; 4) in lamb shoulder aerobically.

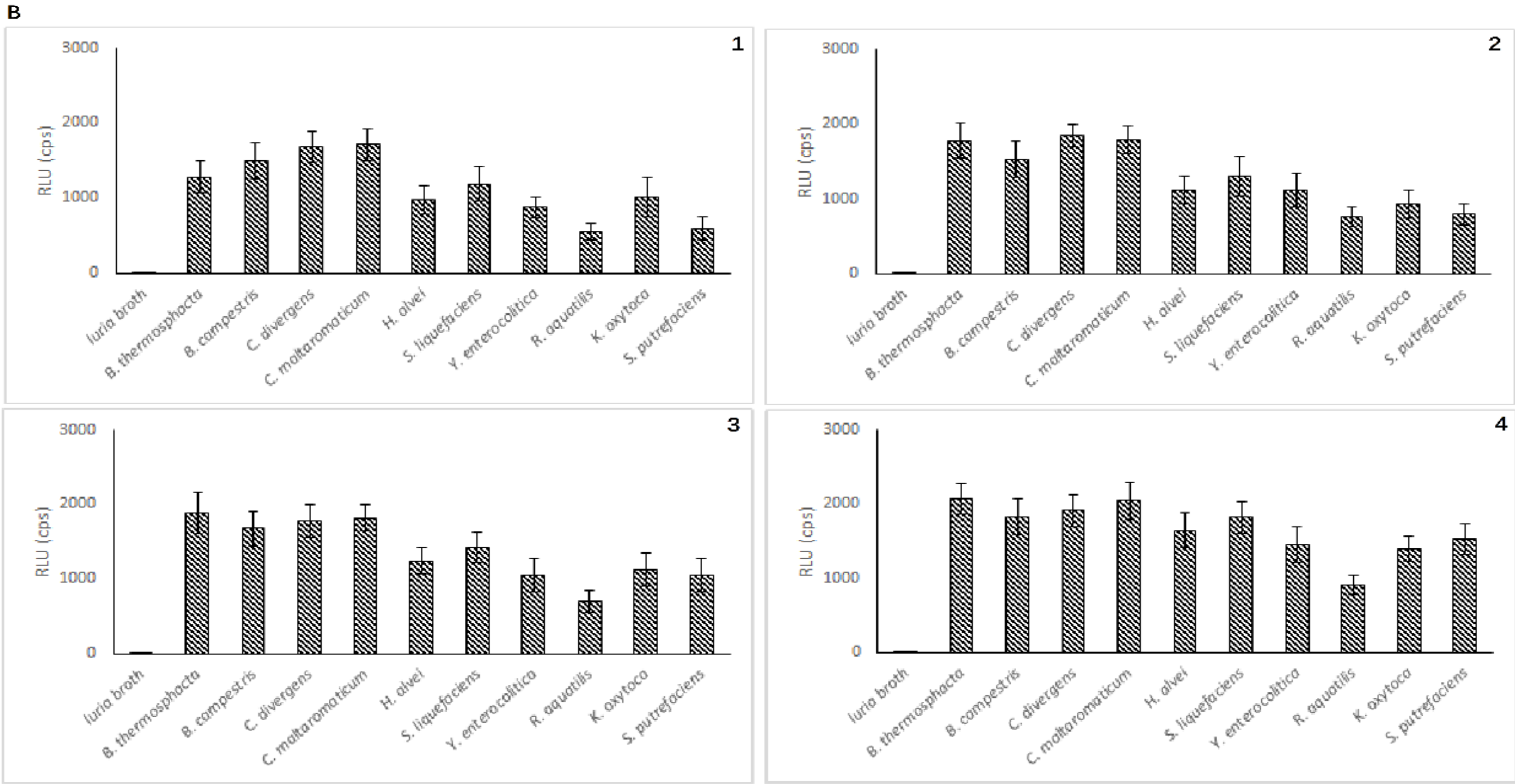


Figure 4.10B. AI-2 production in cell-free extracts of all spoilage bacteria when they were grown at 20 °C overnight, measured in relative light units. 1) In lamb loin anaerobically; 2) in lamb loin aerobically; 3) in lamb shoulder anaerobically; 4) in lamb shoulder aerobically.

Therefore, the production of AI-2 was found to be significantly (p-value < 0.05) affected by pH, oxygen presence and temperature. *H. alvei* was shown in Figure 4.11A as a representative strain. When *H. alvei* was grown in low pH or anaerobic conditions the intensity of bioluminescence was significantly reduced (p-value < 0.05). When the pH was standardized to 6.31, the AI-2 production of *H. alvei* became slightly higher when it was grown in lamb loin with low-fat content. The lowest AI-2 production was found when *H. alvei* was cultivated in an anaerobic environment with a low pH at 0 °C. This agreed with most of the other spoilage bacteria tested in this study. However, the lowest AI-2 production of *R. aquatilis* was found in an anaerobic environment with a low pH at 20 °C, not 0 °C (Figure 4.11B).

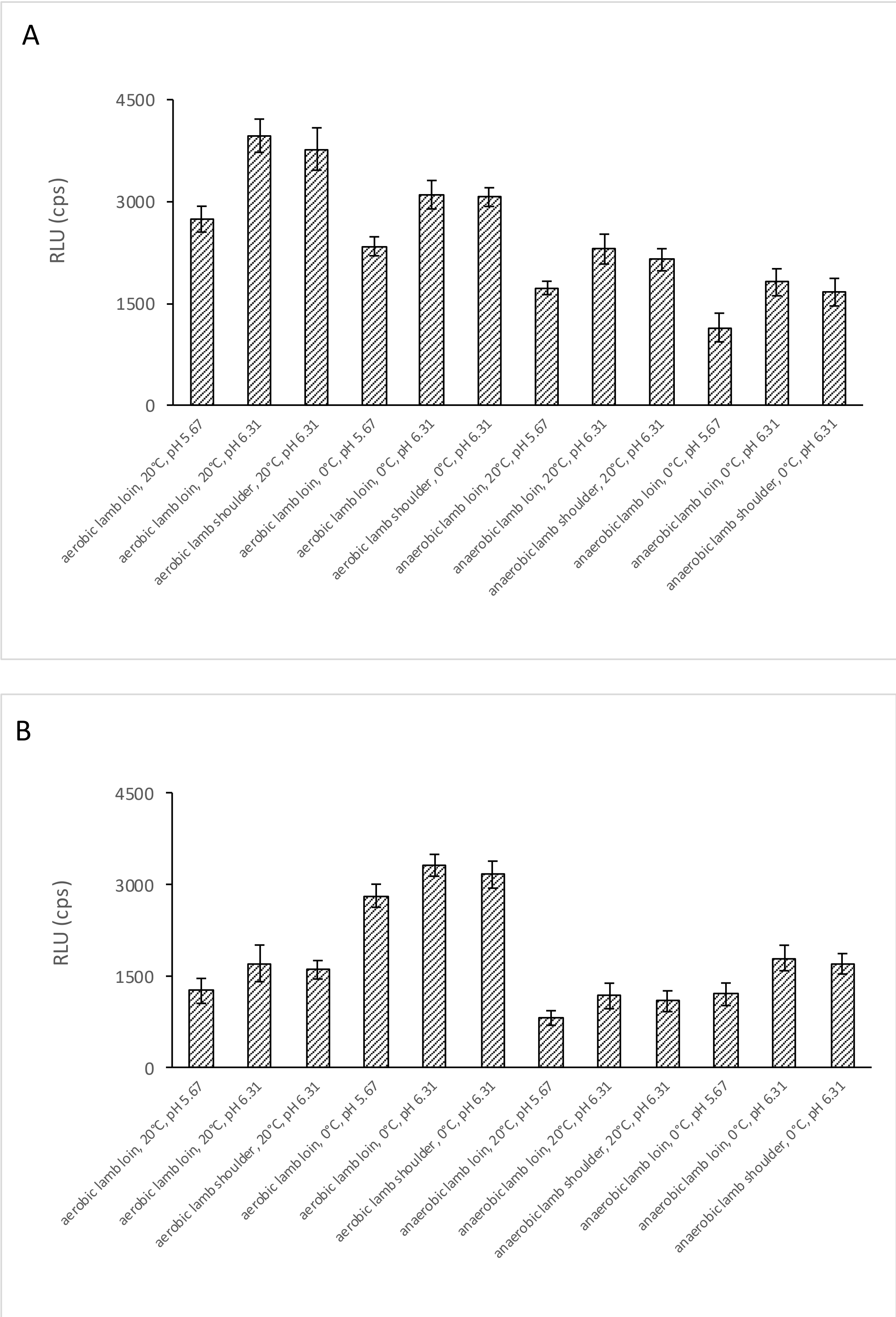


Figure 4.11. AI-2 production in cell-free extracts of spoilage bacteria measured in relative light units. A) *H. alvei* and B) *R. aquatilis*.

4.4 Discussion

Two major types of QS signaling molecules AHLs and AI-2 have been found in many food matrices. In this study, AHLs and AI-2 molecules were found in spoiled chilled VP lamb. To originate the QS signaling molecules, a selection of psychrotrophic anaerobically growing meat spoilage bacteria were used in this study. These microbes can be commonly found in chilled VP lamb, and contribute to microbial spoilage. AHLs and AI-2 molecules were produced by those spoilage bacteria. The microbial production of QS molecules was found to be influenced by growth conditions such as the presence of oxygen, temperature, pH and fat content.

In this study, biosensor strains were used to detect QS signals in a spoiled chilled VP lamb, which were possibly produced by spoilage bacteria. AHL molecules were produced by four *Enterobacteriaceae* species (*H. alvei, S. liquefaciens, R. aquatilis* and *Y. enterocolitica*) when they were cultivated in nutrient broth or lamb, either in aerobic or anaerobic condition. Amongst those four species, a weak AHL production was found in *Y. enterocolitica*, according to the size of the detection zone on agar plates. It is possibly due to the expression of the unique gene *yenS* that is directly regulated by *yenR* after receiving AHL signaling molecules and codes small non-translated RNAs which impede the production of AHL signaling molecules (Tsai and Winans, 2011b).

TLC was used for characterizing AHL signaling molecules (Liu et al., 2006; Nychas et al., 2009). However, as fat content may interfere with the migration of AHLs on TLC plates (Bruhn et al., 2004), the detection range is limited, usually between C4-HSL and

C8-HSL. In this study, TLC was also used to partially identify the presence of AHL molecules, and the results agreed with previous findings that only short and medium-chain AHL signaling molecules were found on TLC plates. It might also be due to the detection limit of the biosensor strain *C. violaceum* CV026 used in this study.

LCMS/MS was thus further used to identify AHL molecules, instead of detecting molecules using a biosensor strain, like TLC does, LCMS/MS directly detects AHL molecules from the cell-free extracts of spoilage bacteria and spoiled chilled VP lamb. Therefore, the detection limit was minimized and a much wider range of AHL signaling molecules, from C4-HSL to C16-HSL, were detected using LCMS/MS. The presence of AHL molecules with different acyl chains detected in spoiled VP chilled lamb (Table 4.3) suggests that the microbial community in a lamb matrix can produce multiple signals. According to the relative abundance of different AHL signaling molecules (Figure 4.8), a greater amount of short and medium-chain unsubstituted AHL molecules were produced by spoilage bacteria *H. alvei* and *S. liquefaciens*, as well as in spoiled lamb. Moreover, long-chained AHL molecules were also detected in spoiled lamb, however with lower abundance. These long-chained AHL molecules might be hydrolyzed into medium or short-chain molecules. By comparing to those produced by an individual spoilage bacterium *H. alvei* or *S. liquefaciens*, AHL signaling molecules found in spoiled lamb were hypothesized to be contributed by a variety of spoilage bacteria. Therefore, the link between lamb spoilage and AHL molecules from the lamb isolates was studied later.

Before detecting AHL signaling molecules, an investigation of bacterial growth under different environmental conditions was carried out to determine the influence of these factors on cell populations. Since the QS process is associated with cell density, the AHL production in different conditions, in which spoilage bacteria were grown, was investigated in this study. The AHL production was compared when spoilage bacteria were in stationary phase thus the cell density was steady and the comparison was rational. As mentioned before, in a chilled vacuum package, the natural microbiota is different, and lamb has a different pH and fat content compared with other meat (Mills et al., 2014), therefore in this study the effect of temperature, oxygen, pH, and fat on AHL molecules production by spoilage organisms was tested. The growth of most tested bacteria was facilitated by ambient temperature, neutral pH and oxygen. In these conditions, a high detection of short-chained AHLs was sensed by *C. violaceum* CV026, with an exception of *R. aquatilis*, which grew better at chilled temperatures compared with ambient temperatures and showed higher AHL production at chilled temperatures. High fat content reduced bacterial growth and AHL production, but the impact was not found to be as much as the other factors. The results indicate that due to the linear regression between bacterial population and AHL production, the impact on bacterial growth correlates with the impact on AHL production. Environmental factors that affect the growth of microorganisms also influence the AHL production in the same manner. However, while using *A. tumefaciens* NTL4/pZLR4 as the biosensor, the detection of AHL did not vary too much. This suggests that similar amounts of AHLs were produced regardless of what environment spoilage bacteria were cultivated in. The contradiction might be because that *A. tumefaciens* NTL4/pZLR4 is able to detect long-chained AHLs that have more than 10 carbon molecules, which were not well sensed by *C. violaceum* CV026. Whether these long acyl chained AHL signaling molecules are associated with spoilage needs to be further defined. Well diffusion bioassay by agar plates provided rapid results for AHL detection but there are also a few potential confounding variables. The diffusion depends on variables such as the size of the signaling molecule, the incubation temperature, and potentially gel strength. The latter may be influenced by the time for agar plates to set and whether the plate has been chilled before use.

AI-2 production was found in all the spoilage bacteria tested in this study, as well as in the extract from spoiled lamb. By a bioluminescence measurement, AI-2 production observed in this study was higher than previous AI-2 findings in poultry and ground beef (Kamlesh A. Soni et al., 2008; Widmer et al., 2007). This could be explained by the longer incubation time used in this study compared with previous studies. A greater amount of biosensor strain *V. harveyi* MM32 was used in this study, which might also influence the result as it could have sensed more AI-2 production by giving higher bioluminescence measurements. However, the most likely explanation is the nature of lamb, which is different from other meat used in previous studies. In this study, the suppression of bacterial density led to lower AI-2 production. Compared to the anaerobes *Carnobacterium* spp., the AI-2 production of facultative anaerobes (*Brochothrix* and *Enterobacteriaceae*) decreased when oxygen was absent. The psychrophilic microorganism *R. aquatilis* has a preference for growth and reproduction at cold temperatures, hence chilled temperatures increased AI-2 production from *R.*

aquatilis in this study. AI-2 detection by induced bioluminescence used in this study was a quick and simple method. In a previous study, the presence of glucose was reported to make the *V. harveyi* BB170 sensor strain grow so well that it started to detect itself (Turovskiy and Chikindas, 2006). In the present study, the biosensor *V. harveyi* MM32 had no capability to produce AI-2, acting only to sense external AI-2 signals. Hence although lamb has a high level of glucose (Gill and Newton, 1978), the confusion for detecting AI-2 produced from spoilage bacteria or *V. harveyi* was minimized. However, fatty acids (linoleic acid, oleic acid, palmitic acid, stearic acid, etc.) found in meat products could inhibit AI-2 sensing (Turovskiy and Chikindas, 2006), and the acidity of the sample may also interfere with the bioassay (DeKeersmaecker and Vanderleyden, 2003b). The pH and the potential presence of fatty acids in lamb juice used in the study could influence the results. The high level of fat and protein in lamb might also interfere with bioluminescence measurement while detecting AI-2 signals. These factors limit the results to a qualitative rather than a quantitative interpretation. In further studies, detection of the AI-2 molecule by LCMS/MS will solve these limitations, as the supernatants are further extracted and reconstituted by chemicals. The relative abundance of signals using LCM/MS can give a rapid and much clearer comparison of AI-2 production in different conditions.

The findings that conditions such as low pH, high fat level, low temperature and oxygen depletion delay the production of both AHL and AI-2 signaling molecules gives a scientific rationale for the use of chilling storage and vacuum packaging in the export and storage of lamb. It also expands our knowledge of environmental effects on lamb spoilage via QS level.

4.5 Conclusion

This part of the study demonstrated that different types of QS signaling molecules were produced in spoiled VP chilled lamb, possibly from a set of anaerobically growing and cold-tolerant spoilage bacteria. This opens the door for further research correlating these QS signals to bacteria causing lamb spoilage. Exogenous temperature and oxygen, as well as endogenous factors in lamb, such as pH values and fat content, were found to influence the population and QS production of these spoilage bacteria.

Chapter Five: Understanding the Role of Quorum Sensing Signals in Chilled Vacuum Packaged Lamb Spoilage

5.1 Introduction

In the last chapter, AHL and AI-2 signaling molecules were found in spoiled chilled VP lamb, potentially produced by spoilage bacteria.

The AHL system is mainly based on a two-component system. AHL signaling molecules are synthesized by LuxI or its homologs, LuxR or its homologs recognize AHL molecules and modulate transcription factors to regulate gene transcription (Rutherford and Bassler, 2012). This LuxI/LuxR and its homologous systems have been identified in Gram-negative bacteria; however, there are “LuxR orphans” which use exogenous AHL signaling molecules to regulate gene expression (Lee et al., 2006). In the marine *Vibrio* spp., LuxM/N-type QS systems are found alongside LuxI/R-type QS systems (Milton et al., 2001); however, no previous literature has referred to LuxM/N-type QS systems in those spoilage bacteria studied in my work.

The AI-2 synthase protein LuxS converts the by-product of the S-adenosylmethionine cycle metabolism, ribose homocysteine, into 4,5-dihydroxy-2,3-pentanedione (DPD), the precursor compound of the AI-2 signaling molecules (Schauder et al., 2001). When bacteria detect a high concentration of AI-2 in the environment, AI-2 signaling molecules bind to a receptor to activate kinase and this phosphorylates a transcription factor, which regulates gene transcription. A LuxP/LuxQ cascade has so far only been identified in *Vibrio* species (Chen et al., 2002). In general, furanosyl borate diester, a so-called BAI-2 form of the AI-2 signal was detected by binding with a binding protein, LuxP. This AI-2/LuxP bound complex interacts with a sensor kinase LuxQ and initiates luciferase production and luminescence (DeKeersmaecker and Vanderleyden, 2003a). For this reason, *Vibrio* species are usually used for AI-2 detection.

Various traits are regulated through QS (Pearson et al., 1994; Zhang et al., 1993). Christensen et al., in 2003 reported that the AHL signaling molecules controlled exoenzymatic activities of *S. proteamaculans* B5a in milk. The production of those hydrolytic enzymes (proteases and lipases) has also been regarded as one of the causes of the deterioration of meat (Dave and Ghaly, 2011). Trappetti et al., in 2017 found that *Streptococcus pneumoniae* sensed exogenous AI-2 signaling molecules via the FruA system, which is highly conserved in Gram-positive bacteria. It enabled the bacterium to assimilate galactose instead of glucose as a carbon source to regulate pathways.

In this part of the study, the aim was to give the idea of whether AHL and AI-2 signaling molecules affect enzymatic spoilage by psychrotrophic and anaerobically growing spoilage bacteria, commonly found in spoiled chilled VP lamb. Mutants of QS related genes of specific spoilage bacteria were developed by random EZ-Tn5 transposon mutagenesis. Transposomics is a fast, trusted and well-established method for generating mutations in live mutant bacteria, including Gram-positive and Gram-negative strains (Goryshin et al., 2000). Amongst many transposomic applications, the EZ-Tn5 system has high efficiency and low bias (Adey et al., 2010). By comparing mutants with wild types of spoilage bacteria, whether AHL and AI-2 signaling molecules are involved in spoilage behavior will be concluded and lead to a later study.

5.2 Materials and methods

5.2.1 Bacterial strains

QS-related mutants were developed by random EZ-Tn5 transposon mutagenesis, as described in Section 3.15. *Hafnia alvei* and *Serratia liquefaciens* were studied as representative *Enterobacteriaceae* spp., which produce both AHL and AI-2 signaling molecules. Reference strains *H. alvei* NZRM860 and *S. liquefaciens* NZRM861 were grown in Luria broth. Lamb isolates *H. alvei* FAM01 (isolated from a lamb shoulder in this study, see Appendix 1 for 16S RNA sequence) and *S. liquefaciens* FAM02 (previously isolated from a spoiled lamb by AgResearch) were cultivated in lamb juice. The genes responsible for synthesizing AHLs (*luxI* homologs), receiving AHLs (*luxR* homologs), and synthesizing AI-2s (*luxS*) of these bacteria were targeted for mutagenesis. *Carnobacterium divergens* FAM03 (previously isolated from spoiled lamb by AgResearch) was selected as a representative Gram-positive bacterium and the mutant of the *luxS* gene was developed.

A kanamycin-resistant transposon was inserted into these spoilage bacteria by random transposon mutagenesis, thus before mutagenesis, wild types of *H. alvei*, *S. liquefaciens*, and *C. divergens* were plated onto kanamycin plates to ensure that these spoilage bacteria were not intrinsically kanamycin resistant.

5.2.2 Phenotypic screening of mutants

The cell-free extracts (CFEs) of *H. alvei* NZRM860 and *S. liquefaciens* NZRM861 mutants were analyzed for AHL production as per Section 3.9. Uninoculated LB was used as the negative control.

Lipase activity was measured to differentiate putative *luxR* homolog mutants of *H. alvei* NZRM860 and putative *luxR* homolog mutants of *S. liquefaciens* NZRM861 from wild types after external AHL standard 3-oxo-C6-HSL was added to the strains. The method was described in Section 3.18. Uninoculated LB was used as the negative control.

AI-2 signal molecules in all CFEs prepared from the *luxS* mutants of *H. alvei* NZRM860, *S. liquefaciens* NZRM861 and *C. divergens* FAM03 were detected using a bioluminescence-based broth bioassay, as described in Section 3.12. The biosensor strain *V. harveyi* MM32 by itself was used as the negative control.

The growth of wild types and putative AI-2 mutants in Luria broth and minimal growth medium with 1% lactose was also investigated and compared. The absorbance was measured at 595nm.

5.2.3 Determination of AHL production using liquid chromatography-tandem mass spectrometry (LCMS/MS)

AHL production of putative mutants obtained after phenotypically screening as well as wild type strains of *H. alvei* NZRM860 and *S. liquefaciens* NZRM861, was detected by LCMS/MS, as described in Section 3.13. Four AHL standards, C4-HSL, C6-HSL, C8-HSL, and C10-HSL were also analyzed and the MS profiles of AHL molecules found in the spoilage bacteria were compared to those of known reference AHL signals

5.2.4 DNA extraction, PCR and gel electrophoresis

The putative QS mutants of *H. alvei* NZRM860, *S. liquefaciens* NZRM861 and *C. divergens* FAM03 were analyzed by PCR and agarose gel electrophoresis. DNA from presumptive mutants was extracted and amplified, as described in Section 3.16. The oligonucleotides used in this study were designed using the NCBI with Primer3 software, and are listed in Table 5.1. The primers for the EZ-Tn5 transposon were demonstrated in Figure 3.2.

Table 5.1. Primers used in this study. All the oligonucleotides were resourced from NCBI.

Target DNA sequence	Accession number	Target Sequence length (bp)	Primer sequence (from 5' to 3')	Primer coordinates	PCR product length (bp)
Serratia liquefaciens AHL synthase *sliI*	AY168877.1 REGION: 3754-4356	603	Forward: CGTGTTGATTGATGCGTGCT	3771-3790	362
			Reverse: AAACATGATCAGCGATGGCG	4113-4132	
Serratia liquefaciens AI-2 synthase *luxS*	NC_009832 REGION: 945680-946195	516	Forward: ATGCCACTGCTGGATAGCTTC	945680-945699	505
			Reverse: GTTCCTGCAGCTTCTCTTTCG	946165-946184	
Serratia liquefaciens AHL receptor *sliR*	AY168877.1 REGION: 2988-3734	747	Forward: TCCAATAGAACGGAGTGGTTTGA	3129-3151	529
			Reverse: TGACGCCAAGCTTTTTGACG	3638-3657	
Hafnia alvei AHL synthase *halI*	NZ_CP009706 REGION: 2669091-2669738	648	Forward: AATGCAGGCGTAACGGCTAT	2669098-2669117	551
			Reverse: CGGTTGGGATGGGATGTTGT	2669629-2669648	
Hafnia alvei AI-2 synthase *luxS*	NZ_CP009706 REGION: 1048836-1049351	516	Forward: TGCGTTTTTGTCACCCGAAC	1048948-1048967	289
			Reverse: TGCATGGAATAGGTTCCGCA	1049217-1049236	
Hafnia alvei AHL receptor *halR*	NZ_CP009706 REGION: 2668370-2669110	741	Forward: CTTCAAACGTACCTCGCCCT	2668588-2668607	498
			Reverse: TCAAGTTCAACGCCCAACCT	2669066-2669085	
Serratia liquefaciens protease synthase *hflB*	NC_021741.1 REGION: 377541-379472	1932	Forward: ATGTCCTTCGGCAAGAGCAA	377934-377953	661
			Reverse: CACGAGCCAGTACAGAAGCA	378575-378594	
Carnobacterium divergens AI-2 synthase *luxS*	OGVA01000030 REGION: 88025-88489	465	Forward: TTGCTGGCAGGAACTTCTGT	88134-88153	306
			Reverse: CAAGACGAACATAGGGGGCT	88420-88439	
Serratia liquefaciens ABC transporter *lipB*	NC_021741.1 REGION: 1146332-1147000	669	Forward: TACGTCTTCTATGCCGACGC	1146419-1146438	515
			Reverse: CCCAAGCCATGCACAACTTC	1146914-1146933	
Hafnia alvei ABC transporter *lipB*	NZ_CP009706 REGION: 1404798-1405469	672	Forward: CCACAGGGGTTAATTCGGCT	1404949-1404968	444
			Reverse: TATGCACACCTTCACCGACC	1405373-1405392	

5.2.5 Analysis of enzymatic activity

The activity of hydrolytic enzymes was investigated and compared between wild types and mutants of spoilage bacteria, *H. alvei* NZRM860, *S. liquefaciens* NZRM861, and *C. divergens* FAM03, when they were grown in lamb juice. AHL synthase knockdown mutants of *H. alvei* NZRM860 and *S. liquefaciens* NZRM861 with the addition of different AHL signaling molecules were also used for comparison.

The enzymatic activities were analyzed by agar plate screening, described in Section 3.17. Dispase, neutral protease from *Bacillus polymyxa* Grade II (Roche Boehringer Mannheim, Germany) and lipase from wheat germ (Sigma-Aldrich, USA) were used as the positive controls. Uninoculated LB and acetonitrile that was used to dissolve AHL standards were used as the negative controls.

The enzymatic activities were compared by absorbance, as described in Section 3.18.

5.2.6 Gene expression investigation by quantitative reverse transcription PCR (RT-qPCR)

Gene expression analyses of genes relevant to QS systems were carried out by RT-qPCR, described in Section 3.19. The analyses were conducted in two parts.

1) In LB, wild types and mutants of *H. alvei* NZRM860 and *S. liquefaciens* NZRM861 were grown at 20 °C overnight with and without the addition of AHL standard 3-oxo-C6-HSL. Wild types were used as the positive controls, AHL mutants and RNase free water were used as negative controls.
2) In lamb juice, wild types and mutants of *H. alvei* NZRM860 and *S. liquefaciens*

NZRM861 were grown with and without the addition of AHL signals from two lamb isolates, *H. alvei* FAM01 and *S. liquefaciens* FAM02. Two types AHL signals were obtained from those two lamb isolates: 1) directly extracted from the cell-free supernatants; 2) extracted the cell-free supernatants by acidified ethyl acetate, nitrogen dried and then reconstituted in acetonitrile. Wild types were used as the positive controls, whereas, AHL mutants and RNase free water were used as negative controls.

5.2.7 Impact of AHL on natural microbial spoilage in lamb loin juice

In lamb loin meat juice, the wild type and *halI* ↓ mutant of *H. alvei* NZRM860 were grown at three different temperatures, -1.5 °C, 4 °C, and 20 °C. In addition, a *halI* ↓ mutant with the addition of AHL standard 3-oxo-C6-HSL was also used for comparison. Enzymatic activities were detected, as described in Section 3.17, every half hour on the first day, and daily from the second day. The starting time points for enzymatic activities and off-odor production were compared amongst all samples.

5.3 Results

5.3.1 Screening of AHL synthase mutant colonies

The AHL production in the CFEs of the mutant colonies was determined using the *A. tumefaciens* NTL4/pZLR4 biosensor strains as reported in Figure 5.1. Different results were found between the putative AHL synthase mutant clones and other mutants. The wild type strain produced a bright green color demonstrating AHL signaling molecules had been produced and sensed by the biosensor strain. This induced *lacZ* β-galactosidase and hydrolyzed the X-gal reagent after incubation (Farrand et al., 1996). Most of the mutant colonies showed a similar green color. Putative clones of AHL synthase gene mutants were unable to synthesize AHL signaling molecules and therefore the X-gal reagent was not hydrolyzed after incubation and gave paler green color, very close to that of the negative control. The screening was repeated in triplicate. Most of the mutants did not show a consistent "pale" color in all the 3 replicates. Hence, only those mutants which consistently showed a pale color, close to the negative control, were selected for further investigations (Figure 5.2).

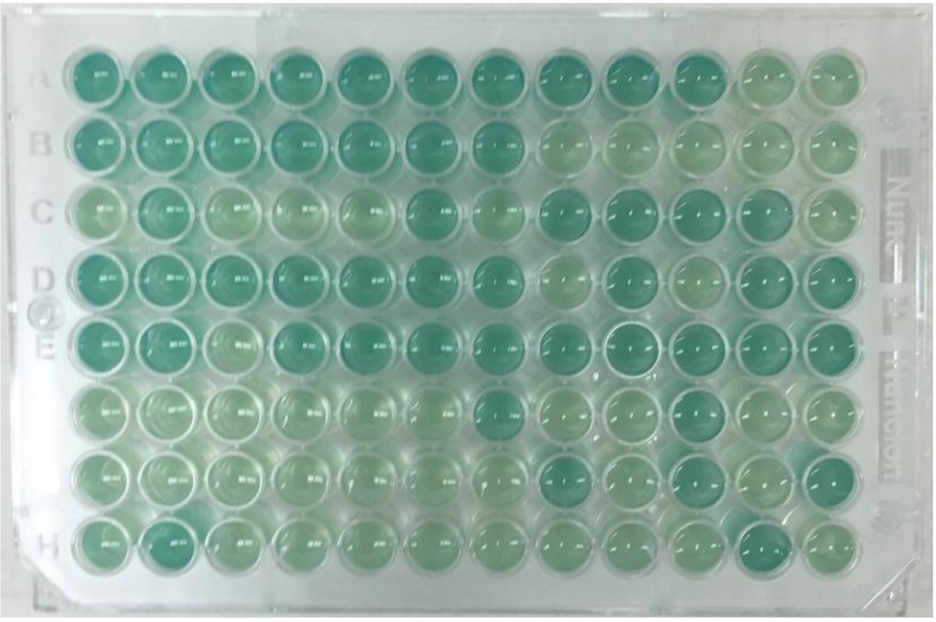


Figure 5.1. The screening of AHL signals in different mutants of *H. alvei* NZRM860 tested, grown overnight at 30 °C with *A. tumefaciens* NTL4/pZLR4 and X-gal solution. The bright green shades resulted from produced AHLs that were detected by the biosensor strain, and the putative AHL synthase gene (*halI*) mutants gave a much lighter green.

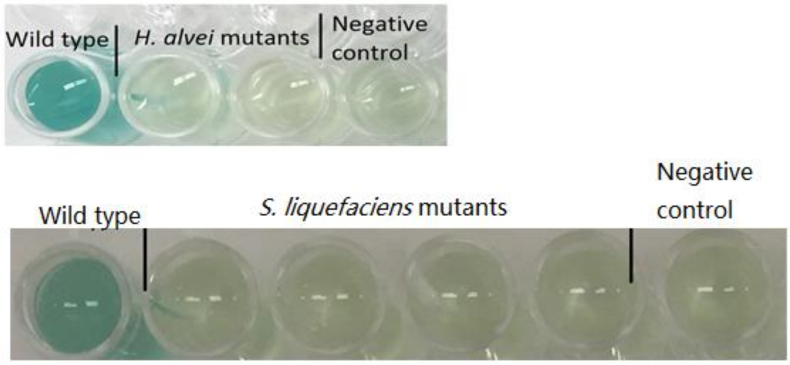


Figure 5.2. The AHL synthase mutant clones of *H. alvei* NZRM860 and *S. liquefaciens* NZRM861, grown overnight at 30 °C with *A. tumefaciens* NTL4/pZLR4 and X-gal solution. These selected mutants did not produce a green color and were similar to the negative control.

5.3.2 Identification of AHL receptor mutant colonies

The lipase activity of the CFEs from the mutant colonies was determined by absorbance at 400 nm (Shirai and Jackson, 1982b). The putative AHL receptor mutants were differentiated after the AHL standard 3-oxo-C6-HSL was added. Mutants with low absorbance values were indicated as putative AHL receptor mutants, as the lipase activity was not activated by AHL. The test was repeated in triplicate and only the mutants that consistently showed low optical density, close to the negative control (Table 5.2) were selected for further investigations. However, these mutants could also be mutants that does not synthesize lipase, so they needed further confirming by sequence analysis.

Table 5.2. Optical density of individual *H. alvei* NZRM860 mutants grown overnight at 30 °C after Tn5 transposition. Mutants showing low absorbance were assumed either *luxR* mutants that lipase activity was not received and activated by AHLs, or mutants not producing lipase.

Sample	Absorbance at 400 nm		
Mutant #1	1.986	2.021	1.997
Mutant #2	2.118	2.191	2.011
Mutant #3	1.089	1.296	1.138
Target Mutant #1	0.017	0.014	0.011
Target Mutant #2	0.038	0.041	0.033
Target Mutant #3	0.016	0.028	0.021
Negative control	0.008	0.006	0.006

5.3.3 Investigation of AI-2 synthase mutants by bioluminescence measurement and bacterial growth

Bioluminescence assay was carried out for AI-2 detection at separate time points after incubation (Figure 5.3). Triplicate data were recorded after 1, 2, 3, 4, 6, 8 h of incubation at 30 °C. These bioluminescence measurements were normalized by the negative control. As shown in Figures 5.3A-C, the bioluminescence reading of CFEs of *luxS* mutants was negligible compared to the wild type. The expression of *luxS* in these tested mutants was suppressed. The AI-2 signals from wild type strains started to be produced between 3 and 4 h of incubation.

Putative AI-2 synthase mutants from bioluminescence screening were further investigated by PCR sequencing.

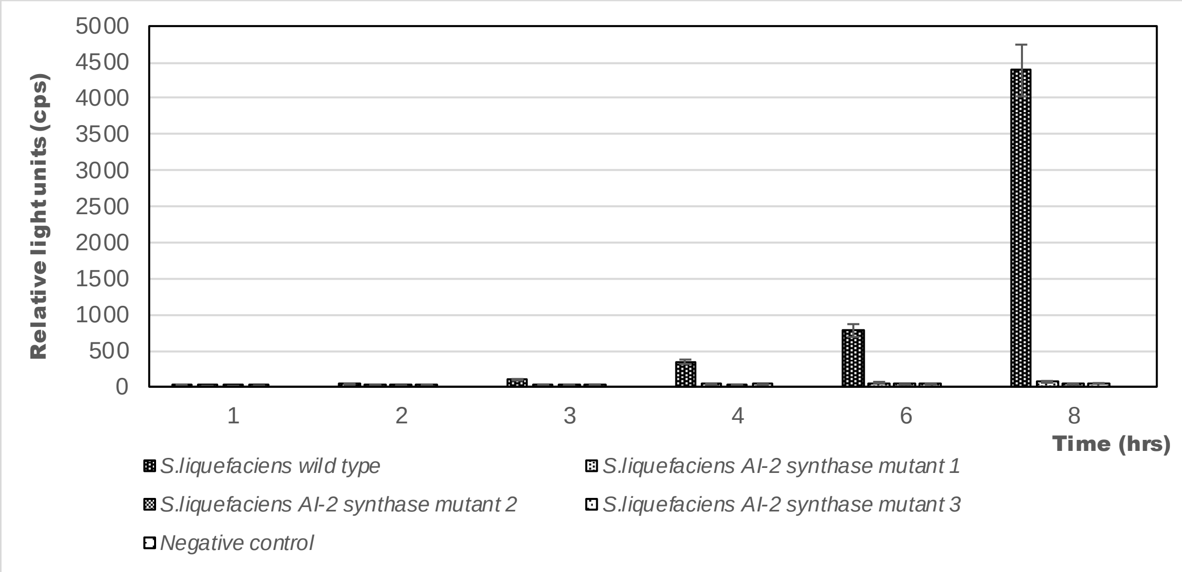


Figure 5.3A. AI-2 production by *S. liquefaciens* NZRM861 wild type and putative AI-2 synthase mutant strains. All the strains were grown overnight at 30 °C, and the bioluminescence was measured at different time points. Mean values and standard deviation of triplicates are indicated.

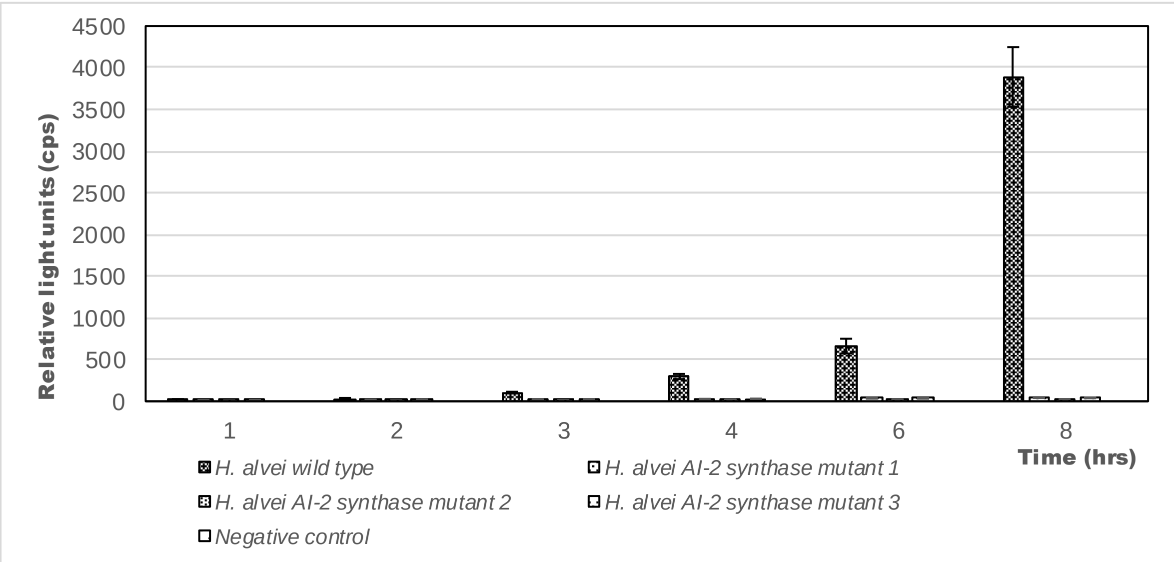


Figure 5.3B. AI-2 production by *H. alvei* NZRM860 wild type and putative AI-2 synthase mutant strains. All the strains were grown overnight at 30 °C, CFEs were measured by bioluminescence at different time points. Mean values and standard deviation of triplicates are indicated.

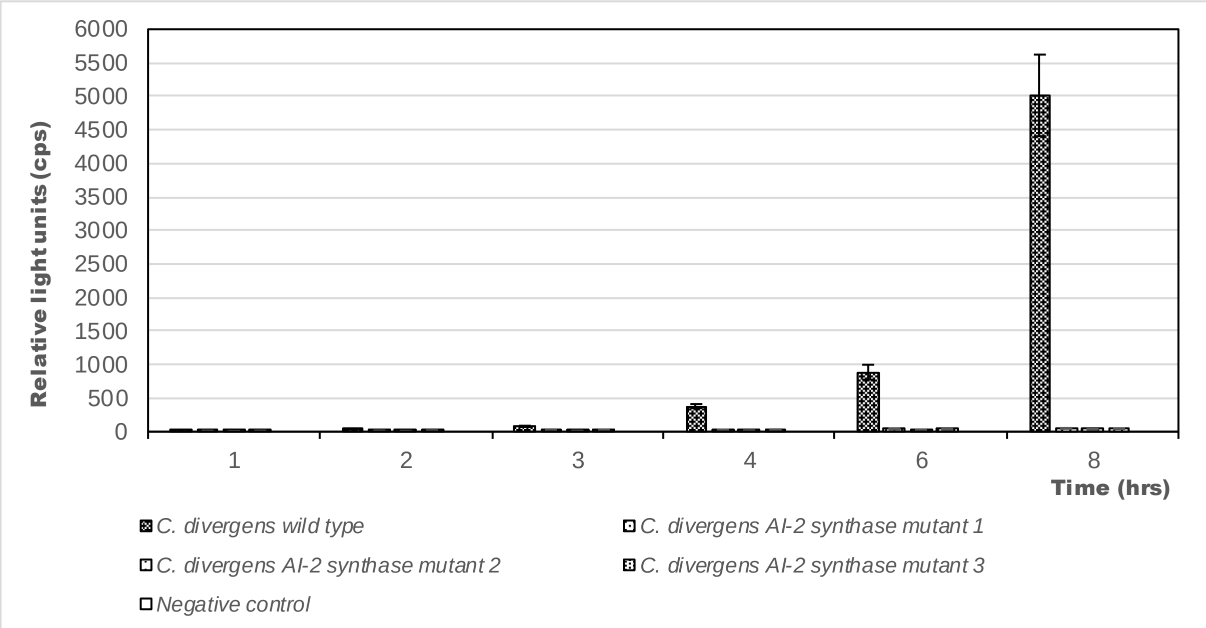


Figure 5.3C. AI-2 production by *C. divergens* FAM03 wild type and putative AI-2 synthase mutant strains. All the strains were grown overnight at 30 °C, and the CFEs were measured by bioluminescence at different time points. Mean values and standard deviation of triplicates are indicated.

Growth curves of the wild type and one selected AI-2 synthase mutant from each bacterium were generated as shown in Figures 5.4A-C. AI-2 production occurred during the exponential growth phase when bacteria were grown in Luria broth. Interestingly, as shown in these figures, AI-2 synthase mutants of *C. divergens* FAM03 showed a significant reduction in growth (p-value < 0.05) in minimal growth medium with 1% lactose compared to the wild types (Figure 5.4C), while the mutants from *H. alvei* NZRM860 and *S. liquefaciens* NZRM861 did not (Figures 5.4A and 5.4B). When the external AI-2 signal ((S)-4, 5-dihydroxy-2, 3-pentandione, Omm Scientific, USA) was added to the *C. divergens* mutants, growth was significantly increased (p-value < 0.01). The growth of the other two bacterial mutants in lactose were not different when the AI-2 signal was added externally.

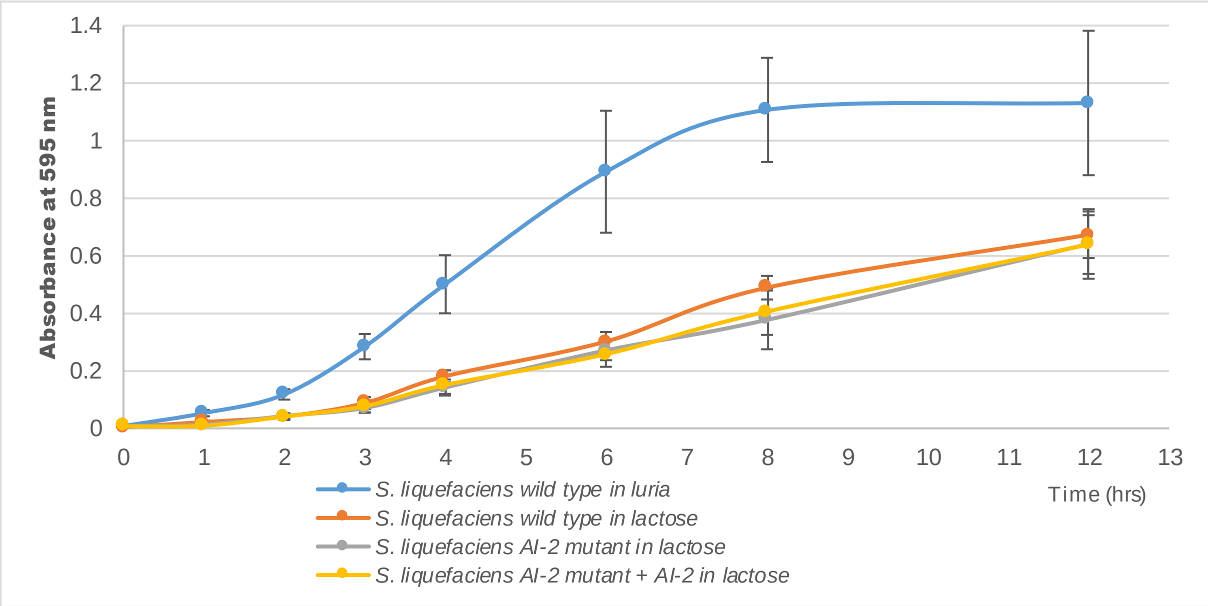


Figure 5.4A. Growth of all *S. liquefaciens* NZRM861 strains in different media. luria: Luria broth; lactose: minimal growth medium with 1% lactose. Mean values and standard deviation of triplicates are indicated.

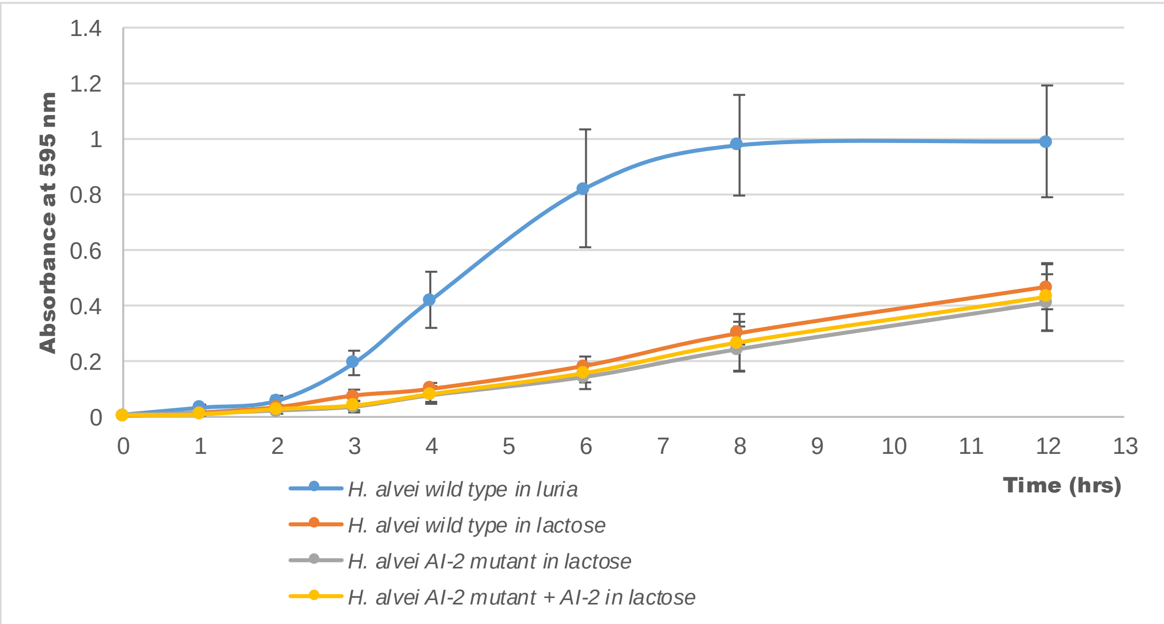


Figure 5.4B. Growth of all *H. alvei* NZRM860 strains in different media. luria: Luria broth; lactose: minimal growth medium with 1% lactose. Mean values and standard deviation of triplicates are indicated.

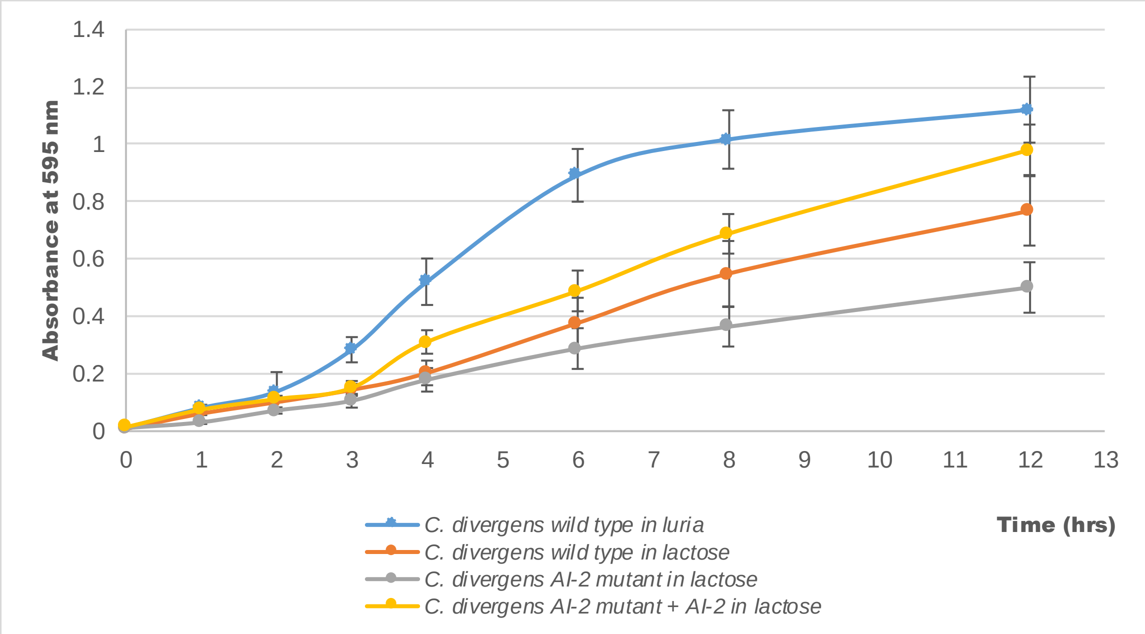


Figure 5.4C. Growth of all *C. divergens* FAM03 strains in different media. luria: Luria broth; lactose: minimal growth medium with 1% lactose. Mean values and standard deviation of triplicates are indicated. Exogenous AI-2 signal helped *luxS*↓ mutant grow in lactose solution.

5.3.4 Detection of AHL molecules by LCMS/MS

The AHL production by spoilage bacteria *H. alvei* NZRM860 and *S. liquefaciens* NZRM861, wild type and AHL synthase mutants (one for each bacterium) when grown in LB, was detected by LCMS/MS (Figures 5.5 and 5.6). In *H. alvei* NZRM860, the primary AHL signals produced were C4-HSL (m/z of 172.92), C6-HSL (m/z of 200.78), 3-oxo-C6-HSL (m/z of 213.17), 3-hydroxy-C6-HSL (m/z of 216.17), and C12-HSL (m/z of 283.08) (Figure 5.5B and Table 5.3). AHL references C4-HSL and C6-HSL were found as peaks with m/z of 172.00 and 199.33 (Figure 5.5C), very close to those peaks produced by the wild type strains. Synthetic C8-HSL and C10-HSL (peaks with m/z of 227.56 and 255.35, shown in Figure 5.5C) were not found in the wild type

(Figure 5.5B). The relative abundances (RA) of C6-HSL and 3-oxo-C6-HSL were over 60%, indicating that substituted and unsubstituted C6-HSL molecules were the major AHL signaling molecules produced by *H. alvei* NZRM860. Although the amount was very low, 3-oxo-C6-HSL (m/z of 213.25) was found in the mutant strain (Figure 5.5A), indicating that the AHL synthase gene (*halI*) was not completely dysfunctional in the *halI* mutant.

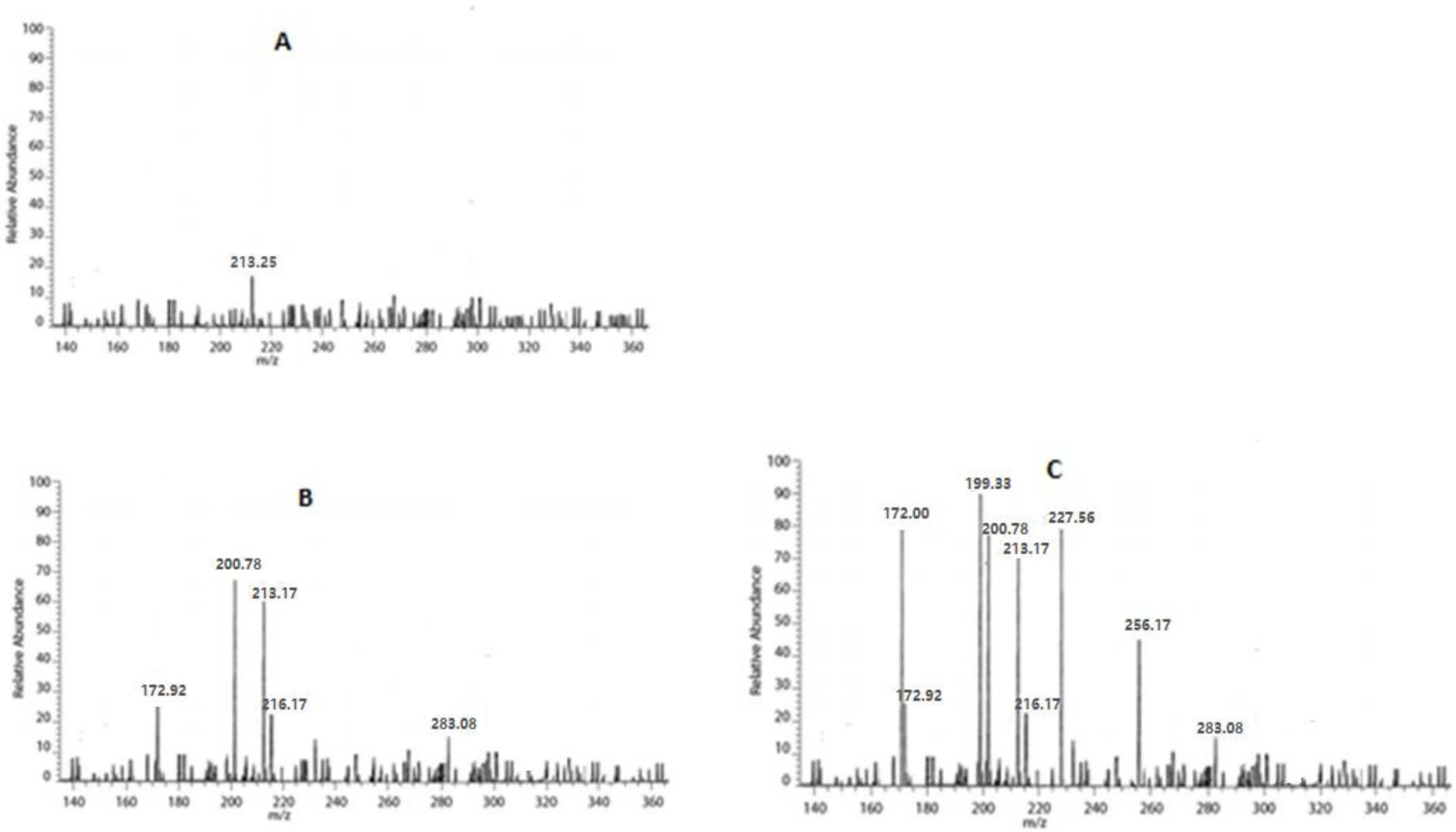


Figure 5.5. AHL profiles of LCMS/MS in *H. alvei* NZRM860 strains with addition of C4-HSL, C6-HSL, C8-HSL and C10-HSL standards. (A) *halI* mutant; (B) wild type; (C) wild type

Table 5.3. The identified AHLs from *H. alvei* NZRM860.

AHL molecules	**Molecular mass**	***H. alvei* wild type**
C4-HSL	171.89 (PubChem reference: 172.00)	172.92
C6-HSL	199.25 (PubChem reference: 199.33)	200.78
3-Oxo-C6-HSL	213.23	213.17
3- Hydroxy-C6-HSL	215.25	216.17
C8-HSL	227.30 (reference: 227.56)	N/A
C10-HSL	255.35 (reference: 256.17)	N/A
C12-HSL	283.41	283.08

In *S. liquefaciens* NZRM861, the primary AHL signals produced were C4-HSL (m/z of 173.00), C6-HSL (m/z of 199.33), 3-oxo-C6-HSL (m/z of 213.08), 3-hydroxy-C6-HSL (m/z of 214.92), C10-HSL (m/z of 255.25), and 3-oxo-C10-HSL (m/z of 268.92) (Figure 5.6B and Table 5.4). The AHL reference C4-HSL produced a peak with an m/z of 171.92, which was very close to the peak produced by the wild type (m/z of 173.00). C6-HSL and C10-HSL standards migrated to the positions with an m/z of 199.25 and 255.25, respectively (Figure 5.6C). A reference C8-HSL was added, with an m/z of 227.30 (Figure 5.6C) but it was not found in the wild type (Figure 5.6B). The relative abundance of C4-HSL and 3-oxo-C6-HSL were over 70%, indicating that these short-chain AHL molecules were produced by the wild type at much higher levels than the rest of the AHL signals. No significant AHL production was found in the mutant strain (Figure 5.6A), indicating that the AHL synthase gene (*sliI*) was disrupted.

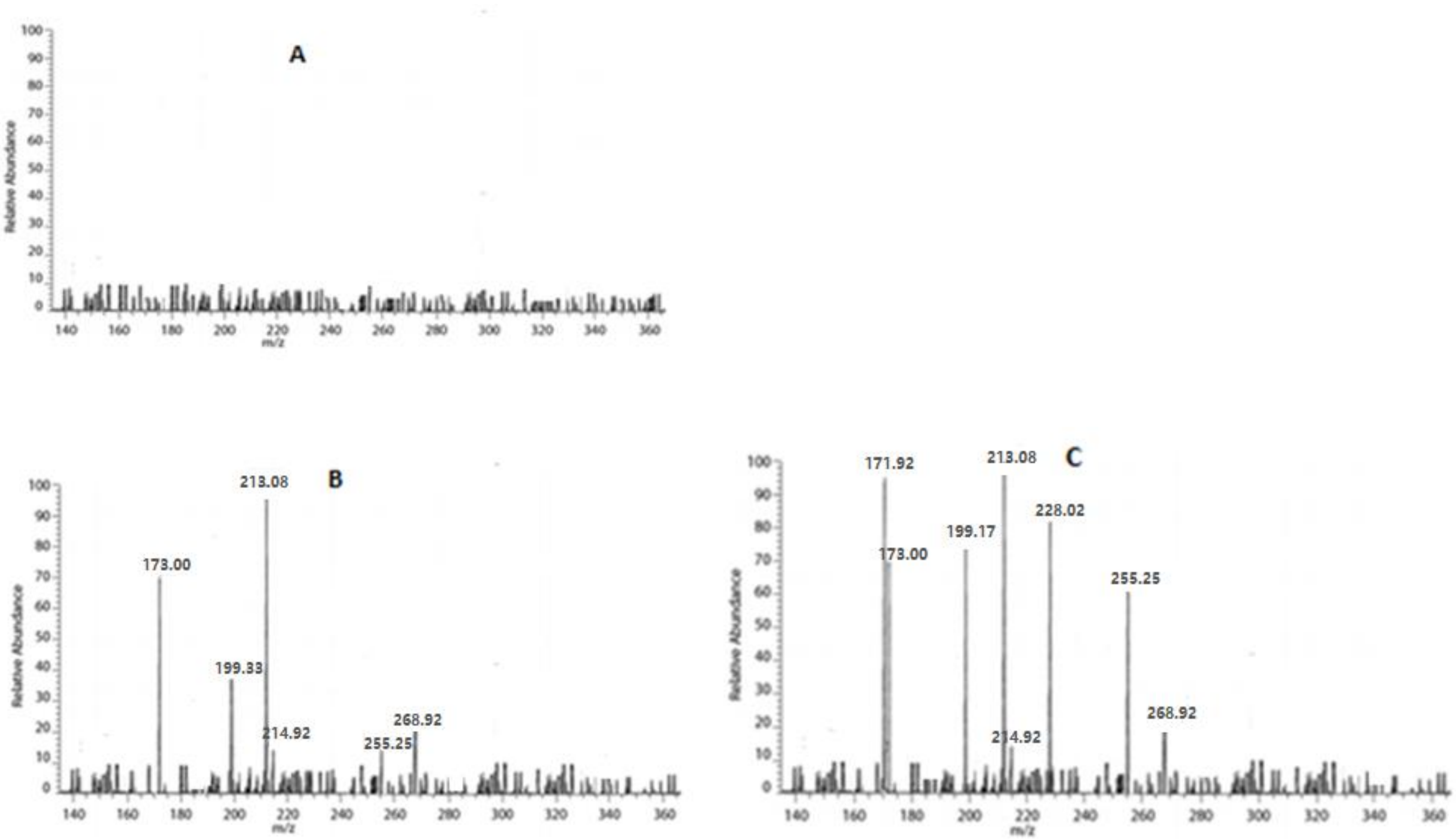


Figure 5.6. AHL profiles of LCMS/MS in *S. liquefaciens* NZRM861 strains with the addition of C4-HSL, C6-HSL, C8-HSL, and C10-HSL standards. (A) *sliI* mutant (B) wild type (C) wild type

Table 5.4. The identified AHLs from *S. liquefaciens* NZRM861.

AHL molecules	**Molecular mass**	***S. liquefaciens* wild type**
C4-HSL	171.89 (reference: 171.92)	173.00
C6-HSL	199.25 (reference: 199.17)	199.33
3-Oxo-C6-HSL	213.23	213.08
3- Hydroxy-C6-HSL	215.25	214.92
C8-HSL	227.30 (reference: 228.02)	N/A
C10-HSL	255.35 (reference: 255.25)	255.25
3-oxo-C10-HSL	269.34	268.92

5.3.5 DNA confirmation of mutants

DNA from all potential mutants of *H. alvei* NZRM860, *S. liquefaciens* NZRM861, and *C. divergens* FAM03 was extracted and analyzed by PCR. After agarose gel electrophoresis, the sizes of PCR products with and without transposon insertion were determined and compared using a 1kb plus DNA ladder. DNase free water was also analysed as blank.

Figure 5.7 shows a ~551 bp PCR product in *H. alvei* NZRM860, indicating the *halI* gene in *H. alvei*. However, the PCR product (~770 bp) was obtained in a putative *halI* mutant. Using the Basic Local Alignment Search Tool (BLAST) to assess similarity, this product showed 76% identity with a query coverage of 62% to *halI* gene in *H. alvei* (NCBI Reference Sequence: NZ_CP009706.1). To check the presence of transposon insertion, the forward primer of *halI* and reverse primer of the transposon were used for PCR and the PCR product was 155 bp, showing an integration of the mosaic end sequence of the EZ-Tn5 transposon. By using the expasy translational toolbox, it was observed that the transposon insertion affected one of the open reading frames (ORF) of the translated protein (See Appendix 2). As the gene was still detectable in the mutant, it was believed that the insertion (containing the mosaic end) of EZ-Tn5 transposon in the *halI* gene may have knocked down gene expression, rather than completely knocked out the gene (hence *halI* ↓ was used instead of Δ*halI*), and have affected the production of AHL signaling molecules in the *halI* ↓ mutant strain.

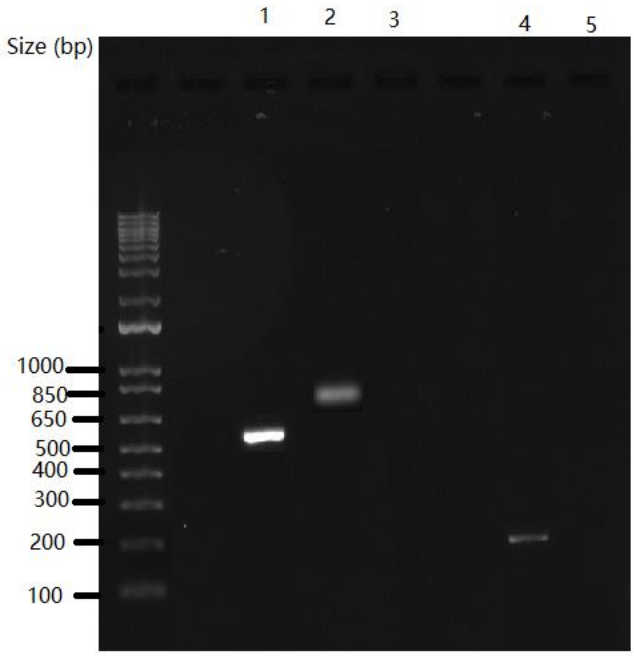


Figure 5.7. Ethidium bromide-stained PCR products after agarose gel electrophoresis. Two sets of primers were used to amplify the target sequence (*halI* gene) from the wild type and putative mutant of *H. alvei* NZRM860. Lane 1: *halI* in wild type*;* Lane 2: *halI* in *halI*↓ strain; Lane 4: Transposon insertion in *halI*↓ strain; Lane 3 & 5: blanks.

Figure 5.8 shows a ~289 bp PCR product in *H. alvei* NZRM860, indicating the *luxS* gene in *H. alvei*. However, the PCR product (~510 bp) was obtained in a putative *luxS* ↓ mutant. BLAST analysis showed 86% identity with 67% query coverage of this product to the *luxS* gene in *H. alvei* (NZ_CP009706.1). The PCR product of the inserted segment was around 220 bp. After sequencing and investigation by expasy, the insertion of the transposon was found to be in one of the ORFs (Appendix 2) and may have knocked down the function of *luxS* in the *luxS* ↓ mutant strain (*luxS* ↓ was used instead of Δ*luxS*).

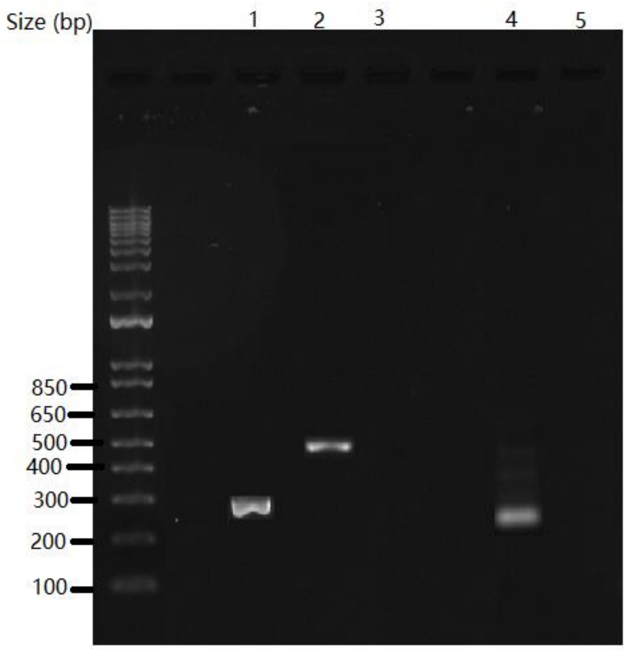


Figure 5.8. Ethidium bromide-stained PCR products. Two sets of primers were used to amplify the target sequence (*luxS* gene) from wild type and putative mutant of *H. alvei* NZRM860. Lane 1: *luxS* in the wild type*;* Lane 2: *luxS* in the *luxS*↓ strain; Lane 4: Transposon insertion in the *luxS*↓ strain; Lane 3 & 5: blanks

Figure 5.9 shows a ~498 bp PCR product in *H. alvei* NZRM860, indicating the *halR* gene. However, the PCR product (~670 bp) was obtained in a putative *halR* ↓ mutant. BLAST showed this product had a 72% identity with 48% query coverage to the *halR* gene in *H. alvei* (NZ_CP009706.1). The PCR product of the inserted segment was around 170 bp. Expasy showed that the insertion of transposon was in one of the ORFs (Appendix 2). The function of the *halR* gene was knocked down by the transposition and it may have affected the AHL reception and regulation in the *halR* ↓ mutant strain (*halR* ↓ was used instead of Δ*halR*).

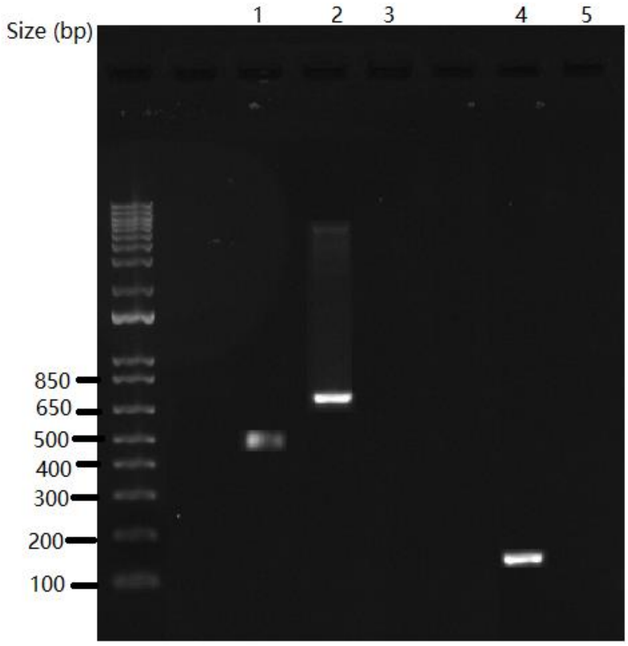


Figure 5.9. Ethidium bromide-stained PCR products. Two sets of primers were used to amplify the target sequence (*halR* gene) from wild type and putative mutant of *H. alvei* NZRM860. Lane 1: *halR* in the wild type*;* Lane 2: *halR* in the *halR*↓ strain; Lane 4: Transposon insertion in the *halR*↓ strain; Lane 3 & 5: blanks

Figure 5.10 shows the PCR product of the *sliI* gene (~362 bp) in *S. liquefaciens* NZRM861. However, the PCR product (~640 bp) was obtained in a putative *sliI* ↓ mutant. A 71% identity with 49% query coverage of this product to the *sliI* gene in *S. liquefaciens* (AY168877.1) was shown by BLAST. The PCR product of the inserted segment was around 280 bp. After sequencing and investigation by expasy, the insertion of the transposon was found to be located in one of the ORFs (Appendix 2) and may have knocked down the AHL synthase gene in the *sliI* ↓ mutant strain (*sliI* ↓ was hence used instead of Δ*sliI*).

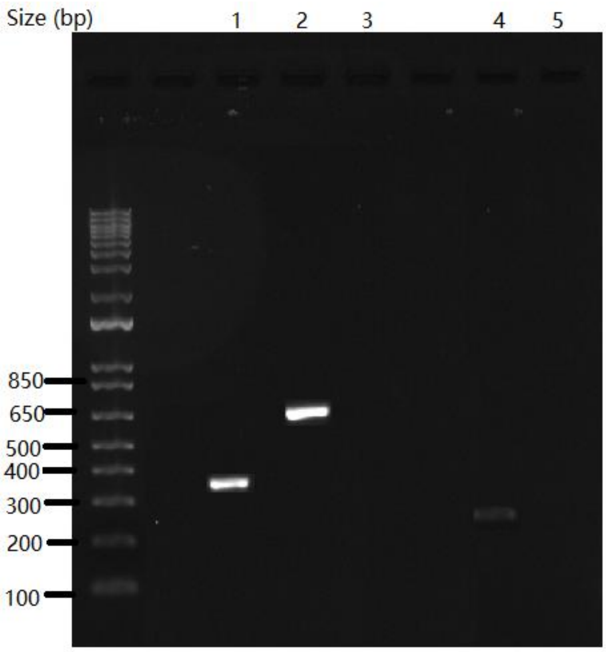


Figure 5.10. Ethidium bromide-stained PCR products. Two sets of primers were used to amplify the target sequence (*sliI* gene) from wild type and putative mutant of *S. liquefaciens* NZRM861. Lane 1: *sliI* in the wild type*;* Lane 2: *sliI* in the *sliI*↓ strain; Lane 4: Transposon insertion in the *sliI*↓ strain; Lane 3 & 5: blanks

Figure 5.11 shows the PCR product of the *luxS* gene (~505 bp) in *S. liquefaciens* NZRM861. However, the PCR product (~785 bp) was obtained in a putative *S. liquefaciens luxS* ↓ mutant. BLAST showed an 82% identity with 68% query coverage of this product to the *luxS* gene in *S. liquefaciens* (NC_009832). The PCR product of the inserted segment was found to be around 280 bp. After sequencing and investigation, the insertion of the transposon was found in one of the ORFs (Appendix 2) and may have knocked down the AI-2 synthase gene in the *luxS* ↓ mutant strain as this gene was not completely knocked out.

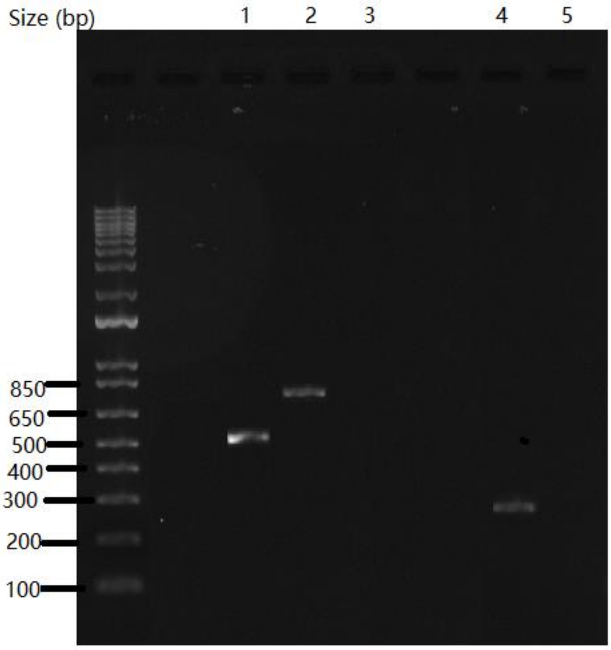


Figure 5.11. Ethidium bromide-stained PCR products. Two sets of primers were used to amplify the target sequence (*luxS* gene) from wild type and putative mutant of *S. liquefaciens* NZRM861. Lane 1: *luxS* in the wild type*;* Lane 2: *luxS* in the *luxS*↓ strain; Lane 4: Transposon insertion in the *luxS*↓ strain; Lane 3 & 5: blanks

Figure 5.12 shows the PCR product of the *sliR* gene (~529 bp) in *S. liquefaciens* NZRM861. The PCR product (~670 bp) was obtained in a putative *sliR*↓ mutant. BLAST showed a 72% identity with 46% query coverage of this product to the *sliR* gene in *S. liquefaciens* (AY168877.1). The PCR product of the inserted segment was found to be around 140 bp. After sequencing and investigation, expasy found that the insertion of transposon was located in one of the ORFs (Appendix 2) and may have affected the AHL reception and regulation in the *sliR*↓ mutant strain (*sliR*↓ was thus used instead of Δ*sliR*).

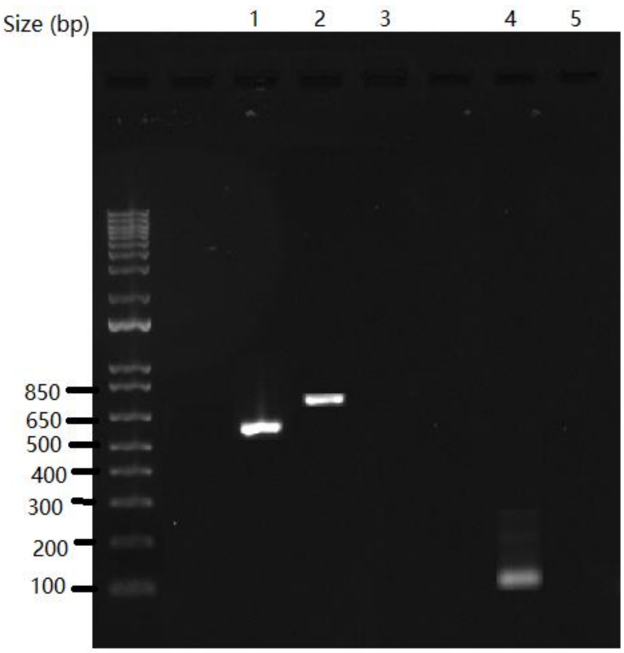


Figure 5.12. Ethidium bromide-stained PCR products. Two sets of primers were used to amplify the target sequence (*sliR* gene) from the wild type and the putative mutant of *S. liquefaciens* NZRM861. Lane 1: *sliR* in the wild type*;* Lane 2: *sliR* in the *sliR*↓ strain; Lane 4: Transposon insertion in the *sliR*↓ strain; Lane 3 & 5: blanks

Figure 5.13 shows the PCR product of the *hflB* gene (~661 bp) in one of the putative *sliI* ↓ mutants of *S. liquefaciens* NZRM861. The PCR product (~860 bp) was obtained in a putative *sliI* ↓ and *hflB* ↓ mutant. BLAST showed a 67% identity with 43% query coverage of this product to the *hflB* gene in *S. liquefaciens* (NC_021741.1). The PCR product of the inserted segment was found to be around 200 bp. Both the *sliI* and *hflB* genes were detectable and expasy found that the insertion of the transposon was in one of the ORFs (Appendix 2). The transposition may have knocked down both the *sliI* and *hflB* genes in the mutant strain.

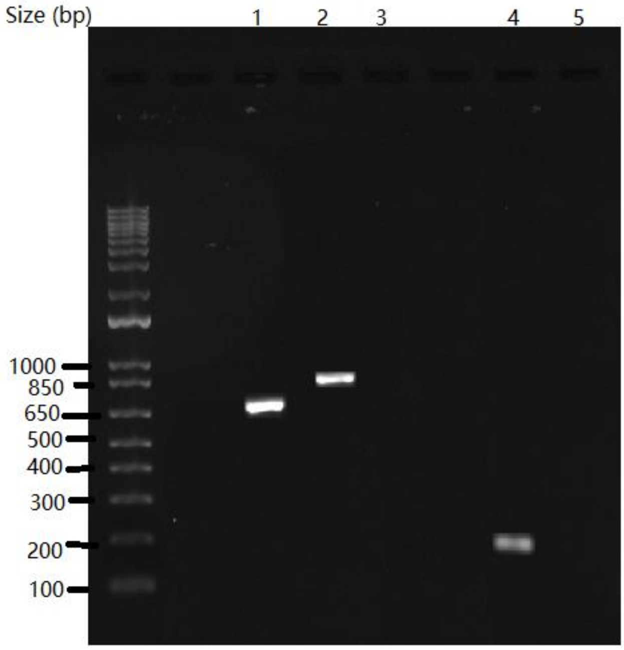


Figure 5.13. Ethidium bromide-stained PCR products. Two sets of primers were used to amplify the target sequence (*hflB* gene) from a *sliI*↓ and putative double *sliI*↓*hflB*↓ mutants of *S. liquefaciens* NZRM861. Lane 1: *hflB* in the *sliI*↓ strain; Lane 2: *hflB* in the *sliI*↓*hflB*↓ mutant strain; Lane 4: Transposon insertion in *sliI*↓*hflB*↓ in the mutant strain; Lane 3 & 5: blanks

Figure 5.14 shows the PCR product of the *luxS* gene (~306 bp) in *C. divergens* FAM03. The PCR product (~580 bp) was obtained in a putative *luxS* ↓ mutant of *C. divergens* FAM03. BLAST showed a 78% identity with 61% query coverage of this product to the *luxS* gene in *C. divergens* (OGVA01000030). The PCR product of the insertion was found around 275 bp. Expasy found that the insertion of the transposon was in one of the ORFs (Appendix 2) and may have affected the AI-2 synthesis in the mutant strain.

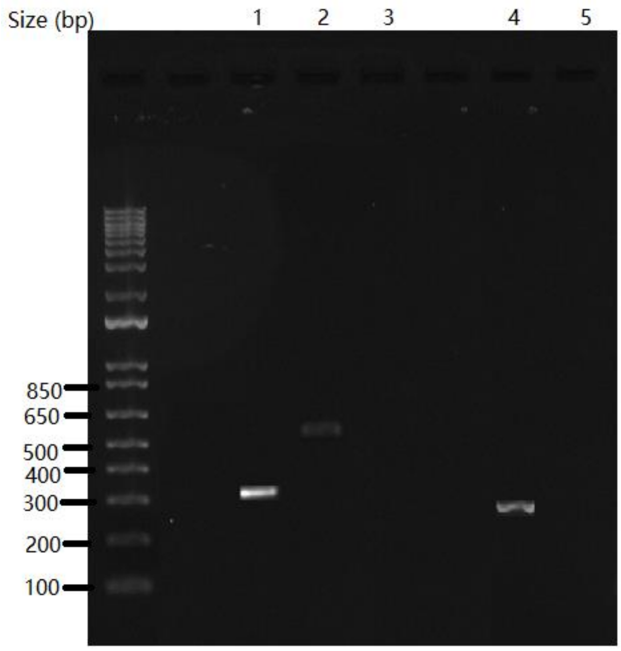


Figure 5.14. Ethidium bromide-stained PCR products. Two sets of primers were used to amplify the target sequence (*luxS* gene) from the wild type and putative *luxS*↓ mutant of *C. divergens* FAM03. Lane 1: *luxS* in the wild type; Lane 2: *luxS* in the *luxS*↓ strain; Lane 4: Transposon insertion in the *luxS*↓ strain; Lane 3 & 5: blanks

5.3.6 Screening of proteases

A positive result of protease activity is a clear zone on a BCG agar plate around the CFEs of bacterial strains. Negative controls (uninoculated LB and acetonitrile) did not show any proteolytic activity. As shown in Figure 5.15A, a clear zone was observed around the positive control dispase, as well as the CFE of the wild type *H. alvei* NZRM860, indicating the presence of proteases. The *halI*↓ mutant did not show proteolytic activity but when different types of external AHL standards were added, a clear zone around the *halI*↓ strain was visualized, indicating the existence of proteases, like the wild type.

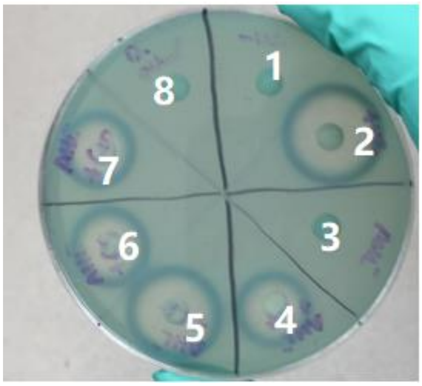


Figure 5.15A. Degradation of protein by *H. alvei* NZRM860 in lamb agar plates containing BCG dye. 1: Negative LB control; 2: Positive control 1 mg/mL dispase; 3: *halI*↓ mutant; 4: *halI*↓ mutant + C4-HSL; 5: *halI*↓ mutant + C6-HSL; 6: *halI*↓ mutant + C8-HSL; 7: *halI*↓ mutant + C10-HSL; 8: Negative acetonitrile control.

Protease production by *S. liquefaciens* NZRM861 and mutants was screened on agar plates. Proteases were secreted by the positive control as well as the CFE of the wild type. However, the *sliI*↓ mutant did not show any proteolytic activity, and when AHL was added to the mutant strain, the proteolytic activity was observed (Figure 5.15B).

Figure 5.15b. Degradation of protein by *S. liquefaciens* NZRM861 in an agar plate containing BCG. 1: *sliI*↓ mutant; 2: *sliI*↓ mutant + C4-HSL; 3: *sliI*↓ mutant + C6-HSL; 4: *sliI*↓ mutant + C8-HSL; 5: *sliI*↓ mutant + C10-HSL; 6: Negative acetonitrile control; 7: Positive control 1 mg/mL dispase; 8: Negative LB control.

In Figure 5.15C, the CFEs of the wild type strains of *H. alvei* NZRM860, *S. liquefaciens* NZRM861, and *C. divergens* FAM03, as well as their *luxS* ↓ mutants were added onto the agar plates for protease screening. All these samples were shown to secrete proteases which degraded the protein inside the plate and showed the halo around the spot.

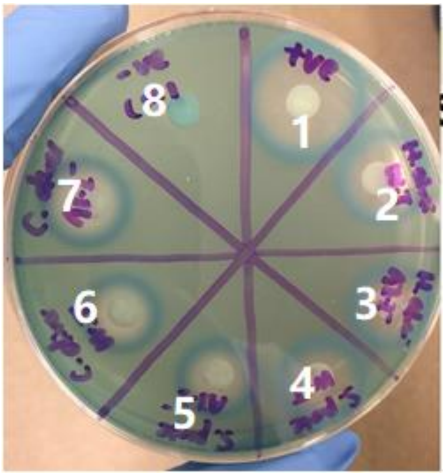


Figure 5.15c. Protease screening of three spoilage bacteria and their AI-2 synthase mutants (*luxS*↓) on an agar plate containing BCG. 1: Positive control 1mg/mL dispase; 2: *H. alvei* wild type; 3: *H. alvei luxS*↓; 4: *S. liquefaciens* wild type; 5: *S. liquefaciens luxS*↓; 6: *C. divergens* wild type; 7: *C. divergens luxS*↓; 8: Negative LB control.

5.3.7 Screening of lipases

A positive result of lipase activity was indicted by a halo under UV light around the CFEs of bacteria on a rhodamine B agar plate. Negative controls did not show any lipolytic activity. As shown in Figure 5.16A, a halo was observed around the positive control as well as the CFE of wild type *H. alvei* NZRM860, under UV detection, indicating the presence of lipases. The *halI*↓ mutant did not show lipolytic activity but when AHL was added, the orange halo was observed around the mutant strain, indicating the existence of lipases.

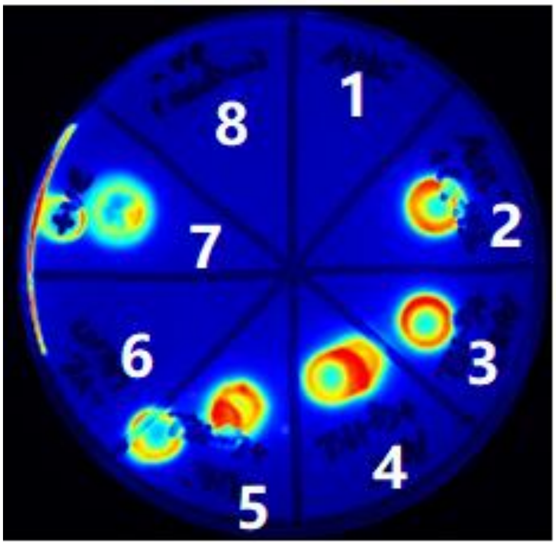


Figure 5.16A. Degradation of lamb fat by lipase secreted by *H. alvei* NZRM860 in agar plates containing rhodamine B. 1: *halI*↓ mutant; 2: *halI*↓ mutant + C4-HSL; 3: *halI*↓ mutant + C6-HSL; 4: *halI*↓ mutant + C8-HSL; 5: *halI*↓ mutant + C10-HSL; 6: Negative acetonitrile control; 7: Positive control 1 mg/mL lipase; 8: Negative LB control.

The screening of lipase production by *S. liquefaciens* NZRM861 and mutants on a rhodamine B plate is shown in Figure 5.16B. Lipases were secreted by the positive control as well as the wild type. However, the *sliI*↓ strain did not show any lipolytic activity, but when AHL was added to the *sliI*↓ strain, the lipolytic activity was observed.

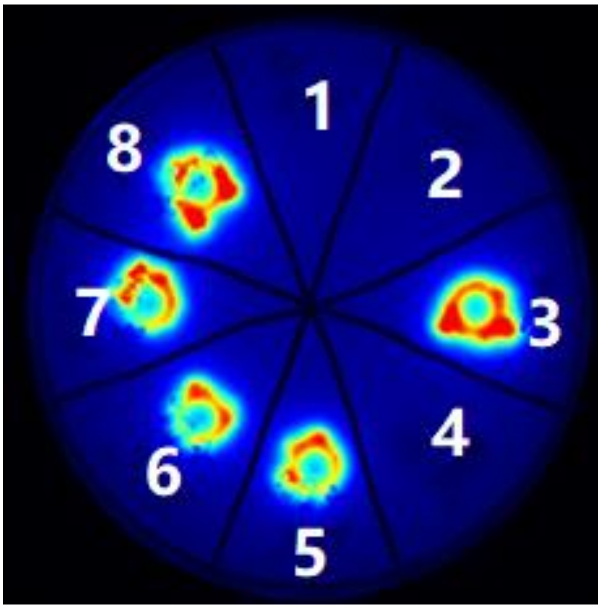


Figure 5.16B. Degradation of lamb fat by *S. liquefaciens* NZRM861 in rhodamine B agar plates. 1: Negative acetonitrile control; 2: Negative LB control; 3: Positive control 1 mg/mL lipase; 4: *sliI* ↓ mutant; 5: *sliI*↓ mutant + C4-HSL; 6: *sliI*↓ mutant + C6-HSL; 7: *sliI*↓ mutant + C8-HSL; 8: *sliI*↓ mutant + C10-HSL.

In Figure 5.16C, similar to the protease screening test, the CFEs of wild type strains of all three spoilage bacteria and their *luxS*↓ mutants were added onto the Rhodamine B agar plate for lipase screening. All these samples were shown to have lipolytic activity which degraded the lamb fat and showed the halo around the spot under UV light.

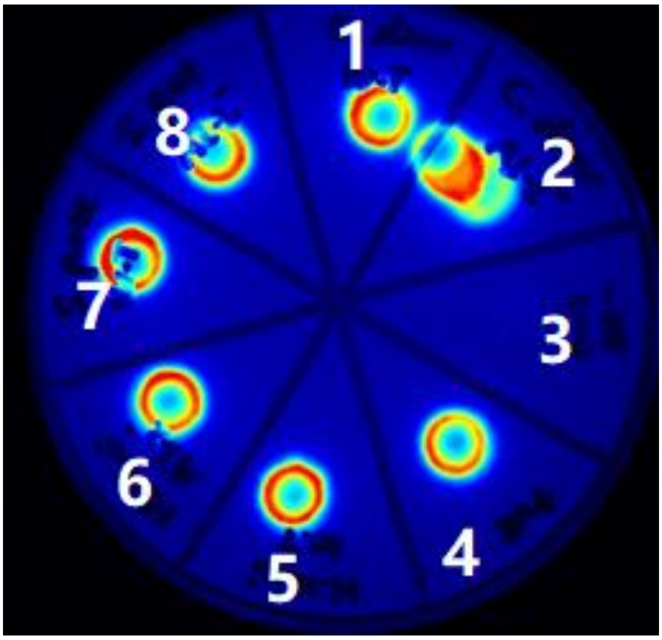


Figure 5.16C. Lipase screening of three spoilage bacteria and their *luxS*↓ mutants in rhodamine B agar plates. 1: *C. divergens* wild type; 2: *C. divergens luxS*↓; 3: Negative LB control; 4: Positive control 1 mg/mL lipase; 5: *H. alvei* wild type; 6: *H. alvei luxS*↓; 7: *S. liquefaciens* wild type; 8: *S. liquefaciens luxS*↓.

5.3.8 Effect of AHL on protease activity

The protease activity of *H. alvei* NZRM860 and *S. liquefaciens* NZRM861, and their AHL synthase mutants, with and without AHL addition were compared by absorbance. Absorbance measurements at 440 nm of *H. alvei* NZRM860 supernatants were compared against the blank LB, the media before bacterial growth, with protease activity represented by relative OD > 1(Figure 5.17A). Protease activity was shown in the wild type, indicating 3.8 times the amount of enzyme compared with the blank LB solution. The protease activity of the *halI*↓ mutant was low, at 1.3 times that of the blank, however when AHL (3-oxo-C6-HSL) was added to the mutant, the protease activity increased to 5.1 times, that of the blank. The protease activity of the *halR*↓

mutant did not change very much with or without the addition of AHL, with the protease activity about 1.8 times that of the blank.

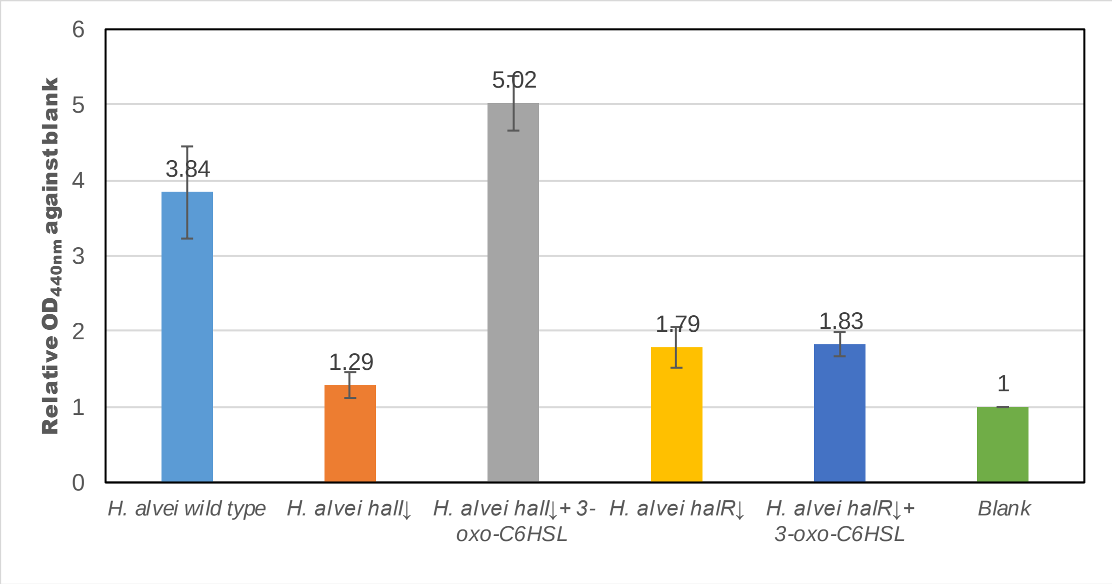


Figure 5.17A. Protease activity of *H. alvei* NZRM860 strains against blank control. It was calculated by relative absorbance at 440 nm. Mean and standard deviation of triplicates are indicated. Exogenous AHL helped to restore the protease activity of the *halI*↓ mutant but not the *halR*↓ mutant.

The wild type strain of *S. liquefaciens* NZRM861, the *sliI*↓ mutant and the mutant with AHL added showed protease levels, compared with the blank of 2.4, 1.6 and 3.3 times respectively (Figure 5.17B). The protease activity of the *sliR*↓ mutant was 1.3 times that of the blank and it did not change very much when external AHL was added. If the *hflB* gene was knocked down in the *sliI*↓ mutant of *S. liquefaciens* NZRM861, the addition of AHL did not influence protease activity and the activity was very close to the blank.

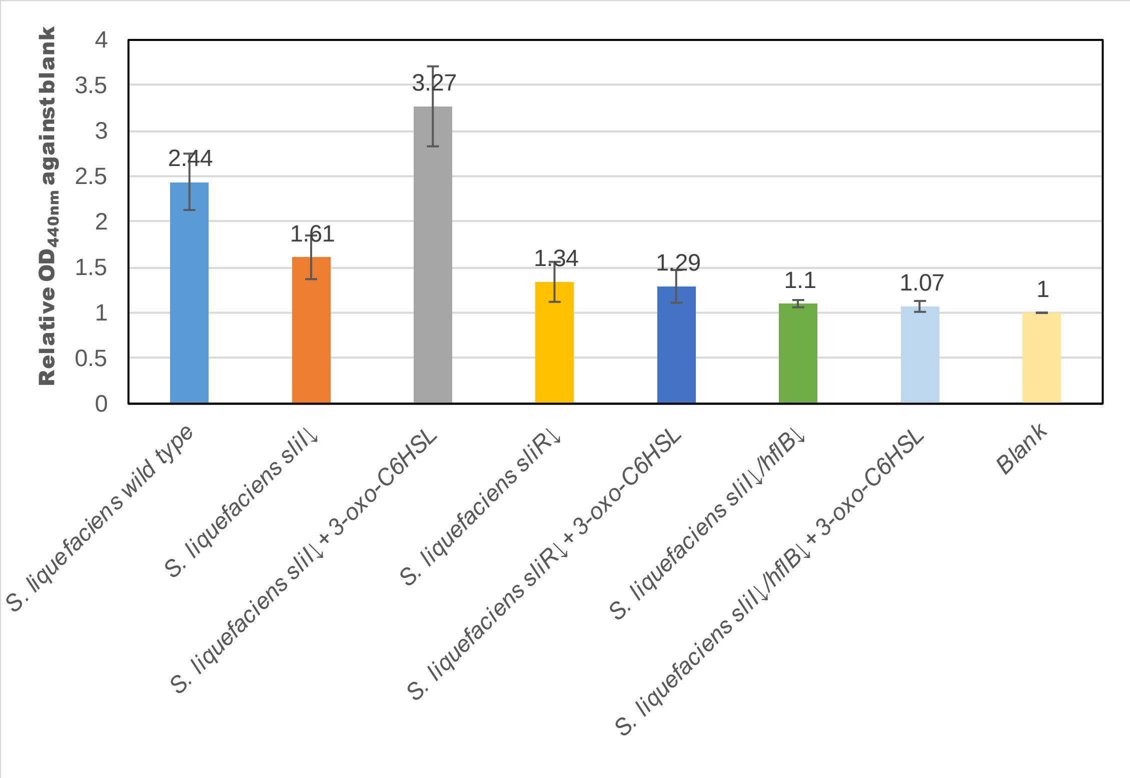


Figure 5.17B. Protease activity of *S. liquefaciens* NZRM861 strains against blank. Mean and standard deviation of triplicates are indicated. Exogenous AHL helped to restore the protease activity of the *sliI*↓ mutant but not the *sliR*↓ mutant. When *hflB* was also knocked down, the AHL signal did not influence the protease activity of the *sliI*↓ mutant.

5.3.9 Effect of AHL on lipase activity

Lipase activity followed a similar trend to the protease activity. AHL addition increased the lipase activity in both Enterobacteriaceae species. In Figure 5.18A, the absorbance of *H. alvei* NZRM860 was 2.3 times that of the blank for lipase activity. The lipase activity of the *halI*↓ mutant was1.2 times that of the blank, significantly lower than that when AHL was added, resulting in 2.1 times that of the blank. The lipase activity of the *halR*↓ mutant did not change very with or without the addition of AHL, with the lipase activity about 1.7 times that of the blank.

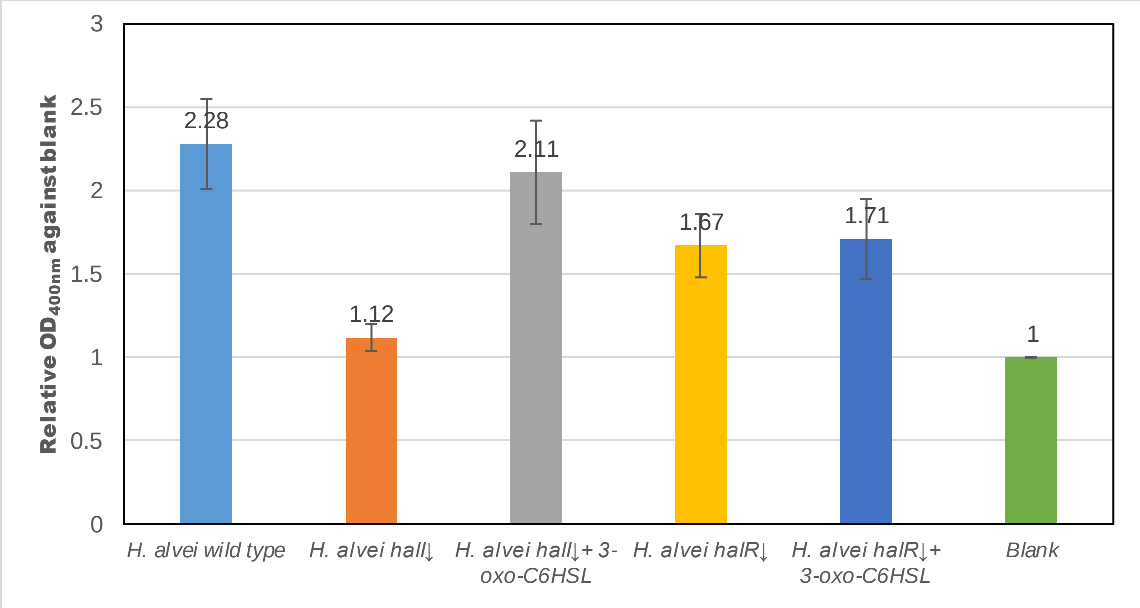


Figure 5.18A. Lipase activity of *H. alvei* NZRM860 strains against blank. Mean and standard deviation of triplicates are indicated. The addition of AHL helped to restore the lipase activity of *halI*↓ mutant but not *halR*↓ mutant.

The lipase activity of *S. liquefaciens* NZRM861 was around 2.3 times that of the blank and the *sliI*↓ mutant showed a reduced level of lipase (1.2 times that of the blank). AHL addition increased the lipase activity of the *sliI* ↓ mutant to 2.2 times that of the blank (Figure 5.18B). Again, when the AHL receptor gene *sliR* was knocked down, the external addition of AHL to the mutant did not influence the lipase activity, about 1.2 times that of the blank.

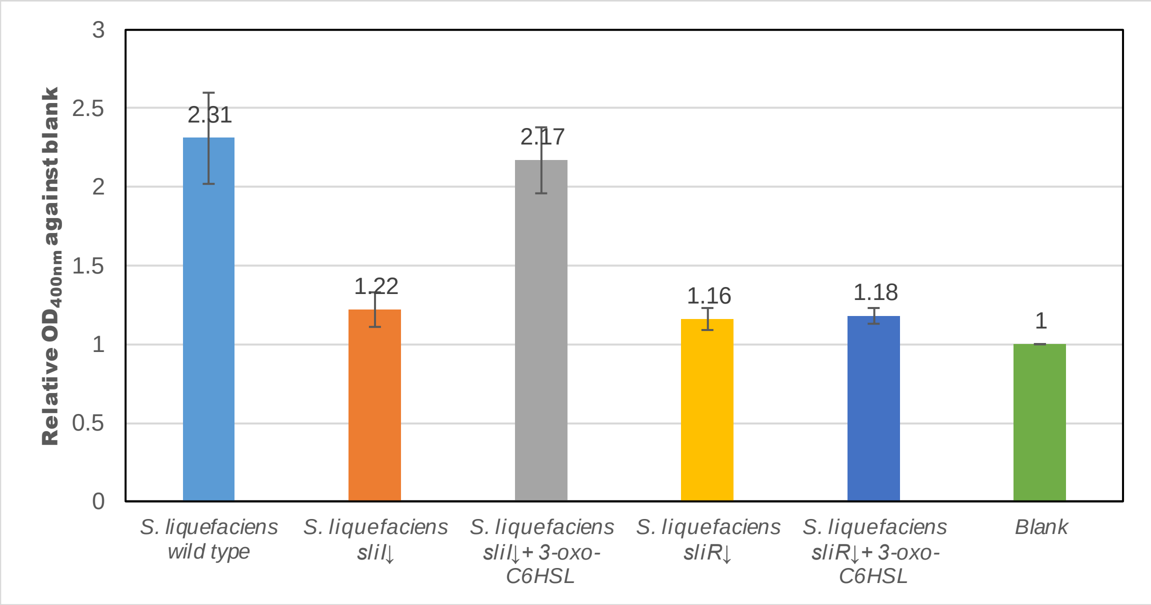


Figure 5.18b. Lipase activity of *S. liquefaciens* NZRM861 strains against blank control. Mean and standard deviation of triplicates are indicated. The addition of AHL helped to restore the lipase activity of the *sliI*↓ mutant but not the *sliR*↓ mutant.

5.3.10 Gene expression of underlying mechanisms

The influence of AHL signals on the gene expression in *H. alvei* NZRM860 and *S. liquefaciens* NZRM861 strains was investigated by RT-qPCR. The wild type and mutant strains of these two *Enterobacteriaceae* species with and without AHL addition were compared. In Figures 5.19A-C, *H. alvei* NZRM860 and its mutant strains were grown in two different media, fresh LB broth and lamb juice. Untreated RNase-free water was used as a negative control. The $\log_{10}$ of relative expression was considered as 0 for the blank (mean and standard deviation of triplicates are indicated). The expression of *halI* did not change significantly (p-value > 0.05) in all strains, except *halI*↓ mutants, irrespective of the growth medium (LB or lamb juice). However, the expression of *halR* was higher when the bacterium was grown in lamb juice rather than in LB, even for *halR*↓ mutants. When AHL was added (in the LB environment, AHL standard 3-oxo-C6-HSL; in the lamb environment, the AHL signals produced by *S. liquefaciens* FAM02), the expression of *halR* increased. The AHL signals produced by the same species *H. alvei* FAM01, which was isolated from lamb did not affect *halR* expression in *H. alvei* NZRM860 and its mutant strains. Interestingly, when the signals from *H. alvei* FAM01 were chemically modified (described as the second type in Section 5.2.5), they enhanced the expression of *halR*. The expression of *lipB* was correlated with *halR* when *halR* was suppressed, the expression of *lipB* decreased; when the expression of *halR* was enhanced, the expression of *lipB* also increased.

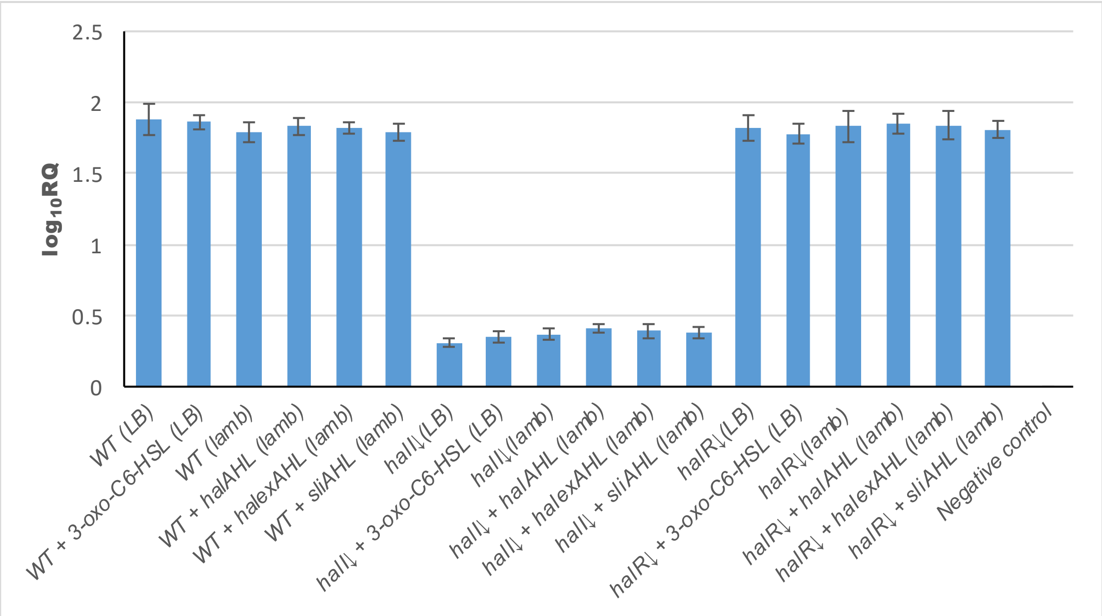


Figure 5.19A. The expression of *halI* in *H. alvei* NZRM860 strains. The wild type and mutant strains were grown in different media with AHLs. In LB, the AHL standard was added, and in lamb juice, AHLs produced by lamb isolates were added. halAHL: AHL signals extracted from lamb-sourced *H. alvei* FAM01; sliAHL: AHL signals extracted from *S. liquefaciens* FAM02; halexAHL: AHL signals extracted from *H. alvei* FAM01 and chemically reconstituted in acetonitrile. Mean values and standard deviation of triplicates are indicated.

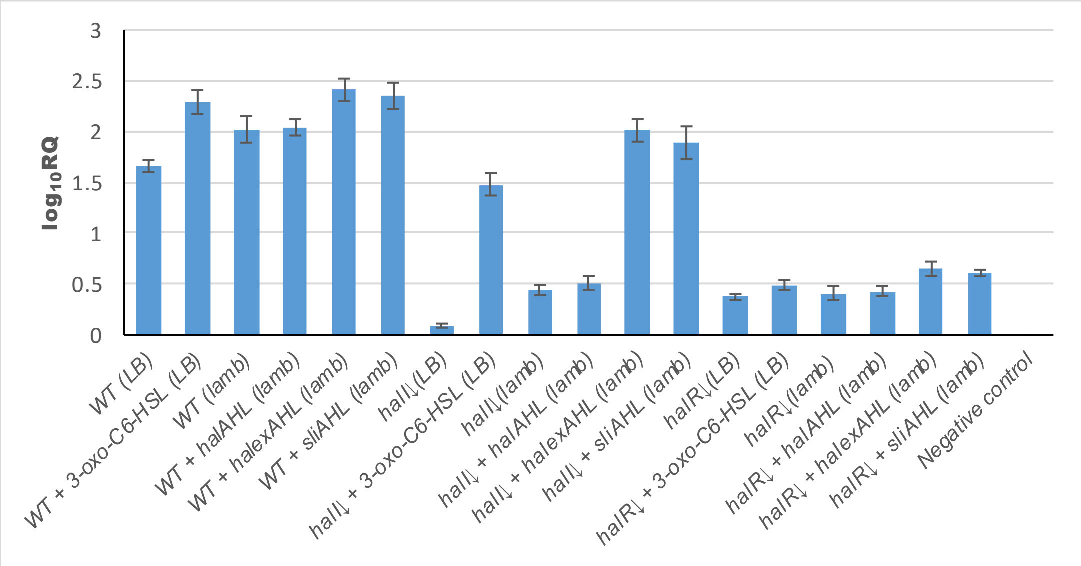


Figure 5.19B. The expression of *halR* in *H. alvei* NZRM860 strains. The wild type and mutant strains were grown in different media with AHLs. In LB, the AHL standard was added, and in lamb juice, AHLs produced by lamb isolates were added. halAHL: AHL signals extracted from lamb-sourced *H. alvei* FAM01; sliAHL: AHL signals extracted from *S. liquefaciens* FAM02; halexAHL: AHL signals extracted from *H. alvei* FAM01 and chemically reconstituted in acetonitrile. Mean values and standard deviation of triplicates are indicated.

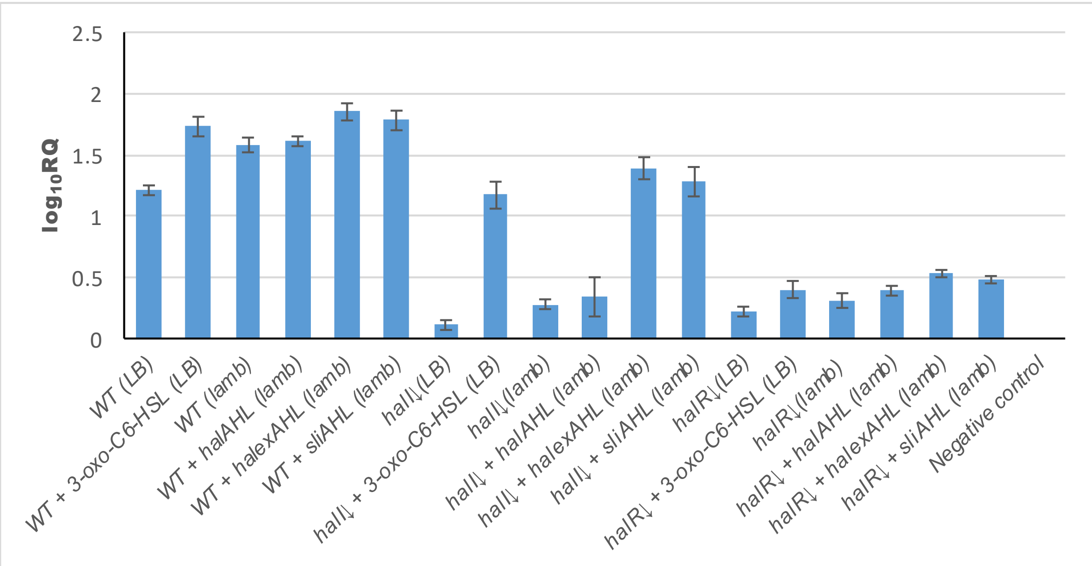


Figure 5.19C. The expression of *lipB* in *H. alvei* NZRM860 strains. The wild type and mutant strains were grown in different media with AHLs. In LB, the AHL standard was added, and in lamb juice, AHLs produced by lamb isolates were added. halAHL: AHL signals extracted from lamb-sourced *H. alvei* FAM01; sliAHL: AHL signals extracted from *S. liquefaciens* FAM02; halexAHL: AHL signals extracted from *H. alvei* FAM01 and chemically reconstituted in acetonitrile. Mean values and standard deviation of triplicates are indicated.

In *S. liquefaciens* NZRM861 and its mutants, the expression of *sliI* did not change significantly (p-value > 0.05), except for the *sliI*↓ mutants in either growth medium. When this bacterium was cultivated in lamb juice, *sliR* expression was higher than when it was grown in LB. When AHL (3-oxo-C6-HSL in LB; AHL signals produced by *H. alvei* FAM01 in lamb) was added, the expression of *sliR* increased, and so did *lipB*. When the AHL signals produced by the same species of *S. liquefaciens* FAM02, which was isolated from lamb, *sliR* and *lipB* expression did not change in *S. liquefaciens* NZRM861 and its mutant strains. However, when the signals were chemically modified, the expressions of those two genes increased (Figure 5.19D-F).

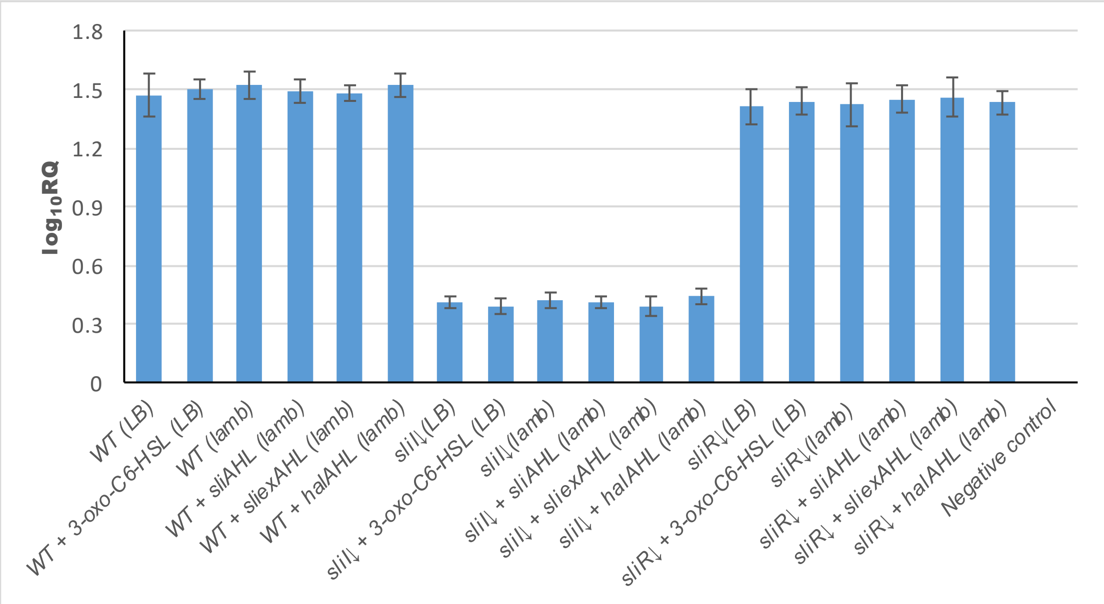


Figure 5.19D. The expression of *sliI* in *S. liquefaciens* NZRM861 strains. The wild type and mutant strains were grown in different media with AHLs. In LB, the AHL standard was added, and in lamb juice, AHLs produced by lamb isolates were added. halAHL: AHL signals extracted from lamb-sourced *H. alvei* FAM01; sliAHL: AHL signals extracted from *S. liquefaciens* FAM02; sliexAHL: chemically reconstituted sliAHL in acetonitrile. Mean values and standard deviation of triplicates are indicated.

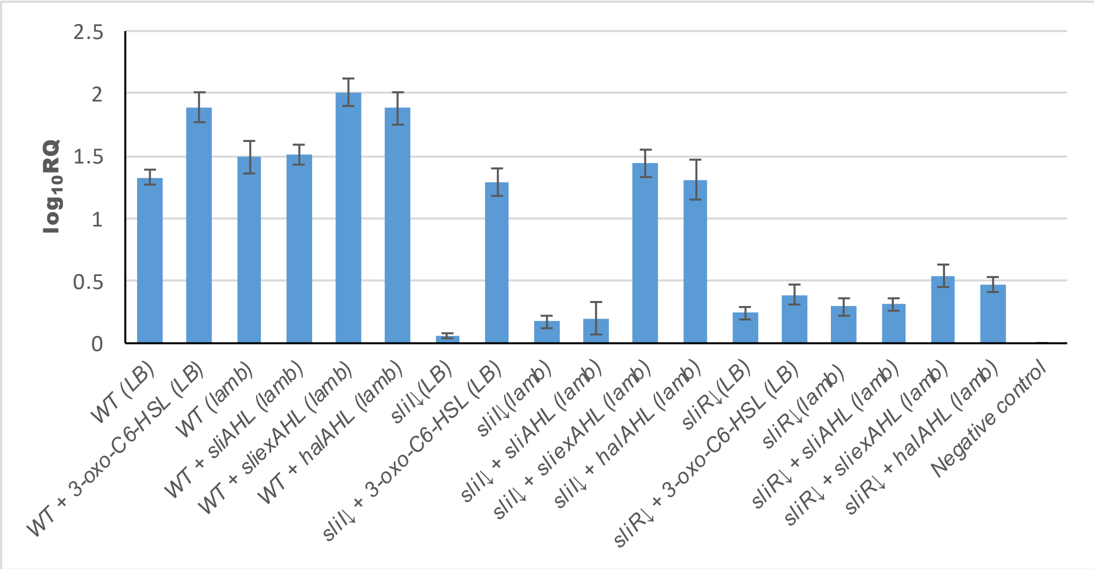


Figure 5.19E. The expression of *sliR* in *S. liquefaciens* NZRM861 strains. The wild type and mutant strains were grown in different media with AHLs. In LB, the AHL standard was added, and in lamb juice, AHLs produced by lamb isolates were added. halAHL: AHL signals extracted from lamb-sourced *H. alvei* FAM01; sliAHL: AHL signals extracted from *S. liquefaciens* FAM02; sliexAHL: chemically reconstituted sliAHL in acetonitrile. Mean values and standard deviation of triplicates are indicated.

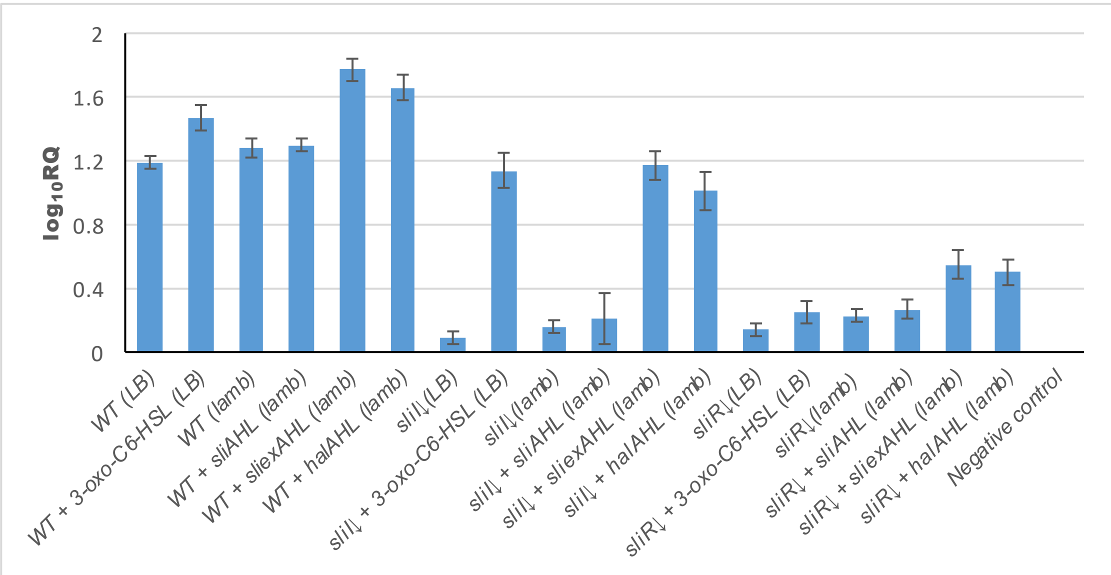


Figure 5.19F. The expression of *lipB* in *S. liquefaciens* NZRM861 strains. The wild type and mutant strains were grown in different media with AHLs. In LB, the AHL standard was added, and in lamb juice, AHLs produced by lamb isolates were added. halAHL: AHL signals extracted from lamb-sourced *H. alvei* FAM01; sliAHL: AHL signals extracted from *S. liquefaciens* FAM02; sliexAHL: chemically reconstituted sliAHL in acetonitrile. Mean values and standard deviation of triplicates are indicated.

5.3.11 Impact of AHL signal on natural microbial spoilage in lamb loin juice

Enzymatic activity (especially lipase and protease activity) of spoilage microorganisms is one of their major cause to meat spoilage, and off-dor is a critical indication for customers to determine the spoilage. In this part of study, a series of tests were undertaken to check the impact of AHL on microbial spoilage of *H. alvei* NZRM860 to lamb. The lipase activity and off-odor production were tested and compared with and without AHL added to the spoilage bacterium. Results are summarised in Table 5.5. When AHL 3-oxo-C6-HSL was added to the *halI*↓ mutant of *H. alvei* NZRM860, lipase activities were found earlier than that without AHL addition, but also in lower cell density at all three temperatures. A mild acidic and sulfurous smell (off-odor) was induced in all the samples, from 1 day to 8 weeks, depending on the storage temperature, with similar cell density (~ 10^9 CFU/mL).

Table 5.5. The effect of AHL signaling molecule on *H. alvei* spoilage activity in lamb.

In cell-free lamb juice, at 20°C			
Sample	**AHL production**	**Lipase activity**	**Off-odor production**
Hafnia alvei	1.5 ± 0.5 h, 10^3 CFU/mL	8 ± 1 h, 10^7 CFU/mL	1 day, >10^9 CFU/mL
Hafnia alvei halI↓	N/D	N/D	1 day, >10^9 CFU/mL
Hafnia alvei halI↓ + 3-oxo-C6-HSL	N/A	3.5 ± 0.5 h, 10^4 CFU/mL	1 day, >10^9 CFU/mL
In cell-free lamb loin meat juice, at 4°C			
Hafnia alvei	6 ± 1 h, 10^2 CFU/mL	2.5 ± 0.5 d, 10^7 CFU/mL	4 days, >10^9 CFU/mL
Hafnia alvei halI↓	N/D	N/D	4.5 ± 0.5 d, >10^9 CFU/mL
Hafnia alvei halI↓ + 3-oxo-C6-HSL	N/A	1.5 ± 0.5 d, 10^5 CFU/mL	3.5 ± 0.5 d, >10^9 CFU/mL
In cell-free lamb loin meat juice, at -1.5°C			
Hafnia alvei	2 weeks, 10^3 CFU/mL	5 weeks, 10^7 CFU/mL	7.5 ± 0.5 w, >10^9 CFU/mL
Hafnia alvei halI↓	N/D	N/D	8 weeks, >10^9 CFU/mL
Hafnia alvei halI↓ + 3-oxo-C6-HSL	N/A	4 ± 1 w, 10^5 CFU/mL	7.5 ± 0.5 w, >10^9 CFU/mL

N/D: not detectable

N/A: not applicable, as AHL standard were added.

5.4 Discussion

In the last chapter, spoilage bacteria were found to produce QS signaling molecules. Those molecules were also found in a spoiled lamb. The link between the QS molecules and spoilage behaviors of spoilage bacteria was investigated in this part of the study. Two *Enterobacteriaceae* species *H. alvei* NZRM860 and *S. liquefaciens* NZRM861 were used as model microorganisms and random mutagenesis was used to construct QS mutants. *C. divergens* FAM03, previously isolated from spoiled lamb by AgResearch was also used as a representative Gram-positive bacterium for this study. After being added to the specific mutants, AHLs were found to impact the enzymatic activities of *H. alvei* and *S. liquefaciens*. These hydrolytic enzymes were also determined as a cause of lamb spoilage.

The EZ-Tn5 transposon was randomly inserted into the genomic DNA of *H. alvei* NZRM860, *S. liquefaciens* NZRM861, and *C. divergens* FAM03 in this study. AHL production was initially detected by biosensor strain *A. tumefaciens* NTL4/pZLR4 and LCMS/MS. When AHL signaling molecules were received by the AHL receptors, it resulted in the secretion of hydrolytic enzymes, such as lipase (Christensen et al., 2003). Therefore, lipase activity using p-nitrophenyl butyrate as the substrate was measured after adding AHL to all the mutants, to detect the AHL receptor mutants of *H. alvei* NZRM860 and *S. liquefaciens* NZRM861. The rationale of this primary screening was that lipases catalyze the hydrolysis of p-nitrophenyl butyrate, and this is detected with absorbance (Shirai and Jackson, 1982b). The screening strategy led to a high number of lipase-producing/secreting clones. Further sequencing confirmed that AHL receptor knockdown mutants of both Enterobacteriaceae species were obtained. As a Gram-positive bacterium, which does not produce AHL signaling molecules, *C. divergens* FAM03 was not studied using these methods.

The AI-2 signaling molecule is a ubiquitous signal that is produced in almost all bacteria (Yu et al., 2013b). Hence a bioluminescence assay was used for selecting AI-2 synthase mutants of all three spoilage bacteria tested. When the AI-2 synthase gene (*luxS*) was mutated, the reduction in AI-2 signals resulted in less bioluminescence. In this part of the study, when AI-2 signals were externally applied to the *luxS* mutant of *C. divergens*, it stimulated bacterial growth in lactose. This supported the findings of Trappetti et al., 2017, showing that exogenous AI-2 was sensed by the FruA system and increased bacterial growth by utilizing galactose as the carbon source. However, this might not be too relevant to my study as plain lamb is usually lactose-free. Blana in 2010 showed that AI-2 signals affected the growth of *S. liquefaciens*. The addition of a low concentration (20% v/v) of AI-2 to cell-free extracts reduced or retained bacterial growth, while no significant growth was observed when a high concentration (50% v/v) of AI-2 was added to cell-free extracts in his study. My study, however, did not show any effect of AI-2 at low concentration (<10% v/v) on bacterial growth.

QS knockdown mutants of *H. alvei* NZRM860, *S. liquefaciens* NZRM861 and *C. divergens* FAM03 were confirmed by sequencing for further analyses. The transposon did not completely knock out the target QS-related genes in all spoilage bacteria but disrupted the expression of those genes. Spoilage bacteria can proliferate and produce extracellular heat-stable hydrolytic enzymes during meat storage. These exoenzymes cause meat spoilage. Agar plate assays allow primary screening of enzymatic activity. The dyes BCG and Rhodamine B did not inhibit the bacterial growth or secretion of enzymes. When hydrolytic enzymes like protease and lipase were produced by the bacteria, they hydrolyzed the protein and fat contained in the agar plate. The dyes bound to unhydrolyzed protein or fat and were used as developing agents to screen for enzymatic activity.

The BCG media plate changes color, giving a blue-greenish color at neutral pH and yellow when the pH is acidic (Thomas, 1998). In my work, the media was made from high pH lamb shoulder extract (pH~6.3), hence when the media was incorporated with BCG dye, it gave a greenish color. Tributyrin agar plates were used for lipase screening in previous studies (Noormohamadi et al., 2013; Ramnath et al., 2017), giving a transparent zone when lipase degrades tributyrin. This was used in the pilot study of my research, but the transparent zone was blurry and did not give a clear indication of enzymatic activity, therefore, lamb juice media with Rhodamine B was used for further investigations.

In this study, both *Enterobacteriaceae* species *H. alvei* NZRM860 and *S. liquefaciens* NZRM861 demonstrated enzymatic activity when they were grown in fresh lamb juice but when the AHL synthase gene was mutated, the mutants did not provide visible enzymatic activity on agar plates. The AHL addition activated the exoenzymatic activity; however, these two *Enterobacteriaceae* cannot respond to the AHL signals produced by themselves. When grown in lamb, they might sense the AHL signals produced by other natural Gram-negative microbes inside the lamb juice, and regulate enzymatic activity.

Agar plating assay provides qualitative detection. A standard curve of ring diameter with substrate-level has been used for a quantitative comparison (Kipps and Whitehead, 1974), but results may be inaccurate due to factors such as gel strength, detection capability of dyes and potential inhibitors inside media. Therefore, in my study cell-free extracts of wild type and mutant strains were investigated by absorbance and compared to the blank LB solution, this method was a fast and sensitive enzyme assay for determining the alteration of the proteolytic and lipolytic activities. The AHL molecule itself did not affect the enzymatic activity but after being added to those spoilage bacteria, the enzymatic activity was enhanced.

Furthermore, an *hflB*↓ mutant was obtained from a *sliI*↓ mutant of *S. liquefaciens* NZRM861. The *hflB* gene is responsible for producing ATP-dependent zinc metalloprotease FtsH. This double knockdown mutant showed no proteolytic activity and the addition of AHL failed to increase proteolytic activity. This suggests that AHL signaling molecules do not restore enzymatic production but may regulate the activity of enzymes which have already been produced in spoilage bacteria (e.g. by *hflB* gene). When the AHL receptor gene was knocked down, enzymatic activity could not be activated in bacteria. In this study, the activity of hydrolytic enzymes by *luxS* mutants was just like wild type strains, indicating that AI-2 signaling molecules do not act as a major regulator of enzymatic activity.

Riedel et al., (2001) found that AHL signals regulate the expression of the *lipB* gene in *Serratia liquefaciens* MG1. This gene is part of the *lipBCD* operon that mediates the secretion of autolytic enzymes such as metalloprotease, protease, and lipase. The impact of AHL signals on the expression of the *lipB* gene was also investigated in this part of the study. The external AHLs are regulated by the AHL receptor genes (*halR* and *sliR*) of *H. alvei* NZRM860 and *S. liquefaciens* NZRM861, and activated the *lipBCD* operon for hydrolytic enzyme secretion by the ATP-binding cassette (ABC). However, lamb is a complex medium that contains natural microbiota, thus when the bacterium was cultivated in lamb juice, AHL receptor and *lipB* genes were expressed at a higher level than when grown in LB broth. AHL signals from other Gram-negative species in lamb might also affect the regulatory mechanisms inside the cultivated spoilage bacteria and influence further physiological activities.

Previous research has found that microbes do not respond to AHLs that are produced by the same species and my study supported this observation. However, in this part of the study, AHL signals from those species were further chemically extracted and reconstituted. When these modified AHLs were added to the bacteria that produced them, the bacteria were found to treat the modified AHLs as foreign signals and responded. It may be because the chemical modification might erase the uniqueness of the bacterium from the signaling molecules and confused the self-recognition.

The impact of AHL on *H. alvei* NZRM860 and its *halI* ↓ mutant growing in lamb juice was also studied in this part. At all three different storage temperatures used in this study, the addition of AHL was found to speed up the enzymatic activity but did not influence off-odor production. Therefore, the off-odor production appears not only to be based on free amino acids or fatty acids hydrolyzed by proteases or lipases, but may also be due to offensive byproducts such as ammonia, amines, and organic sulfides, produced by spoilage bacteria. However, the cell density was > 10^9 CFU/mL when off-odor was detected, and this amount exceeds the threshold level for *H. alvei* to cause meat spoilage (~10^{6-7} CFU/mL). This smell sensory test for the investigation of AHL influence on off-odor production might not be objective, as the bacterial strains had already overgrown and spoiled the meat earlier.

5.5 Conclusion

QS mutants of spoilage bacteria were developed by random transposon mutagenesis. After phenotypic screening, chemical detection, and DNA sequencing, QS knockdown mutants were obtained. The AHL signal was found to upregulate the enzymatic activity of spoilage bacteria *in vitro* and *ex vivo*, and those hydrolytic enzymes further lead to meat spoilage. It to some extent helped connect AHL signaling molecules with lamb spoilage and in the next part of the study, inhibition of AHL signals was to be investigated to see whether it would suppress enzymatic activity and extend shelf-life of lamb.

Chapter Six: Quorum Sensing Inhibitors：Their Role in Lamb Quality Control

6.1 Introduction

Food preservation is achieved through physical or chemical treatment and packaging. Physical methods (e.g. heat) are generally favored over chemical additives as chemical preservation is often associated with health concerns (Antinoro, 2008).

With chemical preservation, there are still issues with microbiological deterioration due to microbial adaption and resistance (Tiwari and Dhama, 2014). To combat this issue, researchers are encouraged to search for natural and alternative additives to enhance food safety (Dhama et al., 2013). One alternative method is the use of QS inhibition, which disturbs the extracellular communication of bacteria. There has been some research on the use of quorum-sensing quenchers or quorum-sensing inhibitors (QSIs) to aid food safety. In the food industry, the use of QSIs is a sensitive issue and any inhibitors used need to be safe for human consumption. Potential biologically active QSI compounds from natural sources have been investigated (Teplitski et al., 2000; Vattem et al., 2007). For example, phytochemicals from traditional medicinal plants have been discovered and investigated as potential new QSI agents to control microbial intervenes (Al-Hussaini and Mahasneh, 2009).

AHL signaling molecules were shown in my study to contribute to hydrolytic enzymatic activities. The involvement of AHL signals in spoilage behaviors from spoilage bacteria, such as *Enterobacteriaceae*, suggests that compounds that can specifically disrupt or quench the impact of AHL signals may be able to prolong the shelf-life of lamb. These compounds may act in a variety of ways:

1. Cease bacterial growth and/or inhibit metabolic activities producing acyl or HSL moieties (precursors of AHL molecules);

2. Inactivate the signaling enzyme AHL synthase;

3. Degrade AHL signaling molecules after they are synthesized;

4. Mimic signaling molecules and block the receptors.

They should be chemically stable and highly effective without any toxic side effects (Rasmussen and Givskov, 2006b).

The objective of this part of the study was to assess the effect of AHL inhibition on spoilage. Five phytochemical compounds or extracts found in fruits, herbs, and spices (cinnamaldehyde, garlic extract, vanillin, curcumin, and ascorbic acid – vitamin C), as well as a type of well-studied AHL analog compound 4-Hydroxy-2(5H)-furanone were selected as potential agents for QS inhibition. A previous study reported that there were at least three different QS inhibitors contained in garlic extract, with an acyclic disulphur compound which exerts a strong antagonistic effect on reception protein in QS (Rasmussen et al., 2005). Cinnamaldehyde and substituted cinnamaldehydes are also inhibitors of AHL and AI-2 activity, by decreasing the DNA-binding activity of LuxR in *Vibrio* spp. (Brackman et al., 2008). Furanone, vitamin C, curcumin, and vanillin have been reported to have significant anti-QS activity (El-Mowafy et al., 2014; Packiavathy et al., 2014; Ponnusamy et al., 2009). The effect of these compounds on the microbial growth, AHL production and regulation were studied in two *Enterobacteriaceae* species *Hafnia alvei* and *Serratia liquefaciens* in this study. Garlic and cinnamaldehyde were also tested *ex vivo* on lamb, to study their effect on preventing spoilage behaviors produced by *H. alvei* and *S. liquefaciens*. The impact of cinnamaldehyde on the natural microbiota in lamb and its use for chilled vacuum-packed lamb preservation was also investigated in this study.

6.2 Materials and methods

A selection of potential AHL inhibiting agents, listed in Section 3.4, dissolved in acetonitrile at a concentration of 1 mg/mL, and concentrated to 10 ppm in the Luria broth (LB) or fresh lamb juice, used in this part of the study.

6.2.1 Effect of inhibitors on the bacterial growth

The effect of inhibitors on bacterial growth of *H. alvei* NZRM860 and *S. liquefaciens* NZRM861 in LB, and *H. alvei* FAM01 and *S. liquefaciens* FAM02 on fresh lamb juice was analyzed as per Section 3.20. The growth of those bacteria with 6% of acetonitrile in respective media at 30 °C for 8 h was also investigated by absorbance at 595nm. The bacteria used in this study are listed in Table 6.1.

Table 6.1. Bacteria used in this part of the study.

Bacterial strain	**Growth medium**
H. alvei NZRM860	Luria broth (LB)
H. alvei FAM01	Lamb juice
S. liquefaciens NZRM861	Luria broth (LB)
S. liquefaciens FAM02	Lamb juice

6.2.2 Measurement of β-galactosidase activity

AHL production was quantitatively compared by β-galactosidase expression in cell-free solutions, measured by a simple absorbance assay. *H. alvei* NZRM860 was used as a representative strain. In a 96 well microplate, 50 µL of cell-free supernatant of overnight grown *H. alvei* NZRM860 and 50 µL of an individual AHL inhibiting compound were grown in 150 µL of LB (supplemented with 50 µg/mL gentamicin) with 50 µL of biosensor *A. tumefaciens* NTL4/pZLR4. The assay was carried out as per as per Section 3.10. The induction of β-galactosidase activity was compared with the control without the addition of AHL inhibiting agents.

6.2.3 Detection of AHL abundance using liquid chromatography-mass spectrometry (LCMS/MS) after adding inhibiting agents

The amount of AHL reduction by each AHL inhibiting compound was determined by LCMS/MS. N-pentanoyl-L-homoserine lactone (C5-HSL) was used as the internal standard and the relative abundances (RAs) of four AHL standards N-butyryl-homoserine lactone (C4-HSL), N-hexanoyl-homoserine lactone (C6-HSL), N-octanoyl-homoserine lactone (C8-HSL) and N-decanoyl-L-homoserine lactone (C10-HSL) were compared directly before and after the addition of AHL inhibiting agents. All these AHL standards were dissolved in acetonitrile at 100 µg/mL. As described in Section 3.13, 10 µL of each sample was injected into the LCMS/MS.

6.2.4 Gene expression studies by quantitative reverse transcription PCR (RT-qPCR)

The mechanism of each inhibitor's action was identified by RT-qPCR as per Section 3.19. The selected genes were *sliI, sliR, lipB* genes of *S. liquefaciens* NZRM861 and *S. liquefaciens* FAM02, as well as *halI, halR, lipB* genes of *H. alvei* NZRM860 and *H. alvei* FAM01 in this part of the study (Table 6.2). These bacteria were grown overnight at 30 °C with agitation (150 rev min-1) in 10 mL LB and lamb juice respectively. Reference strains *H. alvei* NZRM860 and *S. liquefaciens* NZRM861 were grown in LB with 10 ppm of the inhibitor cinnamaldehyde and AHLs (3-oxo-C6-HSL standard). Strains isolated from lamb, i.e. *H. alvei* FAM01 and *S. liquefaciens* FAM02 were cultivated in lamb juice with 10 ppm cinnamaldehyde and AHL signals extracted from each other.

Table 6.2. Primers used in the gene expression study.

Target DNA sequence	Accession number	Primer sequence (from 5' to 3')	Primer coordinates
sliI gene in *S. liquefaciens*	AY168877.1 REGION: 3754-4356	Forward: CGTGTTGATTGATGCGTGCT	3771-3790
		Reverse: AAACATGATCAGCGATGGCG	4113-4132
sliR gene in *S. liquefaciens*	AY168877.1 REGION: 2988-3734	Forward: TCCAATAGAACGGAGTGGTTTGA	3129-3151
		Reverse: TGACGCCAAGCTTTTTGACG	3638-3657
halI gene in *H. alvei*	NZ_CP009706 REGION: 2669091-2669738	Forward: AATGCAGGCGTAACGGCTAT	2669098-2669117
		Reverse: CGGTTGGGATGGGATGTTGT	2669629-2669648
halR gene in *H. alvei*	NZ_CP009706 REGION: 2668370-2669110	Forward: CTTCAAACGTACCTCGCCCT	2668588-2668607
		Reverse: TCAAGTTCAACGCCCAACCT	2669066-2669085
lipB gene in *S. liquefaciens*	NC_021741.1 REGION: 1146332-1147000	Forward: TACGTCTTCTATGCCGACGC	1146419-1146438
		Reverse: CCCAAGCCATGCACAACTTC	1146914-1146933
lipB gene in *H. alvei*	NZ_CP009706 REGION: 1404798-1405469	Forward: CCACAGGGGTTAATTCGGCT	1404949-1404968
		Reverse: TATGCACACCTTCACCGACC	1405373-1405392

6.2.5 Enzymatic activity analyzes

The effect of inhibitors on the activity of hydrolytic enzymes from *Enterobacteriaceae* was investigated. *H. alvei* FAM01, as well as *S. liquefaciens* FAM02, were grown overnight at 30 °C with agitation (150 rev min^{-1}) in 10 mL LB and lamb juice with and without cinnamaldehyde (10 ppm) and AHLs respectively. Proteolytic and lipolytic activities of these *Enterobacteriaceae* were analyzed quantitatively by absorbance, as described in Section 3.18.

6.2.6 Impact of cinnamaldehyde in anaerobically stored lamb juice and vacuum packaged lamb.

Cinnamaldehyde 10 ppm was added to anaerobically stored lamb juice and vacuum packaged lamb as described in Section 3.21, and stored at an abused temperature 20 °C, refrigerated temperature (4 °C) and chilled temperature (-1.5 °C). The experiment also included a control without cinnamaldehyde stored at each temperature. The timing of AHL production, enzymatic activity and off-odour production were compared.

6.3 Results

6.3.1 Effect of inhibitors on the bacterial growth

The effect of selected AHL inhibiting compounds on bacterial growth is shown in Figure 6.1. The effect of bacterial growth was shown by the $\log_{10}$ of relative CFU, which was considered 0 for controls, and the growth reduction of individual bacteria is shown as negative values. All bacteria strains showed a high tolerance to 6%

acetonitrile. There was significant growth inhibition of all bacteria ($p < 0.05$) when they were grown with 10 ppm of most inhibitors overnight at 30 °C with agitation. Ascorbic acid had the strongest ability to inhibit bacterial growth, by reducing 34% of *H. alvei* NZRM860, 40% of *H. alvei* FAM01, 37% of *S. liquefaciens* NZRM861, and 40% of *S. liquefaciens* FAM02 growth. Garlic extracts also showed good inhibition, reducing 27% of *H. alvei* NZRM860, 34% of *H. alvei* FAM01, 31% of *S. liquefaciens* NZRM861, and 34% of *S. liquefaciens* FAM02 growth. The next most powerful inhibitor was cinnamaldehyde (reducing 22% of *H. alvei* NZRM860, 28% of *H. alvei* FAM01, 15% of *S. liquefaciens* NZRM861, and 31% of *S. liquefaciens* FAM02 growth). Vanillin reduced 9% of *H. alvei* NZRM860, 9% of *H. alvei* FAM01, 6% of *S. liquefaciens* NZRM861, and 12% of *S. liquefaciens* FAM02 growth and curcumin reduced 13% of *H. alvei* NZRM860, 19% of *H. alvei* FAM01, 15% of *S. liquefaciens* NZRM861, and 15% of *S. liquefaciens* FAM02 growth. The growth of biosensor *A. tumefaciens* NTL4/pZLR4 was also reduced by 7% - 31% by those inhibitors. Furanone did not seem to affect bacterial growth as much as the rest five inhibitors, reducing the growth by 2% for *A. tumefaciens* NTL4/pZLR4, 2% for *H. alvei* NZRM860, 2% for *H. alvei* FAM01, 1% for *S. liquefaciens* NZRM861, and 3% for *S. liquefaciens* FAM02.

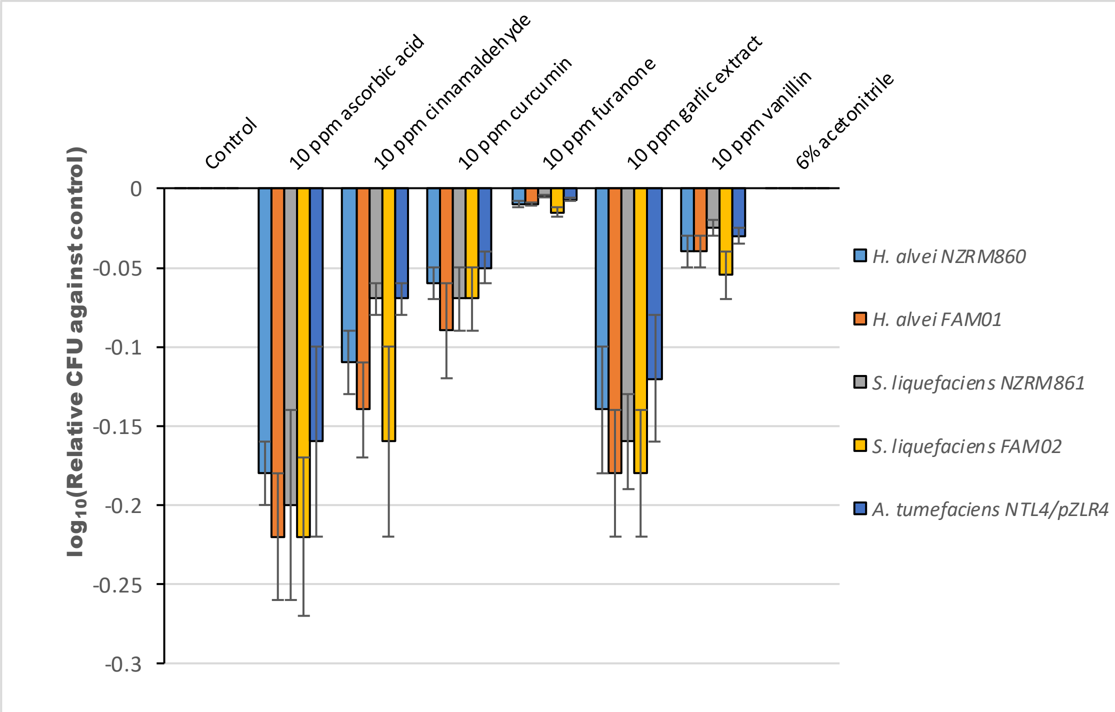


Figure 6.1. The effect of growth when the bacteria were grown with a selection of inhibitors. The tolerance of each bacterium to 6% acetonitrile was also shown. Mean and standard deviation of triplicates are indicated.

6.3.2 β-galactosidase activity measurement by spectrophotometry

The effect of β-galactosidase activity when the bacteria were grown with AHL inhibitors is shown in Figure 6.2. AHL sensed by biosensor induces β-galactosidase activity, which hydrolyzes X-gal to influence absorbance at 635 nm. Hence β-galactosidase activity was used in my study for primary screening of AHL production. By using $\log_{10}$ of relative absorbance, there was a clear impact on β-galactosidase activity of *A. tumefaciens* NTL4/pZLR4 when most of the AHL inhibiting compounds were added and grown with bacteria. Ascorbic acid and garlic extract reduced more than 40% β-galactosidase activity of *A. tumefaciens* NTL4/pZLR4; cinnamaldehyde reduced 35% of β-galactosidase activity; curcumin and vanillin reduced over 25% of β-galactosidase activity. There was no significant effect on β-galactosidase activity when furanone was added ($p > 0.05$).

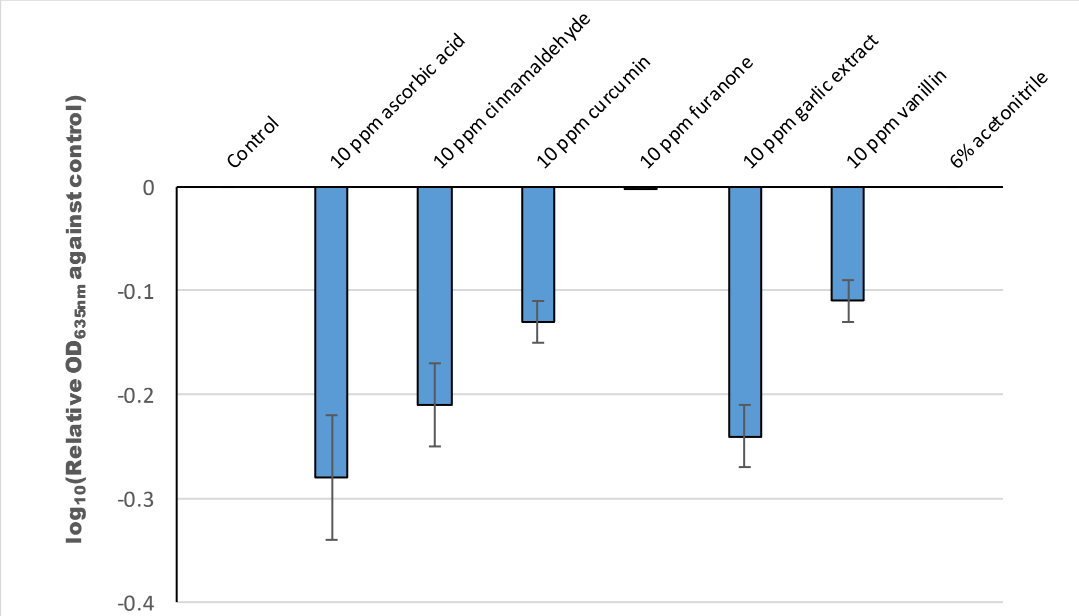


Figure 6.2. The effect of the β-galactosidase activity of *A. tumefaciens* NTL4/pZLR4 when different candidate inhibiting compounds were added. The reduction of β-galactosidase activity indicates the inhibition of AHL production. Mean and standard deviation of triplicates are indicated.

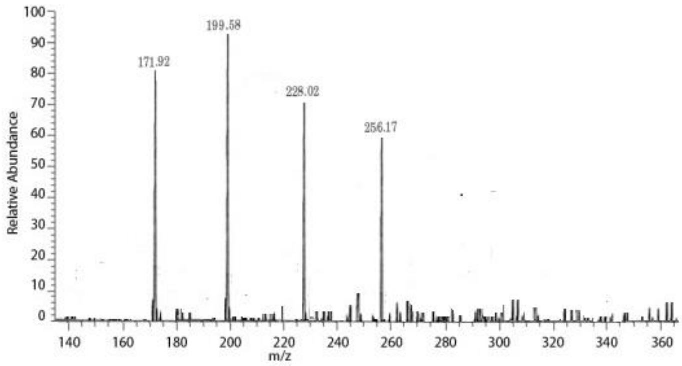
6.3.3 Effect of AHL inhibiting compounds on AHL signal degradation

Four AHL references C4-HSL, C6-HSL, C8-HSL, and C10-HSL in pure solution (acetonitrile) before and after the addition of AHL inhibiting compounds were detected by LCMS/MS. The MS/MS analyses of all the AHL signaling molecules with the addition of two inhibitors, garlic, and cinnamaldehyde, are shown in Figure 6.3A. The reduction in AHLs was demonstrated in $\log_{10}$ of the relative abundances (RAs) compared with the control (Figure 6.3B). These two inhibitors both significantly reduced AHL abundance of each signaling molecule ($p < 0.01$), suggesting these two inhibitors directly affect the AHL signaling molecules without any impact of growth.

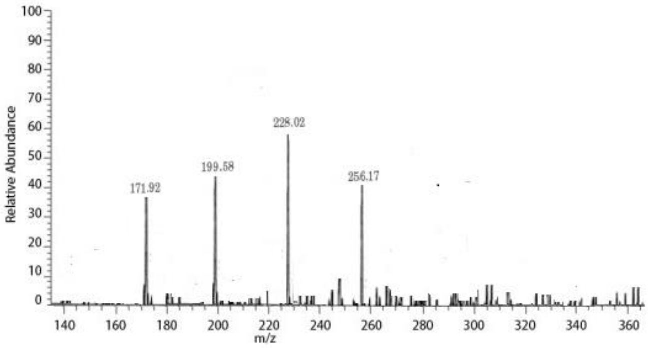
When treated with cinnamaldehyde, the RA of C4-HSL and C6-HSL decreased by 31% and 24%, respectively but the RAs of the longer chained AHL signaling molecules C8-HSL and C10-HSL were influenced to a lesser extent, with 9% and 5% reduction respectively. Garlic was found to degrade about 13% C8-HSL and 9% C10-HSL, lower than the reduction of C4-HSL (37%) and C6-HSL (32%). Hence, treatment with these two inhibitors showed a higher ability to reduce short-chained AHL signaling molecules, than long-chained molecules.

In this part of the study, garlic extracts and cinnamaldehyde were the only two inhibitors found to reduce AHL signaling molecules in pure solution. The AHL signals in pure solution seemed to be disrupted, potentially by the hydrolytic enzyme in those two inhibitors.

1)



2)



3)

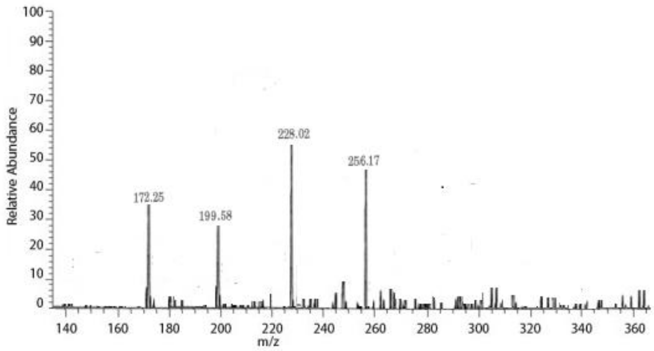


Figure 6.3A. The MS/MS profiles of all the reference AHL signaling molecules with the addition of two inhibitors, garlic, and cinnamaldehyde. 1) Blank (before QSI addition); 2) 10 ppm cinnamaldehyde added; 3) 10 ppm garlic extract added.

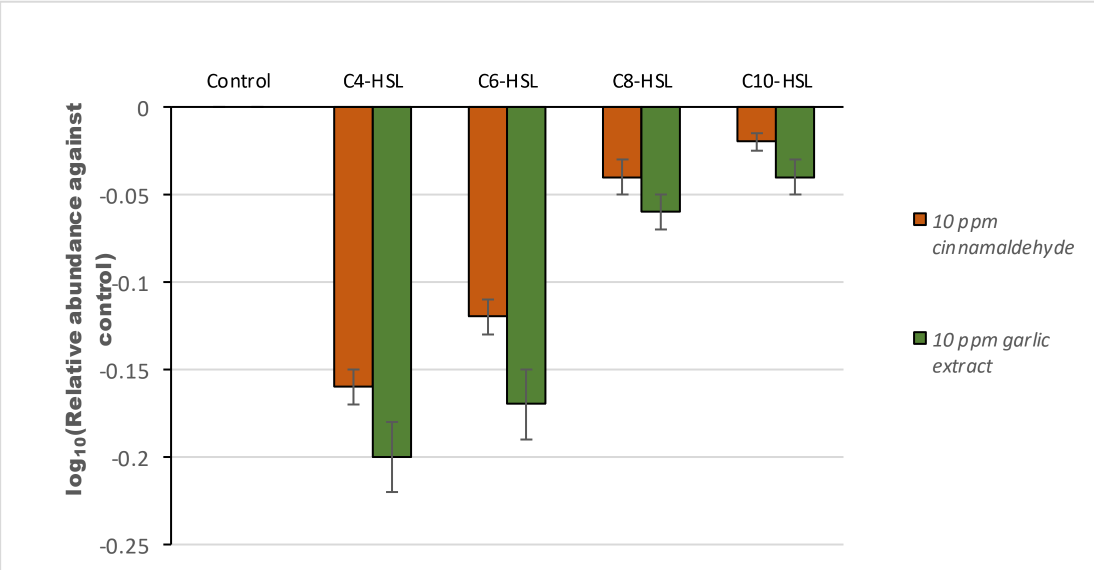


Figure 6.3B. The reduction in the AHL abundance by cinnamaldehyde and garlic extracts, according to the RAs identified by LCMS/MS. Mean and standard deviation of triplicates are indicated.

6.3.4 Effect of cinnamaldehyde on AHL production, reception, and regulation

The expression of *halI*, *halR* and *lipB* genes of *H. alvei* NZRM860 and *H. alvei* FAM01, grown in different media following cinnamaldehyde treatment were compared. The expression of *halI* did not show a significant difference ($p > 0.05$) when external AHL was added (Figure 6.4A). However, when *H. alvei* NZRM860 was cultivated in LB, *halR* expression was less than it when *H. alvei* FAM01 was grown in lamb juice (Figure 6.4B). Similarly, *lipB* expression was less when *H. alvei* NZRM860 grown in LB than it when *H. alvei* FAM01 grown in lamb juice (Figure 6.4C). When external AHL was added, the expression of *halR* and *lipB* from both bacteria was upregulated significantly ($p < 0.05$). Cinnamaldehyde reduced the expression of all these genes in both *H. alvei* strains ($p < 0.01$).

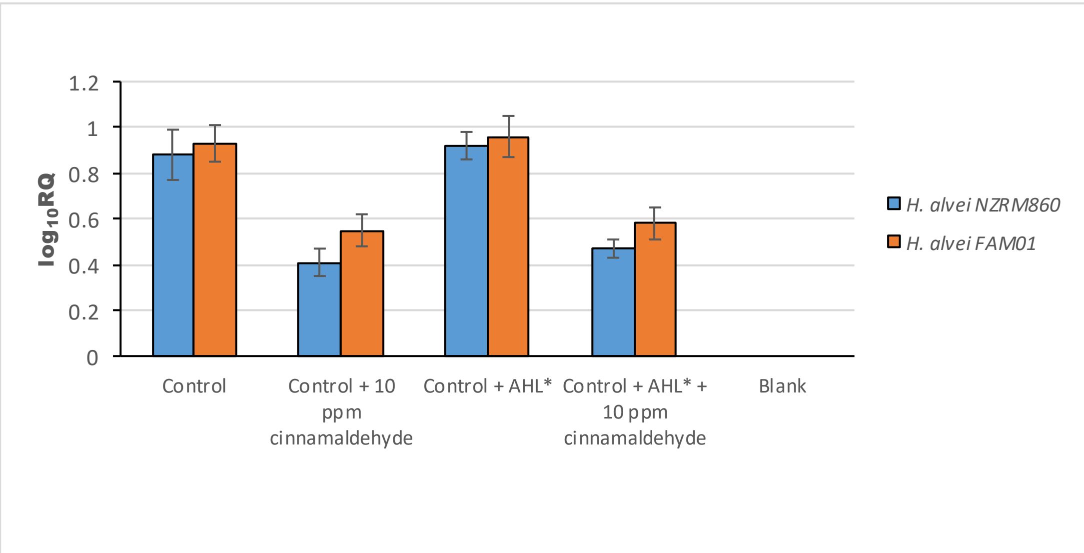


Figure 6.4A. Gene expression of *halI* of *H. alvei* strains. *H. alvei* NZRM860 was grown in LB and *H. alvei* FAM01 was grown in lamb juice. Mean and standard deviation of triplicates are indicated. AHL*: 3-oxo-C6-HSL standard was added to *H. alvei* NZRM860 and AHL signals extracted from lamb isolate *S. liquefaciens* FAM02 were added to *H. alvei* FAM01.

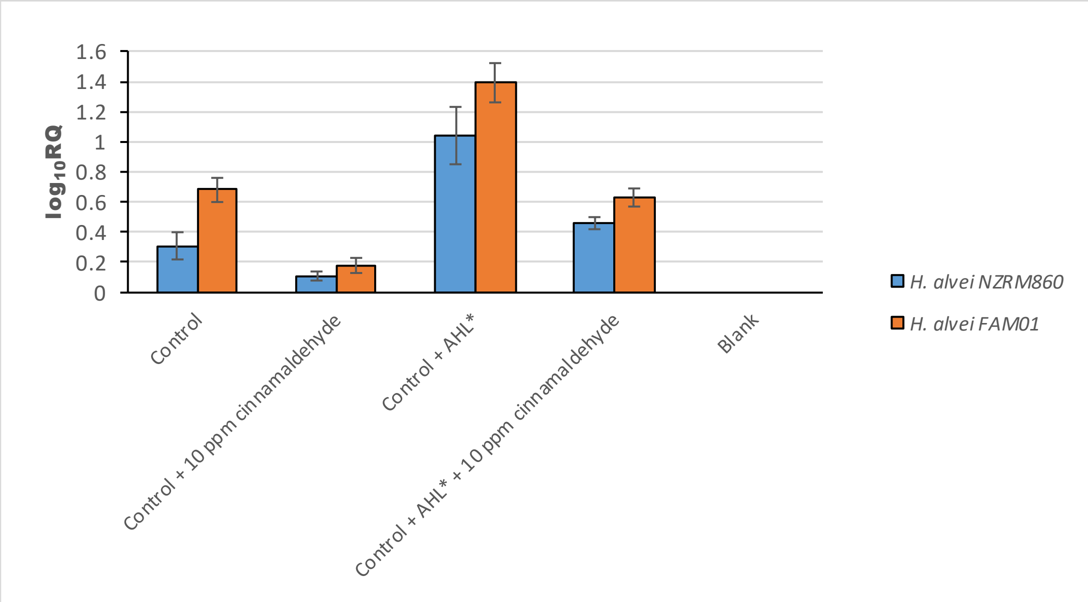


Figure 6.4B. Gene expression of *halR* of *H. alvei* strains. *H. alvei* NZRM860 was grown in LB and *H. alvei* FAM01 was grown in lamb juice. Mean and standard deviation of triplicates are indicated. AHL*: 3-oxo-C6-HSL standard was added to *H. alvei* NZRM860 and AHL signals extracted from *S. liquefaciens* FAM02 were added to *H. alvei* FAM01.

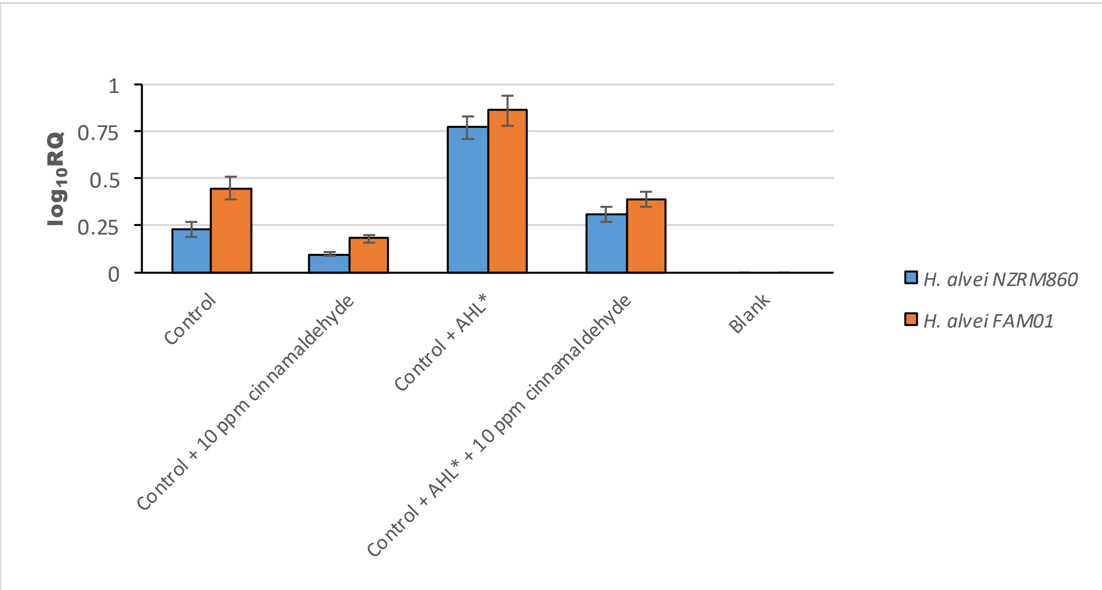


Figure 6.4C. Gene expression of *lipB* of *H. alvei* strains. *H. alvei* NZRM860 was grown in LB and *H. alvei* FAM01 was grown in lamb juice. Mean and standard deviation of triplicates are indicated. AHL*: 3-oxo-C6-HSL standard was added to *H. alvei* NZRM860 and AHL signals extracted from *S. liquefaciens* FAM02 were added to *H. alvei* FAM01.

In the same way, the expression of *sliI*, *sliR* and *lipB* genes in two *S. liquefaciens* strains were compared. Results in Figure 6.5A shows that the expression of *sliI* did not show a significant difference ($p > 0.05$) when AHL was added to both bacteria. The expression of *sliR* and *lipB* in *S. liquefaciens* NZRM861 were lower than the other *S. liquefaciens* strain but not significantly ($p > 0.05$), and external AHL increased the gene expression for both strains ($p < 0.05$) (Figure 6.5B and 6.5C). When cinnamaldehyde was added, all the genes were suppressed in both *S. liquefaciens* strains ($p < 0.01$).

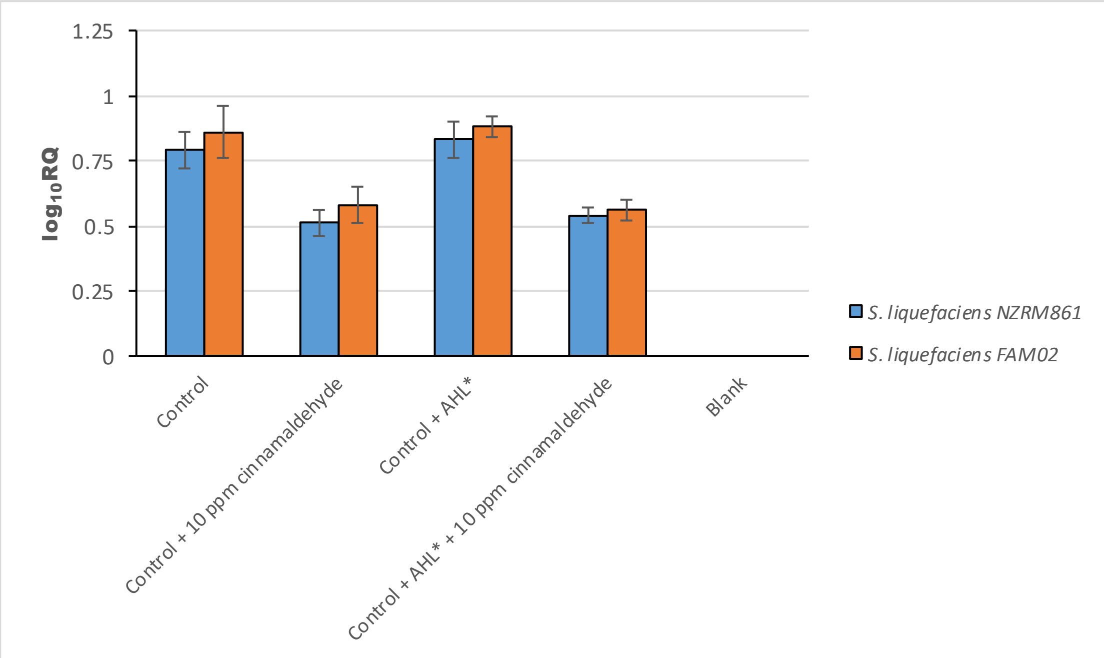


Figure 6.5A. Gene expression of *sliI* of *S. liquefaciens* strains. *S. liquefaciens* NZRM861 was grown in LB and lamb isolate *S. liquefaciens* FAM02 was grown in lamb juice. AHL*: 3-oxo-C6-HSL standard was added to *S. liquefaciens* NZRM861 and AHL signals extracted from *H. alvei* FAM01 were added to *S. liquefaciens* FAM02. Mean and standard deviation of triplicates are indicated.

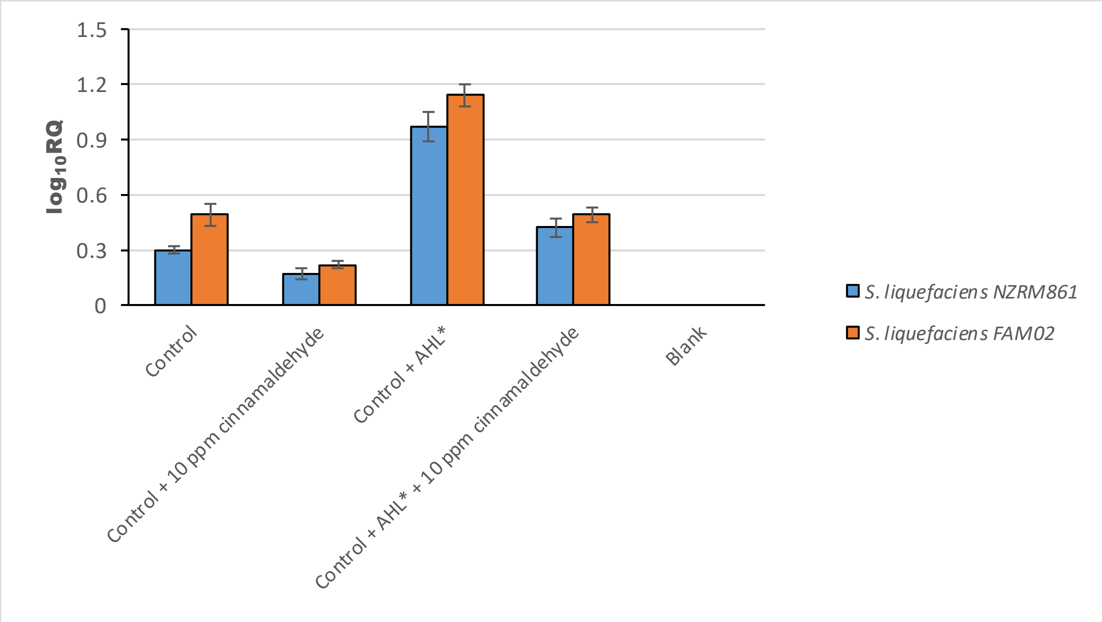


Figure 6.5B. Gene expression of *sliR* of *S. liquefaciens* strains. *S. liquefaciens* NZRM861 was grown in LB and *S. liquefaciens* FAM02 was grown in lamb juice. AHL*: 3-oxo-C6-HSL standard was added to *S. liquefaciens* NZRM861 and AHL signals extracted from *H. alvei* FAM01 were added to *S. liquefaciens* FAM02. Mean and standard deviation of triplicates are indicated.

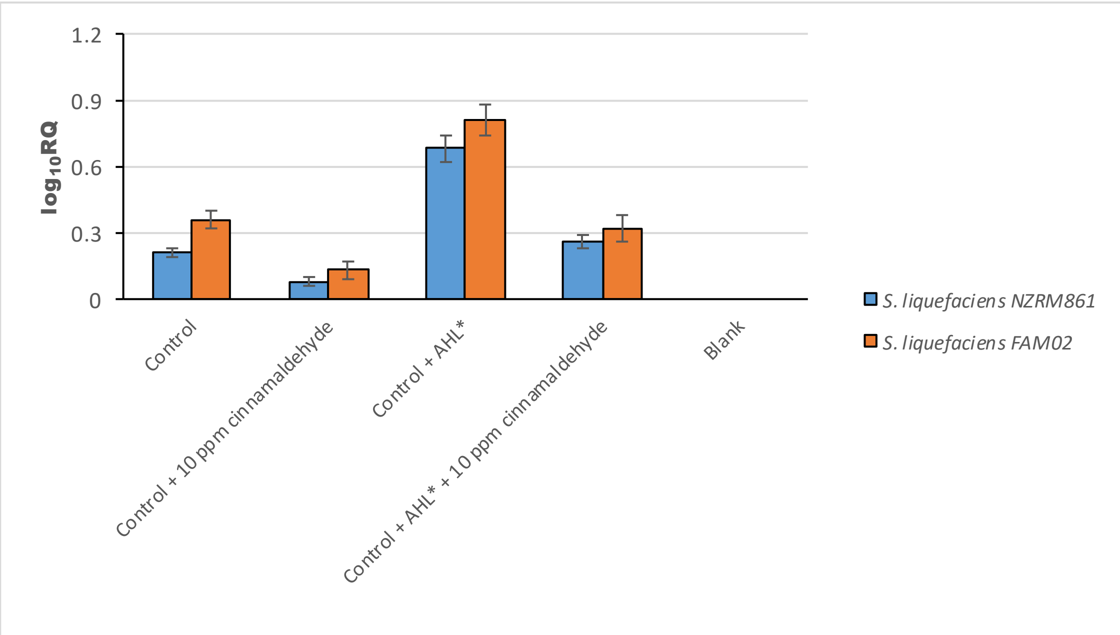


Figure 6.5C. Gene expression of *lipB* of *S. liquefaciens* strains. *S. liquefaciens* NZRM861 was grown in LB and *S. liquefaciens* FAM02 was grown in lamb juice. AHL*: 3-oxo-C6-HSL standard was added to *S. liquefaciens* NZRM861 and AHL signals extracted from *H. alvei* FAM01 were added to *S. liquefaciens* FAM02. Mean and standard deviation of triplicates are indicated.

6.3.5 Effect of cinnamaldehyde on enzymatic activities

The enzyme activities were investigated by the relative absorbance compared against a blank of LB broth, when the two lamb isolates were grown in LB and lamb juice, respectively. Protease activity of both bacteria grown in LB was lower than that in lamb juice (Figure 6.6A and 6.6B), indicating that after being incubated overnight at 30 °C with shaking, the growth of initial microbiota in lamb juice was stimulated and also contributed to the enzymatic activity. When the spoilage bacteria were grown with AHL signals produced by each other, the protease activity increased significantly in both media ($p < 0.01$), about 45% in *H. alvei* FAM01 and 48% in *S. liquefaciens* FAM02. There was no effect of acetonitrile on the protease activity. The AHL signals produced did not affect the enzymatic activity in LB, however, when it was added to lamb juice, the protease activity increased. Cinnamaldehyde decreased the protease activity in both Enterobacteriaceae, regardless of whether they were grown in LB or lamb juice reaching approximately 37% in *H. alvei* FAM01 and 39% in lamb *S. liquefaciens* FAM02, both isolated from lamb.

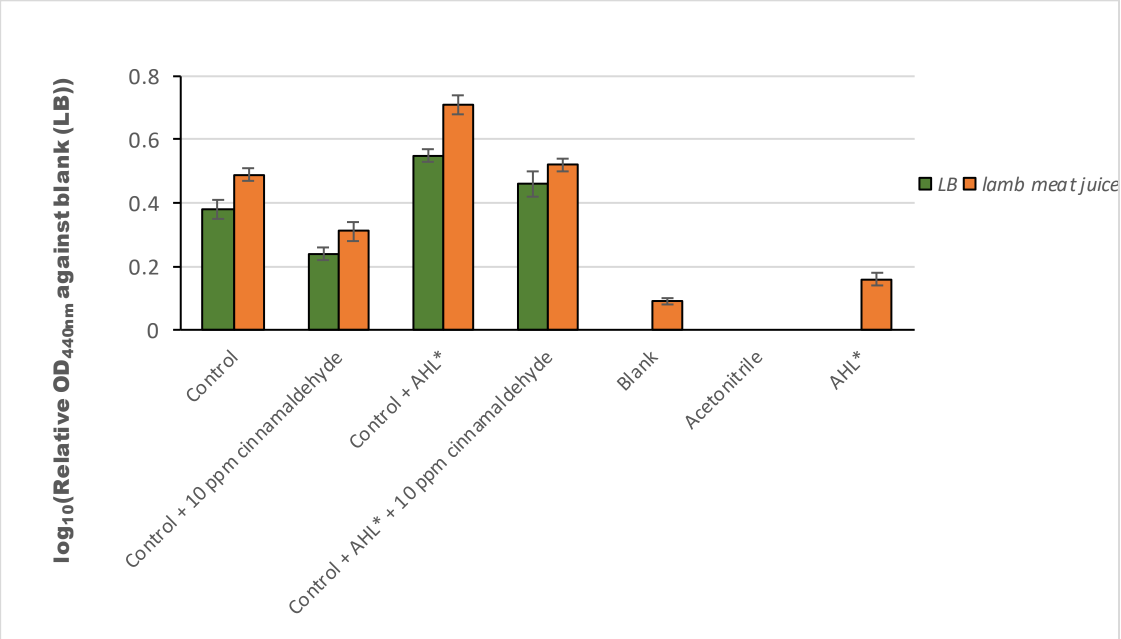


Figure 6.6A. The comparison of protease activity when *H. alvei* FAM01 was grown in LB and lamb juice. The effect of AHL or acetonitrile was also shown when they were added into the respective medium. AHL*: AHL signals extracted from lamb isolate *S. liquefaciens*. Mean and standard deviation of triplicates are indicated.

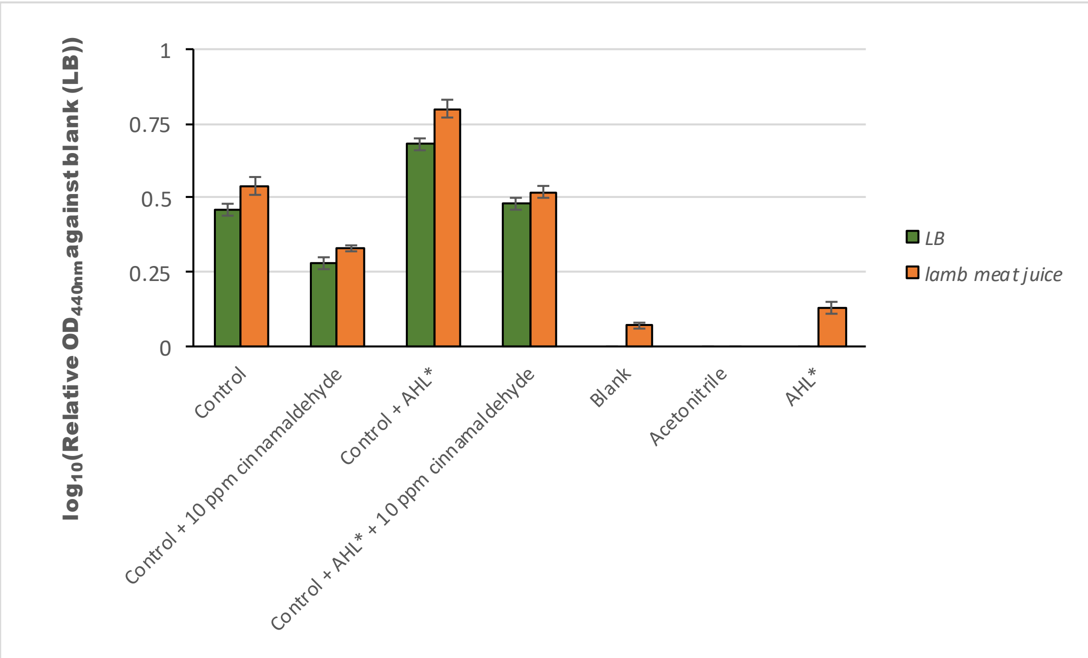


Figure 6.6B. The comparison of protease activity when *S. liquefaciens* FAM02 was grown in LB and lamb juice. The effect of AHL or acetonitrile was also shown when they were added into the respective medium. AHL*: AHL signals extracted from *H. alvei* FAM01. Mean and standard deviation of triplicates are indicated.

The effect of lipase activity is shown in Figures 6.7A and 6.7B. By comparing the absorbance, lipase activity was significantly higher when the spoilage bacteria were grown with AHL signals produced by each other ($p < 0.01$) in both media, with an approximate 43% increase in both bacteria. Lipase activity was also found higher when those Enterobacteriaceae were grown in lamb juice than in LB broth, indicating that lipase activity was possibly influenced by the interaction between those lamb isolates and the initial microbiota inside lamb juice. When the AHL signaling molecule was added to lamb juice, lipase activity increased, confirming that the initial microbiota was affected by the external AHL. Cinnamaldehyde reduced 44% of the lipase activity in *H. alvei* FAM01 and 46% of lipase activity in *S. liquefaciens* FAM02. Acetonitrile did not influence the lipase activity.

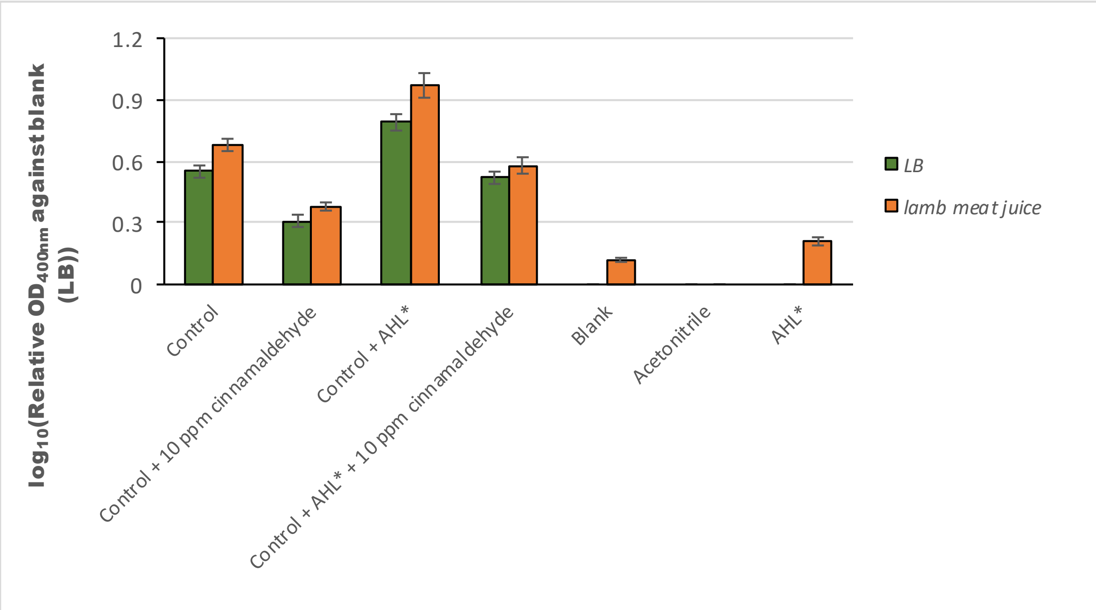


Figure 6.7A. The comparison lipase activity when *H. alvei* FAM01 was grown in LB and lamb juice. The effect of AHL or acetonitrile was also shown when they were added. AHL*: AHL signals extracted from *S. liquefaciens* FAM02. Mean and standard deviation of triplicates are indicated.

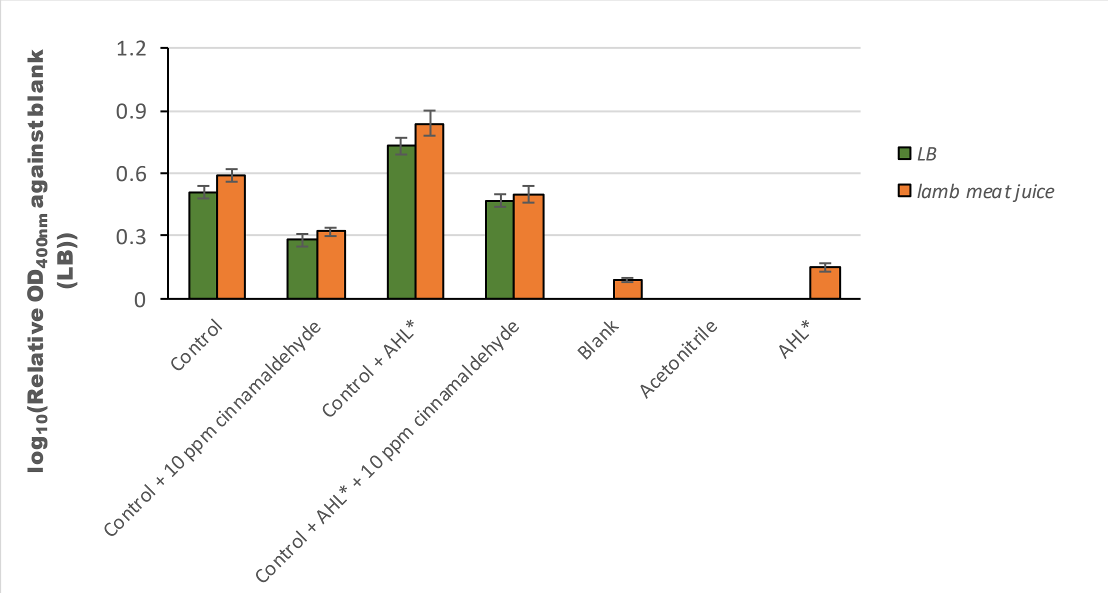


Figure 6.7B. The comparison of lipase activity when *S. liquefaciens* FAM02 was grown in LB and lamb juice. The effect of AHL or acetonitrile was also shown when they were added. AHL*: AHL signals extracted from *H. alvei* FAM01. Mean and standard deviation of triplicates are indicated.

6.3.6 Cinnamaldehyde preservation of lamb

Cinnamaldehyde was added to lamb media under anaerobic conditions and stored at different temperatures to determine the efficiency of cinnamaldehyde on lamb preservation. The overall findings were shown in Table 6.3. A concentration of 10 ppm cinnamaldehyde delayed AHL production and lipase activity in lamb juice under anaerobic conditions for 2 days at 20 °C, 3 - 4 days at 4 °C, and 4 - 5 days at -1.5 °C. When 10 ppm cinnamaldehyde was added to lamb coupons, the production of AHL was delayed along with lipase activity for 1 day at 20 °C, 1 – 2 days at 4 °C, and 3 days at -1.5 °C. The off-odor production was delayed for a maximum of 8 days in anaerobic lamb juice and a maximum of 5 days in vacuum-packed lamb coupons in the presence of cinnamaldehyde.

Table 6.3. The influence of 10 ppm cinnamaldehyde in anaerobic lamb matrices.

	20 °C			**4 °C**			**-1.5 °C**		
	AHL production	lipase activity	Off-odour	AHL production	lipase activity	Off-odour	AHL production	lipase activity	Off-odour
Meat juice (MJ)	10.5 ± 0.5 hours	22 ± 1 hours	2.5 ± 0.5 days	6.5 ± 0.5 days	7.5 ± 0.5 days	9.5 ± 0.5 days	52 ± 2 days	56 ± 1 days	61 ± 2 days
MJ + cinnamaldehyde	2 days	3 ± 1 days	4.5 ± 0.5 days	9.5 ± 0.5 days	11 days	14.5 ± 0.5 days	56 ± 1 days	61 ± 2 days	68.5 ± 2.5 days
Meat coupon (MC)	12.5 ± 0.5 hours	20.5 ± 0.5 hours	3 days	7.5 ± 0.5 days	9 days	10.5 ± 0.5 days	53 ± 1 days	55 ± 2 days	59 ± 1 days
MC + cinnamaldehyde	22.5 ± 0.5 hours	2 days	4 days	9.5 ± 0.5 days	10.5 ± 0.5 days	13 ± 1 days	55.5 ± 0.5 days	58 ± 1 days	63 ± 1 days

6.4 Discussion

Every year there is a food wastage, economic loss and public health concerns due to microbial spoilage. Microbial activity produces a variety of metabolites such as saccharolytic, proteolytic, pectinolytic, and lipolytic enzymes that are associated with food spoilage (Braun et al., 1999b; Loureiro, 2000b; Ragaert et al., 2007) resulting in off-taste and off-odor in the food.

The production of hydrolytic enzymes associated with the deterioration of foods is regulated by QS (Bai and Rai, 2011). In my study, AHL molecules produced by a group of spoilage bacteria were detected in spoiled chilled vacuum-packed lamb. These spoilage bacteria can express specific genes, resulting in enzymatic activity and cause lamb spoilage through AHL regulation. By disrupting the AHL system, gene expression leading to spoilage can be suppressed. A recently developing strategy is called quorum sensing inhibition or the quorum-quenching approach (Dong et al., 2007). A wide range of synthetic and natural QSIs have been developed to target the production of the signaling molecules or block these QS signaling systems that are responsible for food spoilage (Bai and Rai, 2011). For use in food, it is important to ensure there are no toxic effects produced through QSIs. QSIs that originate from natural sources such as dietary phytochemical extracts from fruits, herbs, and spice extracts are preferred (Bai and Rai, 2011; Vattem et al., 2007).

In this part of my study, a few QSIs were considered to investigate their impact on lamb spoilage. The antimicrobial action of these natural extracts has been widely used, but their QS inhibition has not been evaluated as an alternative for lamb preservation. *H. alvei* and *S. liquefaciens* are two facultative anaerobic opportunistic bacteria that are also known to play a role in lamb spoilage, thus they were used as representative bacteria in this study. AHL synthesis was detected using the biosensor *A. tumefaciens* NTL4/pZLR4. Like the previous part of this study, the β-galactosidase expression system was used as a specific indicator of

AHL production. Biosensor screening is a useful, rapid method used to evaluate the QS inhibitory effect of phytochemical compounds (Adonizio et al., 2006; Bodini et al., 2009; Choo et al., 2006). In the present study, AHL production was shown to be reduced by most of the inhibitors tested. However, the antimicrobial activity of those inhibitors was also found in the spoilage bacteria as well as the biosensor strain. Therefore, the results are limited by the effect on bacterial growth. Any effects on AHL production cannot be attributed without controlling for growth effects. Furthermore, due to the reduction of biosensor growth, its effect on AHL detection could also be invalid. The mechanisms of these inhibitors for AHL inhibition needed to be further clarified.

The determination of AHL signals by LCMS/MS is a more sensitive method than the biosensor system to detect and quantify specific AHL signaling molecules without being influenced by bacterial density. In this part of the study, AHL signaling molecules were found to be degraded significantly by garlic and cinnamaldehyde (p-value < 0.05). The degradation of AHL signaling molecules could be through lactone-lysis to cleave the lactone ring and produce acyl-homoserine, or through acy-lysis to produce free fatty acids and shorter chained AHLs (Czajkowski and Jafra, 2009).

The effect of these inhibitors on AHL synthesis and reception was also identified in the present study. A Lux I/R type system is used in AHL communication between *H. alvei* and *S. liquefaciens*. The expression of AHL synthase and reception genes was investigated before and after adding one selected inhibitor cinnamaldehyde, as this inhibitor has been less studied previously than the other inhibitor garlic, so the influences of cinnamaldehyde on AHL communication could be understood in this study. Cinnamaldehyde was found to decrease the expression of *luxI*-type genes of both spoilage bacteria, which are responsible for synthesizing AHL signaling molecules, and also suppressed the expression of the *luxR*-type genes of both bacteria used to receive the AHL signals. These genes were found to express more when AHL signaling molecules were added. The expression of *luxR*-type genes of both bacteria was found higher when lamb sourced strains were grown in lamb, than reference strains that were grown in LB. This might be because of the strain variation, and the possible existence of AHL signals produced from other Gram-negative species growing in lamb juice might also influence the expression.

To investigate the influence of cinnamaldehyde on lamb spoilage, the inhibitor was added to spoilage bacteria *H. alvei* and *S. liquefaciens* when they were grown in lamb juice. The deciding factors for AHL effects on lamb spoilage are based on enzymatic activities (proteolysis and lipolysis) and off-odor production. Previous investigations used agar plating screening and zymograms (electrophoresis for enzyme detection, based on the enzyme-substrate) assays to detect enzymatic activity (Christensen et al., 2003; Li et al., 2018). These methods are rapid but due to factors such as gel strength, diffusion ability and the concentration of substrates, the sensitivity of these two methods may not be good and the zymogram protocol is quite long. In my study, absorbance was used as a sensitive and specific method for enzyme detection and comparison. The natural microbiota in lamb juice showed enzymatic activity, without inoculating with Enterobacteriaceae. The enzymatic activity increased after AHLs were added. Cinnamaldehyde reduced the enzymatic activity. In my study, the regulation of *lipB* by AHL signals was found to modulate the secretion of lipolytic and proteolytic enzymes and led to lamb spoilage. Understanding the influence of AHL inhibitors on the expression of *lipB* helped determine the effect in AHL communication and enzyme secretion. Cinnamaldehyde, used as an AHL inhibitor, reduced the expression of the *lipB* gene of both *Enterobacteriaceae* species. The expression of the *lipB* gene correlated with the expression of *luxR*-type genes, suggesting that the reduction in the concentration of reception proteins is the major mechanism for all these selected AHL inhibitors to control the AHL-dependent phenotypes. AHL addition enhanced the expression of *lipB*, probably through the regulation system of *luxR*-type genes. This is supported by the results of enzymatic activity in this study.

In this part of the study, cinnamaldehyde was applied to the natural microbiota in vacuum-packed lamb and lamb juice in an anaerobic tube (to mimic the anaerobic condition inside vacuum packaged meat). The *ex vivo* study showed that cinnamaldehyde delayed lipase activity with the length of time depending on the storage temperature. To the majority of customers, off-odor production is the main indicator of food rejection. In this study, cinnamaldehyde was found to delay off-odor production and extend the shelf-life of vacuum-packed lamb. Hence it can be considered as a good use in lamb preservation due to its safety for human consumption. It disrupts the microbial activity leading to food spoilage while maintaining the nutritional quality of packaged and ready to eat (RTE) food.

6.5 Conclusion

Cinnamaldehyde was found to reduce bacterial growth and AHL production from *H. alvei* and *S. liquefaciens* grown *in vitro* and *ex vivo*. AHL signaling molecules in pure solution were degraded by cinnamaldehyde, and this compound suppressed AHL synthase and AHL reception genes in both spoilage bacteria. Cinnamaldehyde also decreased the expression of *lipB* in both *H. alvei* and *S. liquefaciens*, even though the addition of exogenous AHL signal increased the expression of *lipB*. Protease and lipase activities of both spoilage bacteria, grown *in vitro* and *ex vivo*, were inhibited by cinnamaldehyde. Furthermore, when applied to anaerobically stored lamb and lamb juice, cinnamaldehyde extended the shelf-life for 2-8 days at different temperatures. This could be due to its ability to reduce bacterial growth, degrade AHL signals or deactivate AHL regulation. Cinnamaldehyde could be used as a natural agent to extend the lamb shelf-life.

Chapter Seven: Overall Summary

7.1 Origin of study

Bacterial growth on meat during processing, distribution, storage, and transportation is primarily responsible for meat spoilage (Nychas et al., 2007). Although meat may be attacked by a wide range of microbes, the spoilage of meat is often caused by a few specific microbial groups including lactic acid bacteria, *Brochothrix* spp., *Enterobacteriaceae*, and *Shewanella putrefaciens* (Borch et al., 1996; Ercolini et al., 2006; Lambert et al., 1991; Nychas et al., 2007). These microorganisms produce extracellular enzymes that hydrolyze proteins and fats resulting in meat spoilage (Tsigarida and Nychas, 2001; Blana, 2010).

The shelf-life of aerobically stored fresh meat is variable depending on the storage temperature. Vacuum packed meat can extend the shelf-life from days to weeks or months. In anaerobic storage, the microbiota of stored meat consists of lactic acid bacteria and *Enterobacteriaceae*, suggesting that meat spoilage in vacuum-packed products is a result of an interaction between *Enterobacteriaceae* and lactic acid bacteria (Bruhn et al., 2004). Various QS signaling molecules such as AHLs and AI-2s are produced through this cell-to-cell interaction (Bruhn et al., 2004; Lu et al., 2004a).

AHL signals cause milk deterioration by regulating the secretion of hydrolytic enzymes (Christensen et al., 2003). In vacuum-packed meat, *Hafnia alvei* and *Serratia* spp. are the predominant *Enterobacteriaceae* species, and they produce AHL signaling molecules (Bruhn et al., 2004; Gram et al., 1999; Ravn et al., 2001). However, very limited study investigated the AHL production in lamb, and the effect of AHL signals on spoilage of vacuum-packed meats was not previously reported.

Lu et al., (2004) reported AI-2 production in meat but the activity was found to be very low in a high indigenous bacterial population. AI-2 production is found universally in bacteria but the production is variable, affected by the presence of fatty acids in meats and food additives (Lu et al., 2004a; K. A. Soni et al., 2008a; Widmer et al., 2007). Hence the influence of AI-2 on meat spoilage at the start of this project was unclear. Blana (2010) reported that the presence of AHL signal molecules encourage the growth of *Pseudomonas fluorescens*, and partly increase the growth of *S. liquefaciens*, whereas AI-2 reduces bacterial growth of both species.

The objectives of my research were to:

1. Identify, for the first time, QS signaling molecules produced in spoiled vacuum-packed lamb
2. Understand the influence of these signaling molecules on lamb spoilage, which has not been well studied.
3. Determine the potential of QS inhibitors on lamb preservation.

7.2 Overall conclusions

The findings of this study are summarised as follows:

- ✓ *Hafnia alvei*, *Serratia liquefaciens*, *Yersinia enterocolitica*, *Rahnella aquatilis*, *Klebsiella oxytoca*, *Shewanella putrefaciens*, *Brochothrix thermosphacta*, *Brochothrix campestris*, *Carnobacterium divergens* and *Carnobacterium maltaromaticum* were used for initial part of the research. Some of these spoilage bacteria were isolated from spoiled VP chilled lamb. AHL signaling molecules were produced by *H. alvei*, *S. liquefaciens*, *R. aquatilis*, and *Y. enterocolitica*. AI-2 production was found in all the bacteria. Both AHL and AI-2 signaling molecules were found in spoiled VP chilled lamb. Extrinsic factors such as storage temperature and oxygen, as well as intrinsic factors in lamb, such as pH values and fat content, influenced the QS activities of these spoilage bacteria.
- ✓ AHL production was high in *H. alvei* and *S. liquefaciens*, and because they were reported as the dominant *Enterobacteriaceae* species in lamb spoilage, knockdown mutants of these

two bacteria were developed by random transposon mutagenesis. In addition, knockdown mutants of *C. divergens* were developed. These mutants were used for further investigations into QS molecule production and inhibition. AHL upregulated the hydrolytic enzymes produced by spoilage bacteria and agreed with the findings of Christensen and Bruhn.

- ✓ Cinnamaldehyde, garlic, curcumin, vanillin, ascorbic acid and furanone were investigated to study the influence of AHL inhibition on lamb preservation. My results using different detection methods agreed with previous findings that all these compounds could be used as AHL inhibitors (Brackman et al., 2008; El-Mowafy et al., 2014; Packiavathy et al., 2014; Ponnusamy et al., 2009; Rasmussen et al., 2005). Cinnamaldehyde and garlic extract were found to reduce 25-40% of bacterial growth and hydrolyzed 30-40% of the AHL signaling molecules. By gene expression assay, AHL synthesis was reduced 10% and 35%, by cinnamaldehyde and garlic respectively. 50% and 80% reduction of AHL reception was found by the addition of cinnamaldehyde and garlic, respectively. The expression of the *lipB* gene was investigated in *H. alvei* and *S. liquefaciens* before and after the addition of inhibitors. The expression of *lipB* was found to be modulated by the AHL regulatory system leading to enzyme secretion and lamb spoilage.
- ✓ When added to anaerobically stored lamb and lamb juice, cinnamaldehyde helped extend the shelf-life for 2-8 days according to different storage temperatures. My results demonstrated the possibility that natural extracts like cinnamaldehyde could be used as natural agents, instead of synthetic chemical additives, to slow lamb spoilage.

7.3 Recommendations for future work

A number of questions have been raised during my research. Further work is needed in the topics below:

- AI-2 detection by induced bioluminescence used in our study was quick and simple, but as AI-2 production could be affected by certain meat matrices and the bioluminescence used in the study could be influenced providing a deviation in quantitative interpretation. Liquid chromatography (LC) coupled to mass spectrometry (MS/MS) is a more sensitive method to detect and quantify specific AI-2 signaling molecules, the signaling molecules will be chemically extracted from meat matrices or growth medium which normally interferes the sensing of signals, and reconstituted into pure solution, it should provide more reliable results and thus be used in the future to explore AI-2.
- The agar plating assay used in this study is a good phenotypic interpretation of the effect of various parameters on QS molecule production, however, this did not explain how these factors influence the QS system. Gene expression of synthase genes and reception genes of QS on different spoilage bacteria when they are grown under different conditions will be useful in the future. This may give an idea about how different growth factors like temperatures and pH, affect QS communication at genotypic level.
- Novel detection techniques for QS signaling molecules to simplify detection will be useful to identify bacterial communication early in field studies or meat manipulation. A rapid, easy, and portable test using microdots of immobilized whole cell-based biosensors on paper (Wynn et al., 2018) could be a useful tool for future investigation.
- Random transposon mutagenesis used in this study developed knockdown mutants to help explain phenotypic analyzes. The *halI*↓ mutant retained β-galactosidase activity resulting in reduced AHL production. In future studies, *in vivo* site-directed mutagenesis could be used to develop gene knockouts so that the comparison between knockout mutants and wild type strains to provide a stronger interpretation.
- Mutants were developed from Enterobacteriaceae type strains *H. alvei* NZRM860 and *S. liquefaciens* NZRM861. As these two reference strains used in mutagenesis in the study

did not originate from lamb, they may differ from isolates obtained from lamb. In the future, the mutants should be developed from spoilage bacteria isolated from lamb such as *H. alvei* FAM01 and *S. liquefaciens* FAM02, instead of using type reference strains.

- Cinnamaldehyde solution used in this study was sent for encapsulation in lamb packaging, for further investigation as an intervention to delay food spoilage. Further research should include the concentration of cinnamaldehyde by natural carrier oil, such as vegetable oils, instead of acetonitrile used in this research to enable the safety in the food industry.
- The strong odor of cinnamaldehyde could affect the flavor and aroma of meat and become unacceptable to consumers. Further studies are needed to develop active packaging that prevents odor issues. An active packaging system including an inner surface coated with a chitosan film with thyme essential oil was designed by Quesada et al., 2016. Without direct contact with meat, the essential oil in the film enhanced the meat color and chitosan reduced the impact of thyme on meat sensory properties. However, in the same study, they found that *Enterobacteriaceae* were not affected by the presence of the essential oil in the films. Further studies are needed for more strategies, two of my assumptions are 1) developing oil blends so that the essential oil molecules can directly kill bacteria. Essential oil is also safe to enter human body and enhance the immunity; or 2) using air technologies like atomization or ionizers to bond with the essential oil scent molecule and deactivate the scent.
- As QS activity could be either problematic or beneficial, limiting the bad and promoting the good will be a major challenge in meat quality control in the future.

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Appendix

Appendix 1

16S RNA gene sequence of *H. alvei* FAM01. The sequence shown 5' to 3'.

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TCGAGCGGTAGCACAAGAGAGCTTGCTCTCTGGGTGACGAGCGGCGGACGGGTGAGTAATGT
CTGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATGAYGT
CTTCGGACCAAAGTGGGGGACCTTCGGGCCTCACGCCATCAGATGTGCCCAGATGGGATTAG
CTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCTCTAGCTGGTCTGAGAGGATGACCA
GCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA
CAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAG
TACTTTCAGCGAGGAGGAAGGCATTGTGGTTAATAACCACAGTGATTGACGTTACTCGCAGA
AGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGATTAAGTCAGATGTGAAATCCCCGAGCT
TAACTTGGGAACTGCATTTGAAACTGGTCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCA
GGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGA
CAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCTGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACG
CGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGG
CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCT
TGACATCCAGAGAATTTRSYAGAGATAGYTTAGTGCCTTCGGGAACTCTGAGACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TATCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAAGGAGACTGCCGGTGATAAACCGGA
GGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACA
ATGGCATATACAAAGAGAAGCGAACTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAG
TCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGA
ATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGT
TGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCACTT
```

Appendix 2

Sequences of translated proteins of all test mutant strains from Section 5.3.5.

Areas associated with transposon insertion are highlighted in yellow, open reading frames that have the ability to be translated are highlighted in green.

halI↓ strain of *H. alvei* NZRM860

L M Q A * R L * A A P G N S P D K E R P H * A A D V R Q T P H H E I
P E N C R H G * L C Q C * V C R A G A A H C L G A S L R L T E S C K
P S G A N N K L P S T * I T G F * * V R L E R C * I Y S F G M V N S
V V S N I G S L L * N T D G T L N K L A Q H C F V M F X T K M L Q T
W K A Y L * * S K L G A N L L F A E T S I P A S A I L R S * I S F R
L Q L L C T P R S L L A L I S M Y L L K L V V I S D S L * R E S K F
D K A L S L I H I S T I I

luxS↓ strain of *H. alvei* NZRM860

G X X * R G I H T L E H L F A G F M R S R Q H R S G C S I N G * * L
S R K Y K E * N D G T V T A R A D K D W Q L V V S M R N W L T A K S
Y F W L E C T S P Q S S A C C N R P Y Q Q G A S * S I F S S X N V P
L F A S I K A L S L T L S T P K R R L P C C C K S K L L Q S T I T K
X P F R P D S V C N K A A S L L L P L X L S X S I L M A A S W V I X
X * X I L X R W * I * * L L V T X E R * S V S L X S X S W L M S E E
R N S M V G G

halR↓ strain of *H. alvei* NZRM860

G R P H * A A D V R Q T P H H E I P E N C R H G * L C Q C * V C R A
G A A M H C L G A S L R L T X X * R G I H T L * X I L X R W * I * *
L L S R Q H R S G C S I N G * * L S R K Y K E * N D * I Y S F G M V
N A R A D K D W Q L V V S M H I S T I I A K S Y F W L E C T S P Q S
S A C C N R P Y Q Q G A S * S I F S S X G

sliI↓ strain of *S. liquefaciens* NZRM861

X X Q A * R M L Q A A A * * P G N S P D K E R L L Q R K K E M * V R
Q T P H H E I P E N C R H G * L C Q C * V C R A G A A H M I S V V L
C L G A S L R L T E S C K P S G A N N K L P S T * I T G F * * V R L
E R C * I Y S F G M V N S V V S N I L R S * I S F R L I E S C K P S
G A N N K L P S T * I T G F * * V R L E R C * I Y S F G M V N S V V
S N I G S L L * N T D G T L N K L A Q H C F V

luxS ↓ strain of *S. liquefaciens* NZRM861

A S T T E G K K D T L E H L F A G F M R S R Q V L S S V Q A A S H G
* * L S R K Y K E * N D G T V T A R A D K D W Q L V V S M R N W L T
A K S Y F W L E C T S P Q S S A C C N R P Y Q Q G A S * S I F S S X
N V P L F P L X LA A S W V I X I L X R W * I * * L L V T X E R * S V
S L X S X S W L M S E E R N S M V G G

sliR ↓ strain of *S. liquefaciens* NZRM861

M R N W L T A K S Y F W L E C T S P Q S S A C C N R P Y Q Q G A S *
S I F S S X N V P L F A S I K A L S L T L S T P K R R L G I H T L *
X I L X R W * I T S P Q S S A C C N R P * Q H R S G C S I N G M P P
W F N A R A D K D * W Q L V V S

sliI↓ *hflB*↓ strain of *S. liquefaciens* NZRM861

G I H T L E H L F A G F M R S R Q H R S G C S I N G * * L S R K Y K
E * N D G T V T A R A D K D W Q L V V S M R N W L T A K S Y F W L E
C T S P Q S S A C C N R P Y Q Q G A S * S I F S S X N V P L F A S I
K A L S L L V V S M R N W L T A K S Y F X I L X R W * I * * L L V T
X E R * L M S R L E R C * I Y S F

luxS ↓ strain of *C. divergens* FAM03

G F M E E Q L R * L S R K Y K E * N T D D Y S S Q P F F D M T K X P
F * R P D S V C N K A A S L L L P D K D W Q L V V L X L S X S I * I
* * L L V T X E R * S V S L M S E E R N S M V G X S X S W L M S E E
R N S M V * G G K R R L P C C C K S K L L Q S T I T K X P F R P D S
V C N K A A S L I I