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NEAR EAST UNIVERSITY
INSTITUTE OF GRADUATE STUDIES

ANTI-QUORUM SENSING ACTIVITY OF HIGH-INTENSITY SWEETENERS

VICTOR MARKUS

DOCTORAL THESIS
DEPARTMENT OF MEDICAL BIOCHEMISTRY

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Hereby I declare that this thesis study is my study. I had no unethical behavior in all stages from the planning of the thesis until writing thereof. I obtained all the information in this thesis in academic and ethical rules. I provided reference to all the information and comments which could not be obtained by this thesis study and took these references into the reference list and had no behavior of breeching patent rights and copyright infringement during the study and writing of this thesis.

Victor Markus
Name and Surname of Student


Signature

30th/6/2021
Date

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ABSTRACT

Name of Student: Victor Markus

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The human gut harbors trillions of microorganisms including bacteria, viruses, archaea, and fungi. Of these gut microbial species, Firmicutes and Bacteroidetes are the most abundant phyla. Quorum sensing (QS), a sophisticated system of bacterial communication, is crucial in the maintenance of gut microbial balance. Several factors including diet have been shown to cause dysbiosis, leading to diseased conditions such as diabetes, obesity, and inflammatory bowel disease (IBD). High-intensity sweeteners (HIS), widely used as sugar substitutes in foods and beverages, have been implicated in modulating gut microbiota composition and function, yet the mechanism of action of these agents remains poorly understood. Since QS has a role in normobiosis, it was hypothesized that one mechanism by which HIS could induce dysbiosis is through the inhibition of QS. This study aimed to investigate the anti-QS activity of HIS. Using recombinant bioreporter strains carrying N-acyl homoserine lactone (AHL)-mediated QS systems, the anti-QS activities of three widely used FDA approved artificial sweeteners (aspartame, saccharin, and sucralose) and stevia components (stevia extract, stevioside, rebaudioside A, and steviol), were assessed. To further clarify the mode of action of HIS, a representative LuxR-type receptor (LasR) was expressed in the presence of the artificial sweeteners and/or the AHL native ligand. The expressed protein was purified using nickel-affinity chromatography and monitored by SDS-PAGE and western blotting. The results obtained show that the HIS exert significant inhibitory effects on the AHL-dependent communication machinery, presumably by interfering with the receptor:AHL interaction. The evidence in this study provides insight into the molecular events that may occur in the gut microbiota exposed to these HIS.

Keywords: gut microbiota, quorum sensing, high-intensity sweeteners, dysbiosis.

ÖZET

Öğrencinin Adı: Victor Markus

Danışmanı: Doç. Dr. Kerem TERALI

Anabilim Dalı: Tıbbi Biyokimya

İnsan bağırsağı; bakteri, virüs, arke ve mantar dahil trilyonlarca mikroorganizmayı barındırır. Bu bağırsak mikrobiyal türlerinden Firmicutes ve Bacteroidetes en bol bulunan filumlardır. Gelişmiş bir bakteri haberleşme sistemi olan nisap algılama (QS), bağırsak mikrobiyal dengesinin korunmasında çok önemlidir. Diyeti de içeren çeşitli faktörlerin disbiyoza neden olduğu ve bunun da diyabet, obezite ve inflamatuar bağırsak hastalığı (IBD) gibi patolojik durumlara yol açtığı gösterilmiştir. Yiyecek ve içeceklerde şeker ikameleri olarak yaygın şekilde kullanılan yüksek yoğunluklu tatlandırıcıların (HIS) bağırsak mikrobiyota bileşimini ve işlevini modüle etmeden sorumlu olduğu bilinse de bu ajanların etki mekanizmaları henüz tam olarak anlaşılamamıştır. QS'nin normobiyozda bir rolü olduğundan, HIS'in disbiyozu indükleme mekanizmalardan bir tanesinin QS inhibisyonu yoluyla olduğuna inanılmaktadır. Bu çalışmada HIS'in QS karşıtı aktivitesinin araştırılması amaçlanmıştır. N-asil homoserin lakton (AHL) aracılı QS sistemleri taşıyan rekombinant biyoraportör suşlarından faydalanılarak, yaygın olarak kullanılan üç FDA onaylı yapay tatlandırıcının (aspartam, sakarin ve sukraloz) ve stevia bileşenlerinin (stevia özütü, steviosit, rebaudiosit A ve steviol) QS karşıtı aktiviteleri değerlendirilmiştir. HIS'in etki modunu daha da belirginleştirmek için temsili bir LuxR tipi reseptör (LasR), yapay tatlandırıcıların ve/veya AHL doğal ligandının varlığında ifade edilmiştir. İfade edilen protein, nikel afinite kromatografisi kullanılarak saflaştırılmış ve SDS-PAGE ve western blot ile saptanmıştır. Elde edilen sonuçlar, HIS'in büyük olasılıkla reseptör:AHL etkileşimini bozarak, bakterilerin AHL temelli haberleşme düzeneği üzerinde anlamlı inhibitör etkiler uyguladığını göstermektedir. Bu çalışmadan elde edilen kanıtlar, söz konusu HIS'e maruz kalan bağırsak mikrobiyotasında meydana gelebilecek moleküler olaylar hakkında fikir vermektedir.

Anahtar kelimeler: Bağırsak mikrobiyotası, nisap algılama, yüksek yoğunluklu tatlandırıcılar, disbiyoz.

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ABBREVIATIONS

3-Oxo-C12-HSL:	N-3-oxo-dodecanoyl-L-homoserine lactone
Ace-K:	Acesulfame potassium
ACP:	Acylated-acyl carrier protein
ADI:	Acceptable daily intake
AHLs:	N-Acyl homoserine lactones
AI-1:	Autoinducer-1
AI-2:	Autoinducer-2
AI-3:	Autoinducer-3
AIPs:	Autoinducing peptides
APS:	Ammonium persulfate
BSA:	Bovine serum albumin
C4-HSL:	N-butyryl-L-Homoserine lactone
CYP2D1:	Cytochrome P450 2D1
CYP3A4:	Cytochrome P450 3A4
DDW:	Double distilled water
DHPD:	4,5-dihydroxy-2,3-pentanedione
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
Dulc A:	Dulcoside A
EHEC:	Enterohemorrhagic *Escherichia coli*
FAO:	Food and Agriculture Organization
FDA:	United States Food and Drug Administration
GIT:	Gastrointestinal tract

GLP-1:	Glucagon-like peptide 1
HIS:	High-intensity sweeteners
HRP:	Horseradish peroxidase
IBD:	Inflammatory bowel disease
IPTG:	Isopropyl β-D-1-thiogalactopyranoside
LasR-LBD:	LasR ligand-binding domain
LB:	Luria-Bertani
LCS:	Low-caloric sweeteners
LuxS:	S-ribosylhomocysteinase
Na_2HPO_4:	Disodium hydrogen phosphate
NaCl:	Sodium chloride
NCS:	Non-caloric sweeteners
Ni-NTA:	Nickel-nitrilotriacetic acid
NNS:	Non-nutritive sweeteners
OD:	Optical density
P-gp:	P-glycoprotein
PMSF:	Phenyl methane sulfonyl fluoride
QS:	Quorum Sensing
RCT:	Randomized controlled trials
Reb:	Rebaudioside
RLU:	Relative light unit
SAH:	S-adenosylhomocysteine
SAM:	S-adenosylmethionine

SD:	Standard deviation
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRH:	S-Ribosylhomocysteine
TEMED:	Tetramethylethylenediamine
TRPM5:	Transient receptor potential channel M5
WHO:	World Health Organization

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Chapter one

1. INTRODUCTION

HIS, also known as non-caloric sweeteners (NCS), low-caloric sweeteners (LCS), or non-nutritive sweeteners (NNS), are sugar substitutes present in day-to-day food products and beverages such as soft drinks, desserts, yogurts, and gum, recommended for sufferers of type 2 diabetes mellitus, obese and glucose intolerance (Gardner *et al*., 2012). Although most HIS have been approved by the United States Food and Drug Administration (FDA) (FDA, 2018), European Food Safety Authority (Mortensen, 2006), and other government regulatory authorities around the world, current scientific data shows that there is still no consensus in the scientific community regarding the safety status of the agents. The first concern about the safety of HIS came from the evidence that the agents may cause cancer. In 1969, cyclamate was stroke out of the list of food additives due to safety concerns that the chemical causes bladder cancer (Oser *et al*., 1975). Discovered in 1937 (Du Bois & Prakash, 2012) and approved for use in 1958 (Baker-Smith *et al*., 2019), cyclamate was used extensively in the 1960s, mostly in a 10:1 mixture with saccharin (Kroger *et al*., 2006). After several extensive studies in mice, rats, hamsters, dogs, and monkeys, the link between cyclamate and cancer was refuted (Ahmed & Thomas, 1992; Baker-Smith *et al*., 2019). After cyclamate-cancer fears, saccharin was the next sweetener implicated in causing bladder cancer (Garland *et al.*, 1989; Hagiwara *et al*., 1980; Murasaki & Cohen, 1981; Schoenig *et al*., 1985). But like cyclamate, the likelihood of saccharin to cause bladder cancer in humans was refuted (Elcock & Morgan, 1993; Morgan & Wong, 1985; Okamura *et al*., 1991). Other sweeteners such as aspartame (Marinovich *et al*., 2013), sucralose (Magnuson *et al*., 2017), advantame (Opinion, 2013), and stevia (Toyoda *et al*., 1997) were also cleared not to have any connection with cancer.

In recent times, the effects of HIS on the gut microbiota have gained considerable attention, igniting a new angle of debate. The ability of HIS to disrupt gut microbial balance needed to be clarified since diet is among the major factors that cause dysbiosis

(Garcia-Mantrana *et al*., 2018; Kho & Lal, 2018; Toor *et al*., 2019). Both independent researchers and relevant regulatory agencies, such as the European Food Safety Authority, have reported the induction of gastrointestinal disturbances by some HIS (Opinion, 2013; Suez *et al.*, 2015). While the proponents of HIS focus on the advantages of the agents in weight and diabetes management, the issues with HIS appear to be beyond just their low-caloric content. HIS have been implicated in contributing or exacerbating metabolic diseases such as diabetes and obesity that they were intended to reduce (Becker *et al.*, 2020; Nettleton *et al.*, 2019, 2020; Soffritti *et al*., 2016; Suez *et al*., 2014, 2015). A recent study reveals that maternal consumption of artificially sweetened beverage during gestation increases infant body mass index (BMI) and decrease *Bacteroides* spp. (Laforest-Lapointe *et al.*, 2021). Samples of breast milk taken from 20 lactating mothers revealed that sucralose, saccharin, and acesulfame-potassium were in 65% of the mothers' milk (Sylvetsky *et al*., 2015). Because of their reduced ability to remove xenobiotics from their system (Lu & Rosenbaum, 2014), infants may be more affected by HIS than adults. It is known that gut bacteria are involved in the regulation of the immune system, production of vitamins, facilitation of dietary substrates digestion, and repression of pathogens expansion (C. Landman & Quévrain, 2016; Lane *et al.*, 2017). These beneficial functions of the gut bacteria are likely to be hampered in the event of HIS-induced dysbiosis. Indeed, the long-term impact of HIS on health, especially infants who may have been exposed to these chemicals during gestation and lactation, is still unclear.

The gut microbial communities regulate their activities via QS (Jimenez & Sperandio, 2019). Studies show that there is a connection between QS and normobiosis (Bivar Xavier, 2018; Cecilia Landman *et al.*, 2013; Le Balc'h *et al.*, 2017), implying that alteration in QS could influence gut microbiota equilibrium and induce disease conditions. Artificial sweeteners pass through the human gastrointestinal tract (GIT) unaffected (Buerge *et al*., 2009; Lethco & Wallace, 1975; Roberts *et al.*, 2000; Suez *et al*., 2014), indicating they directly encounter the intestinal microbiota in the form they were ingested. Although the alteration of gut microbiota composition and function by artificial sweeteners are well-documented, the mechanism of action of these synthetic agents remains poorly understood. For the natural sweeteners steviol glycosides, they are metabolized in the colon (Gardana *et al.*, 2003; Hutapea *et al*.,

1997; Nikiforov *et al*., 2013). However, while the action of gut bacteria on steviol glycosides is known, the action of the glycosides on the gut microbiota community is not yet clear. Since QS plays an important role in regulating the group behavior of gut bacteria, it was hypothesized that one mechanism HIS may alter gut microbiota homeostasis is by disrupting QS. This study was aimed to evaluate the anti-QS activity of HIS.

Chapter two

2. LITERATURE REVIEW

2.1. Quorum sensing

QS is the generic name used to describe a sophisticated system of bacterial communication (**Figure 2.1**). Via QS, bacteria interact with each other and adjust the expression of their genes according to their population density, either for the advantage of the entire community or for the benefit of one species over another (Azimi *et al*., 2020; Mukherjee & Bassler, 2019; S. Wang *et al*., 2020). QS occurs through QS signals – chemical molecules synthesized by the bacterial cells.

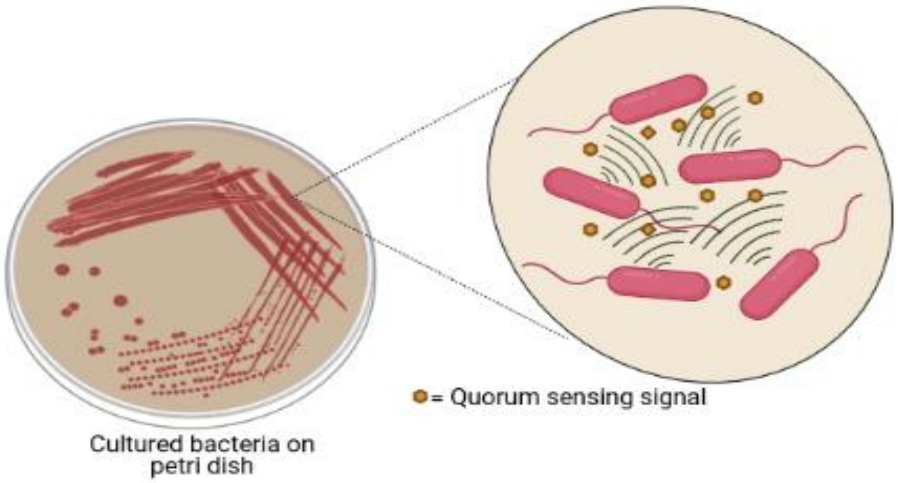

Figure 2. 1. Quorum sensing.

2.2. QS discovery

Until the discovery of QS that transformed scientists' thinking, bacteria were thought to act separately (O'Toole, 2016). Although unicellular, bacteria have demonstrated the ability to live a multicellular life using QS to coordinate and regulate group behaviors that would normally not be possible to accomplish by an individual cell (Rutherford & Bassler, 2012). First discovered in 1970 by Hastings and colleagues

(Nealson *et al.*, 1970), QS has revolutionized the study of bacteria. In their study, Hastings and colleagues observed rapid luminescence by *Vibrio fischeri* (called, at the time, *Photobacterium fisheri*) which was not a function of cell growth but a consequence of "conditioning" of medium by the growing cells, a phenomenon they called "autoinduction". The study further showed that light production by the luminescence bacteria was absent in the freshly inoculated culture and was delayed until the middle of the exponential (logarithmic) growth phase. Interestingly, subsequent studies indicate that the "autoinduction" phenomenon is conserved across different microbial species, including pathogenic ones (An *et al*., 2014; Azimi *et al*., 2020; Bassler *et al*., 1997; X. Chen *et al*., 2002; Galloway *et al*., 2011; Mukherjee & Bassler, 2019; Smith & Iglewski, 2003; S. Wang *et al*., 2020; Xavier & Bassler, 2005). Currently, QS systems are being considered as potential targets for addressing drug resistance in bacteria. In 1994, the term "quorum sensing" was first coined by Greenberg and colleagues to describe the "autoinduction" phenomenon by bacteria that helped to convey the concept to a wide audience (Fuqua *et al.*, 1994).

2.3. QS mechanism in bacteria

The QS mechanism in bacteria consists of a series of steps as represented in **Figure 2.2**. Essentially, it involves: (i) the synthesis and secretion of small chemical signal molecules (called autoinducers) by the constituted bacteria as they grow (**Figure 2.2 a and b)**; (ii) accumulation of the autoinducers in an extracellular environment until a concentration threshold is attained (at a specific cell population density or "quorum") (**Figure 2.2 c)**; (iii) detection of the autoinducers by the neighboring cells through specific receptors (on the membrane or in the cytoplasm); (iv) response to signals through the activation of the signal transduction cascade and expression of the target genes (Thoendel *et al.*, 2011; Zhao *et al*., 2017).

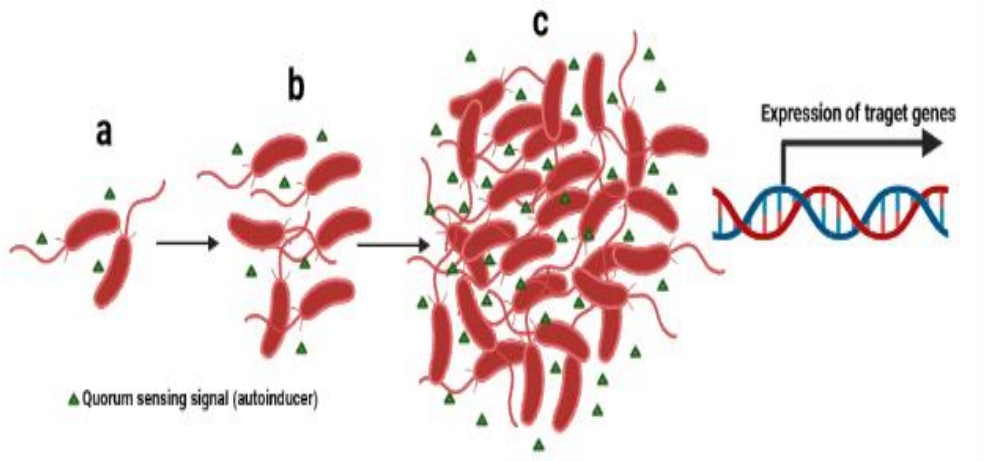

Figure 2. 2. QS mechanism in bacteria.

2.4. Canonical bacterial QS systems

Gram-positive and Gram-negative bacteria utilize different kinds of QS systems. These QS systems can be broadly divided into two: (i) intraspecies QS system, and (ii) interspecies QS system (Barnard *et al.*, 2007; Miller & Bassler, 2001). Using these QS systems, bacteria can distinguish between species ('self' from 'non-self') in a complex mixed population.

The Gram-positive bacteria use oligopeptides, called autoinducing peptides (AIPs), as autoinducers (Zhao *et al.*, 2017). The AIPs vary in structure and sequence (Lazazzera, 2001; Okada *et al.*, 2005; Thoendel *et al.*, 2011). Once synthesized, the AIPs are processed and released out of the cell through a transporter (Rutherford & Bassler, 2012). At a threshold concentration, corresponding to a specific bacterial population, the AIPs bind to a membrane two-component histidine kinase receptor (Thoendel *et al.*, 2011; Wei *et al.*, 2012). The binding of the AIPs to the receptor activates the receptor's kinase activity, causing autophosphorylation, and subsequent passage of a phosphate to the cytoplasmic regulator (Rutherford & Bassler, 2012). The phosphorylated response regulator drives the transcription of the QS target genes (Rutherford & Bassler, 2012; Zhao *et al.*, 2017), as represented in **Figure 2.3A**. In some Gram-positive bacteria (Hawver *et al.*, 2016; Ziemichód & Skotarczak, 2017), AIPs are taken back into the cytoplasm of cells through a transporter where they bind

to the transcription factors to form regulatory assemblies that drive gene expression (**Figure 2.3B**).

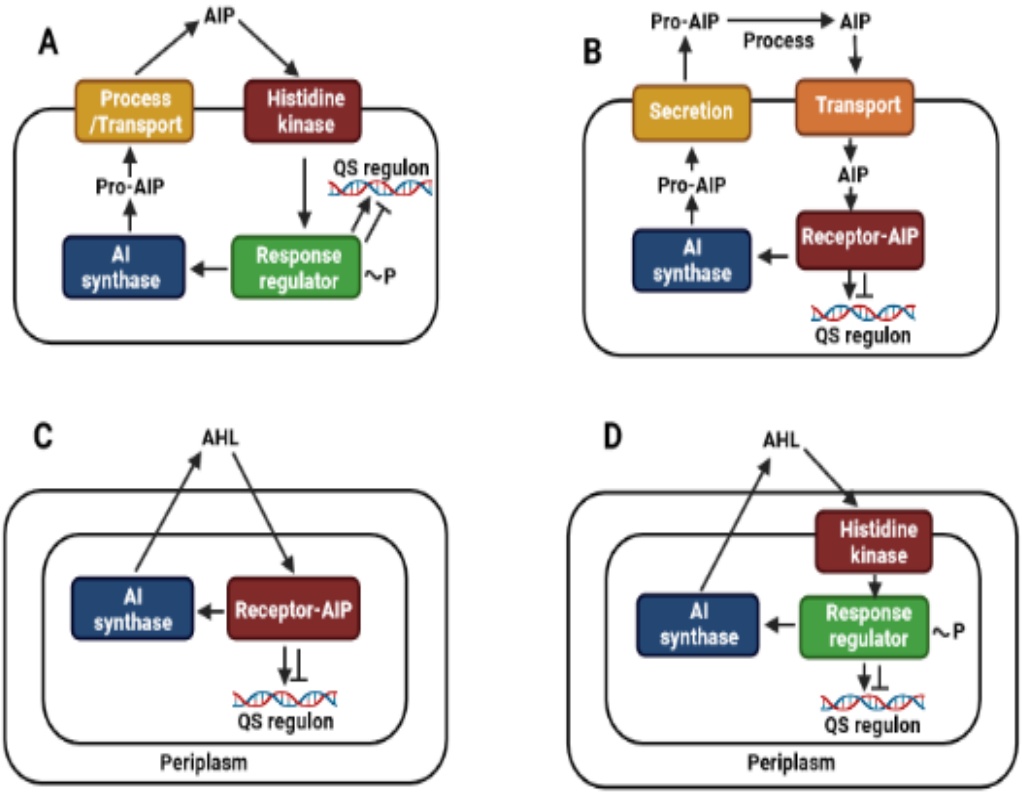

Figure 2. 3. Canonical bacterial QS systems**.**

AHLs (**Figure 2.4**) are the most common category of autoinducers in Gram-negative bacterial QS systems (Whitehead *et al*., 2001). Different AHLs are synthesized by different bacterial species with varying lengths or substitution in the acyl chain but with the same important moiety, homoserine lactone (Dong *et al.*, 2001; Martin Schuster *et al*., 2013). AHLs are produced by a LuxI protein family from S-adenosyl methionine (SAM) and an acylated-acyl carrier protein (ACP) based on bacterial population density (Parsek *et al.*, 1999). In *Vibrio fischeri*, the QS regulatory protein LuxR or its analog in other Gram-negative bacterial species has two observable binding domains: the N-terminal domain which interacts with AHL, and the C-terminal domain with interacts with DNA (Jayaraman & Wood, 2008). Produced and secreted by the cells, the AHLs diffuse in and out the cell membrane freely (Galloway *et al*., 2011; Whitehead *et al*., 2001). At sufficient (threshold) concentration, the autoinducers interact with the cytoplasmic receptors to generate regulatory complexes that drive the expression of QS target genes (**Figure 2.3C**). In some Gram-negative bacteria (Papenfort & Bassler, 2016; Rutherford & Bassler, 2012; Wei *et al*., 2011, 2012), the autoinducers are identified by two-component histidine kinase receptors that operate in a similar way to the one described for Gram-positive two-component QS system (**Figure 2.3D**).

An important autoinducer considered as a "universal" signaling molecule for interspecies interaction is furanosyl borate diester, commonly known as autoinducer-2 or AI-2 (**Figure 2.4**) (X. Chen *et al.*, 2002). Until the discovery of AI-2 (Bassler et al., 1993, 1994; X. Chen *et al.*, 2002), QS was thought to be intraspecies in which AIPs (in Gram-positive bacteria) or AHLs (in Gram-negative bacteria) are employed as signaling molecules. AIPs and AHLs are generally known as autoinducer-1 or AI-1. Credit to Bassler laboratory (Bassler et al., 1993, 1994; X. Chen *et al.*, 2002), now we know that bacteria in a mixed population have an alternative mechanism that can sense the presence, identify and interact with each other. AI-2 was first discovered in *Vibrio harveyi* (Bassler *et al*., 1993, 1994) as one of two autoinducers (the first being AHL) that co-regulate bioluminescence in the marine bacterium. Across several Gram-negative and Gram-positive bacteria, AI-2 is conserved (Miller & Bassler, 2001; S. Schauder & Bassler, 2001; Stephan Schauder *et al.*, 2001; Surette *et al.*, 1999). AI-2 is synthesized by S-ribosylhomocysteinase (LuxS) (Stephan Schauder *et al.*, 2001).

The utilization of S-adenosylmethionine (SAM) as a methyl donor during the methyl-transfer process in cells yields S-adenosylhomocysteine (SAH), which is then converted to adenine and S-ribosylhomocysteine (SRH) by nucleosidase Pfs (Cornell *et al.*, 1996; Wnuk *et al*., 2007). Next, SRH is cleaved at the thioether linkage by LuxS to yield 4,5-dihydroxy-2,3-pentanedione (DHPD) and homocysteine (X. Chen *et al*., 2002). DHPD undergo cyclization and further rearrangements to form AI-2 (Stephan Schauder *et al*., 2001). The reaction catalysed by LuxS at least serves two purposes for bacteria: detoxification of SAH and the synthesis of AI-2 molecule (Pei & Zhu, 2004).

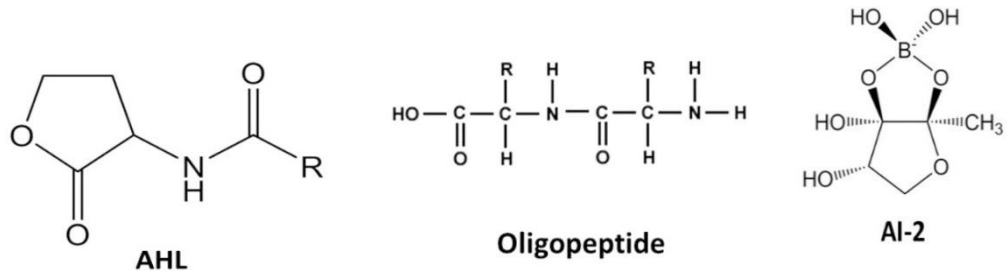

Figure 2. 4. General structures of autoinducers

2.5. Processes controlled by QS in different bacterial species

QS provides different bacteria with a significant advantage to carry out various activities including synthesis of virulence factors (Kim *et al*., 2020; Smith & Iglewski, 2003; Whitehead *et al*., 2001), formation of biofilms (L. Chen & Wen, 2011; Harmsen *et al*., 2010; O'May & Tufenkji, 2011; Poli *et al*., 2018), bioluminescence (Nealson *et al*., 1970; Winson *et al*., 1998), sporulation (Perchat *et al*., 2016; Rai *et al*., 2015), horizontal transfer of DNA (Rai et al., 2015; L. Zhu et al., 2020), glucose uptake (An *et al*., 2014; Goo *et al*., 2015; Ha *et al*., 2018), biosynthesis of nucleotides (Goo *et al*., 2015), production of antibiotics (El-Sayed *et al*., 2001), synthesis of secondary metabolite (Barnard *et al*., 2007), homeostasis and adaptation to hostile environments (Kang *et al*., 2017; Pena *et al*., 2019).

2.6. QS and gut microbiota

Microorganisms represent the most abundant living things on earth, with the gut environment containing more and diverse microorganisms than any setting in nature (Turnbaugh & Gordon, 2009). It was estimated that about 10^{30} microorganisms exist in the universe (Turnbaugh & Gordon, 2008) of which 10^{14} bacteria and several other microorganisms such as viruses, archaea, and fungi are said to harbor the human gut (C. Landman & Quévrain, 2016). The activities of these gut microorganisms are governed by different factors, including QS (Jimenez & Sperandio, 2019). Using QS, the gut microorganisms regulate group behavior. Scientific evidence from the human gut shows a link between normobiosis and the presence of 3-oxo-C12:2-HSL, an AHL signaling molecule (Cecilia Landman *et al.*, 2013; Cécilia Landman *et al.*, 2018; Le Balc'h *et al.*, 2017). Other signaling molecules found in the gut are AI-2 (Bassler *et al.*, 1993, 1994; X. Chen *et al.*, 2002), and autoinducer-3 (AI-3) which is responsible for the enterohemorrhagic *Escherichia coli* (EHEC) pathogenesis (Hernandez & Sintim, 2020; Kendall *et al.*, 2007; Kim *et al*., 2020; Sperandio *et al*., 2003; Walters & Sperandio, 2006).

2.7. High-intensity sweeteners

HIS can be broadly classified into two: (1) Artificial sweeteners, and (2) Natural sweeteners. Six artificial sweeteners (acesulfame potassium or Ace-K, advantame, aspartame, neotame, saccharin, and sucralose) and two natural sweeteners (*Siraitia grosvenorii* fruit extracts and steviol glycosides) have been approved for use in the United States by the FDA (FDA, 2018) (**Table 2.1**). The first discovered artificial sweetener was saccharin, introduced in 1879 as a food additive (FDA, 2018) and used for many years without regulation until the Food Additives Amendment of 1958 in the United States (Baker-Smith *et al*., 2019; Kroger *et al*., 2006). Subsequently, the FDA approved the use of aspartame, Ace-K, sucralose, neotame, and advantame in 1981, 1988, 1998, 2002, and 2014 respectively (FDA, 2018). It is generally considered that these chemicals are not metabolized, but pass through the GIT unaffected (Buerge *et al*., 2009; Lethco & Wallace, 1975; Roberts *et al.*, 2000; Suez *et al*., 2014).

Among the natural sweeteners, stevia is the most popular (Samuel *et al.*, 2018). Stevia is a common name for *Stevia rebaudiana* Bertoni, a shrub plant widely consumed and used as a sugar substitute in foods and beverages. The plant is considered to have originated from South America where it has been used by the indigenous people for centuries as a sweetener in drinks and hypoglycemic agent (Samuel *et al.*, 2018). The most abundant steviol glycoside in the stevia leaves responsible for the characteristic sweet taste is stevioside first isolated in the early 1930s (Brandle *et al.*, 1998). Subsequently, other steviol glycosides such as dulcoside A (Dulc A), rebaudioside (Reb) A, B, C, D, and E were isolated from stevia leaves. On a dry weight basis, the predominant glycosides in the stevia leaves are stevioside (~9.1% w/w), Reb A (~3.8% w/w), Reb C (~0.6% w/w), and Dulc A (~0.3% w/w) (Brandle *et al.*, 1998). The two prominent steviol glycosides, stevioside, and Reb A differ by only one glucose moiety from each other. While stevioside has two molecules of glucose, Reb A has three molecules of glucose (Samuel *et al*., 2018). In literature, the majority of the data about steviol glycosides comes from studies conducted on stevioside and Reb A, which are metabolized in the colon to their aglycon steviol (Gardana *et al*., 2003; Hutapea *et al*., 1997; Nikiforov *et al*., 2013), Other non-predominant glycosides such as steviolbioside, Dulc A, Reb B, C, D, E, F, and M, have been reported to have the same metabolic fate with stevioside and Reb A (Purkayastha *et al*., 2016). Mammals cannot metabolize steviol glycosides since they do not have the necessary enzymes. The degradation of steviol glycosides into steviol is done in the colon by gut microbiota, primarily *Bacteroides* via their β-glucosidase activity (Gardana *et al*., 2003).

Table 2. 1. FDA-approved HIS

Sweetener	Status	Some brand names that contain sweetener	Sweetness compared sucrose	Acceptable daily intake (ADI) (mg/kg bw/d)	Sweetener packets equivalent to ADI[a]
Acesulfame Potassium (Ace-K)	Approved for use generally except in meat and poultry	Sunett®, Sweet One®	200 x	15.0	23
Advantame	Approved for use generally except in meat and poultry		20,000 x	32.8	4,920
Aspartame	Approved for use generally	Equal®, Nutrasweet®, Sugar Twin®	200 x	50.0	75
Neotame	Approved for use generally except in meat and poultry	Newtame®	7,000-13,000 x	0.30	23 (sweetness intensity at 10,000 x sucrose)
Saccharin	Approved for use only in special foods and purposes	Sweet Twin®, Sweet and Low®, Sweet'N Low®, Necta Sweet®	200-700 x	15.0	45 (sweetness intensity at 400 x sucrose)
Siraitia grosvenorii Swingle (Luo Han Guo) fruit extracts (SGFE)	Generally regarded as safe	Monk Fruit in the Raw®, Nectresse®, PureLo®	100-250 x	NS[b]	ND
Stevia rebaudiana (Bertoni) Bertoni	Generally regarded as safe	PureVia®, Truvia®, Enliten®	200-400 x	4.0[c]	9 (sweetness intensity at 300 x sucrose)
Sucralose	Approved for use generally	Splenda®	600 x	5.0	23

[a]The amount a 60 kg individual would have to consume to reach the acceptable daily intake (ADI). Calculations consider that a packet of HIS is equivalent in sweetness as 2 sugar teaspoons. [b]NS = not specified. [c]Approved by the Joint Expert Committee on Food Additives (JECFA) of FAO/WHO. FDA = United States Food and Drug Administration. HIS = High-intensity sweeteners. FAO = Food and Agriculture Organization. WHO = World Health Organization

2.8. Impact of HIS on health

2.8.1. Artificial sweeteners

Although artificial sweeteners increase the palatability of food products with no increase in caloric content (Baker-Smith *et al.*, 2019), current data in the literature indicate no consensus in the scientific community concerning the safety status of the substances. Besides the induction of hematopoietic neoplasias (Soffritti *et al.*, 2016), sucralose was reported to cause an increase in fecal pH and a decrease in crucial fecal microflora (Abou-Donia *et al.*, 2008). Other findings from the study of Abou-Donia *et al.* showed that P-glycoprotein (P-gp), Cytochrome P450 3A4 (CYP3A4), and Cytochrome P450 2D1 (CYP2D1) were upregulated by sucralose. P-gp, CYP3A4, and CYP2D1 are important proteins reported to decrease the bioavailability of drugs administered orally (Breedveld *et al*., 2006; J. Chen *et al*., 2021; Fromm, 2000; Guengerich, 2021; Mitschke *et al*., 2008; van Waterschoot & Schinkel, 2011). Other studies showed that consumption of saccharin (Bian, Tu, *et al*., 2017) and sucralose (Bian, Chi, *et al.*, 2017) increase the pro-inflammatory products of the gut microbiome in mice with potential induction of systemic inflammation, shifting cells to a pro-inflammatory state. Additionally, secondary bile acids (which possess antimicrobial properties) were decreased during sucralose consumption, corresponding to an increase in multidrug resistance genes in the gut microbiome (Bian, Chi, *et al*., 2017). It, therefore, suggests that sucralose may lead to an increase in pathogens and multidrug-resistant bacteria in the gut. Like antibiotics, recent studies have shown that Ace-K, aspartame, saccharine, and sucralose may promote the spread of antibiotic-resistant genes between different and same phylogenetic bacteria strains (Yu *et al*., 2021). Earlier, in 2019, Ace-K was reported to induce oxidative stress (Cruz-Rojas *et al*., 2019). Available evidence showed that artificial sweeteners may accumulate in adipose tissue. Using rats fed with sucralose, Schiffman and colleagues revealed two biotransformation products (both acetylated forms of sucralose) in their feces and urine as well as an accumulation in adipose tissue (Bornemann *et al*., 2018). The authors noted that their studies differed from the earlier metabolism studies used for receiving FDA approval for the food additive that claimed it was not metabolized or stored in the body. Other studies indicated that aspartame, saccharin, and sucralose could induce

glucose intolerance (Suez *et al.*, 2014, 2015) through the disruption of gut microbiota. Palmnäs *et al.* reported that aspartame consumption may increase the abundance of Enterobacteriaceae, bacteria that produce metabolites associated with inflammation and insulin resistance (Palmnäs *et al*., 2014). Compared to the control, the neotame-treated mice showed a significant increase in Bacteroidetes, decrease in Firmicutes, and decrease in butyrate synthetic genes (Chi *et al.*, 2018). Chi *et al*. also showed that the concentration of multiple fatty acids and cholesterol were significantly increased, while glyceric acid and malic acid were significantly reduced in the neotame-treated mice feces compared to the control. As for advantame, relevant regulatory authorities such as the European Food Safety Authority (Opinion, 2013) and independent researchers (Otabe *et al.*, 2011) have tagged the chemical safe for consumption. Although stable under normal storage conditions, there are indications that advantame is unstable in thermally-treated foods and acidic beverages (Opinion, 2013). This data suggests a need for advantame to be reassessed.

While most studies using rodent models suggested artificial sweeteners have negative effects on metabolic health (Abou-Donia *et al*., 2008; Bian, Chi, *et al*., 2017; Bian, Tu, *et al*., 2017; Bornemann *et al*., 2018; Chi *et al*., 2018; Soffritti *et al*., 2016; Suez *et al*., 2014, 2015), data from humans randomized controlled trials (RCT) are conflicting regarding the effects of the agents on bodyweight (Higgins *et al.*, 2018; Higgins & Mattes, 2019; Rogers & Appleton, 2021; Sylvetsky & Rother, 2018) and metabolic health (Ahmad *et al*., 2020; Bonnet *et al*., 2018; Serrano *et al*., 2020). It appears that the issue of artificial sweeteners is more complicated than just sweetness without calories since all artificial sweeteners do not produce the same effects (Blundell, 2019). More long-term human intervention studies with detailed reports of the intervention period, treatments, comparators, and outcomes are required to assess the effects of artificial sweeteners on health (Toews *et al*., 2019).

2.8.2. Natural sweetener stevia

As the safety of artificial sweeteners is questionable, the attention of food manufacturers and consumers has tilted toward natural sweeteners like stevia. Although some studies suggest that steviol glycosides may play a role in glycemic control by inducing the release of glucagon-like peptide 1 (GLP-1) from the

enteroendocrine cells (Glycoside *et al*., 2014; Wielen *et al*., 2016) and stimulating the release of insulin through the activation of the transient receptor potential channel M5 (TRPM5) in pancreatic β-cells and type-II taste receptor cells (Philippaert *et al.*, 2017), other studies could not ascertain these claims. A recent study showed that stevia could not rescue glucose-intolerant mice or alter weight gain, caloric intake, or liquid consumption (Becker *et al*., 2020). Also, some studies implicate stevia in disrupting gut microbial balance (Nettleton *et al.*, 2019, 2020) similar to what was observed using saccharin (Becker *et al.*, 2020). Nettleton *et al.* demonstrated in rat dams and their new-born that stevia disrupts gut microbiota, metabolism, and mesolimbic reward system (Nettleton *et al.*, 2020). The authors earlier reported that Reb A, the second most abundant steviol glycosides, disrupted gut microbiota equilibrium and at the same time reduced nucleus accumbens tyrosine hydroxylase and dopamine transporter mRNA levels compared to the control (Nettleton et al., 2019).

2.9. Consumption of HIS

In the absence of any definite conclusion on the safety status of HIS, more people including children and adolescence are increasingly being exposed to these chemicals in larger amounts (Baker-Smith *et al*., 2019). Governments' regulations regarding sugar consumption force food manufacturers to increase the proportion of HIS in their products. The number of products in the market containing HIS has been shown to quadruple in recent times (Baker-Smith *et al*., 2019). Because manufacturers may not specify the content of HIS in their products, it has become challenging to assess the concentration of these sweeteners in such products. Although food manufacturers list the HIS in the ingredient list, however, they do not indicate the amount of the HIS per serving. For this reason, it has become difficult to better evaluate the health effects of these sweeteners at the level of an individual or population (Baker-Smith *et al*., 2019).

It is commonly known that athletes pay attention to their diet and use supplements to improve their performance in training sessions and competitions. Like in food products and beverages, the use of HIS in sports supplements has been unprecedented in recent years. Because sportsmen and sportswomen consume much of these supplements which contain HIS in undisclosed amounts, it suggests that this demographic group may be the highest consumers of HIS. Perhaps more worrisome is the lack of public

awareness of HIS. Studies suggest that parents have difficulty in identifying food products that contain artificial sweeteners. Although 72% of parents do not think that artificial sweeteners are safe for their children, most of them (77%) were not able to distinguish which products have artificial sweeteners (Sylvetsky *et al*., 2014). Indeed only 24% of the children tested were able to differentiate between the taste of artificial sweeteners and sugar in food products (De Ruyter *et al.*, 2014). What compounds the problem is that even our drinking water now contains these artificial sweeteners (Lange *et al*., 2012; Mawhinney *et al*., 2011). Many people who would think they have not been exposed to artificial sweeteners are likely to be surprised that they already have these agents in their body circulation.

2.10. HIS as emerging environmental pollutants

The designation of artificial sweeteners as emerging pollutants because of their chemical stability and persistence in the environment has generated a new facet of debate in the scientific community (Praveena *et al*., 2019). Artificial sweeteners have been identified in soil, air (Gan *et al.*, 2014), groundwater, surface water, seawater, lakes, and tap water in different locations all over the world (Praveena *et al.*, 2019). Because artificial sweeteners consumed in foods and beverages by people are not metabolized, they are excreted in urine and feces and reaching the environment via wastewater (Buerge *et al*., 2009). Additionally, due to their resistance to wastewater treatment (Sang *et al*., 2014), artificial sweeteners are introduced continuously into the environment. A study in the United States reported that some water treatment plants from 19 states serving more than 28 million people contain sucralose in 15 out of 19 water source of the treatment plants (47–2,900 ng L^{-1}), and in 13 out of 17 "finished" water of the treatment plants (49–2,400 ng L^{-1}) and in 8 out of the 12 distribution system water of the treatment plants (48–2,400 ng L^{-1}) (Mawhinney *et al.*, 2011). In Germany, Lange *et al*. reported the presence of Ace-K (7 μg L^{-1}) and sucralose (2.4 μg L^{-1}) in drinking water (Lange *et al*., 2012). The amounts of the artificial sweeteners reported in the air and soil samples are also considerable, ranging from 6450 to1280 ng g^{-1} in some areas (Gan *et al.*, 2014). This data suggest that many people get artificial sweeteners into their body without their knowledge and in amounts possibly beyond the ADI.

Chapter three

3. MATERIALS AND METHODS

3.1. Materials

Difco Luria-Bertani (LB) Broth, Miller (10 g L^{-1} tryptone; 5 g L^{-1} yeast extract; 10 g L^{-1} NaCl) and Difco LB agar, Miller (10 g L^{-1} tryptone; 5 g L^{-1} yeast extract; 10 g L^{-1} NaCl; 15 g L^{-1} agar) were obtained from Becton Dickinson & Company (Le Pont-de-Claix, France). Phenyl methane sulfonyl fluoride (PMSF), lysozyme, isopropyl β-d-1-thiogalactopyranoside (IPTG), disodium hydrogen phosphate (Na_2HPO_4), tetramethylethylenediamine (TEMED), kanamycin A monosulfate, trimethoprim, N-3-oxo-dodecanoyl-L-homoserine lactone (3-oxo-C12-HSL)**,** N-butyryl-L-Homoserine lactone (C4-HSL), saccharin, and aspartame were purchased from Sigma-Aldrich (St. Louis, MO, USA). Glycerol, sodium chloride (NaCl), ammonium persulfate (APS), 10x TG-transfer buffer, 10x TBST/TTBS washing solution, methanol, 10-180 kDa molecular mass marker, 40% acrylamide/bisacrylamide (37.5:1) were purchased from Bio-Lab (Hanapach Ashkelon, Israel). Nickel-nitrilotriacetic acid (Ni-NTA) beads were purchased from ADAR biotech (Rehovot, Israel). Poly prep chromatography columns, nitrocellulose membrane, and transfer filter papers were obtained from Bio-Rad (Haifa, Israel). Instant Blue (a coomassie-based staining preparation for protein gels) was purchased from Expedeon (Cambridgeshire, UK). Sample (loading) buffer for SDS-PAGE and bovine serum albumin (BSA) ≥ 98% were procured from Carl Roth (Karlsruhe, Germany). Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific (Leics, United Kingdom). Imidazole and sucralose were obtained from Alfa Aesar (Massachusetts, USA). Primary antibody (anti-mouse IgG) and the secondary antibody (horseradish peroxidase (HRP)-conjugated) were purchased from Cell Signaling Technology (Massachusetts, USA). Chemiluminescent substrate for western blotting was procured from Cyanagen (Bologna, Italy). Stevia extract and stevioside were procured from HWI Pharma Services GmbH (Rülzheim, Germany). Reb A was purchased from PhytoLab (Vestenbergsgreuth, Germany).

3.2. Bacterial strains

PAO-JP2 (pKD201-*lasI*), a *lasI-rhlI* double mutant of *Pseudomonas aeruginosa* PAO1 harboring a pKD201 plasmid infused with a QS *lasI* promoter upstream to the *luxCDABE* operon. PAO-JP2 (pKD-*rhlA*), a *lasI-rhlI* double mutant of *Pseudomonas aeruginosa* PAO1 harboring a pKD plasmid infused with *rhlA* promoter upstream to the *luxCDABE* box. *Escherichia coli* BL21, containing a pETM-11 vector encoding for a shortened His_6-tagged LasR construct (LasR-LBD), spanning residues Met-1 to Lys-173. All strains were graciously provided by professor Michael M. Meijler, Ben-Gurion University of the Negev, Israel (Bukelman *et al*., 2009), and stored at –80°C in 50% (v/v) glycerol, a cryoprotectant additive. **Figure 3.1** shows the features of a pETM-11 plasmid (Dümmler *et al*., 2005). The maps of the pKD and pKD201 plasmids were not made available by the original designers (Duan & Surette, 2007).

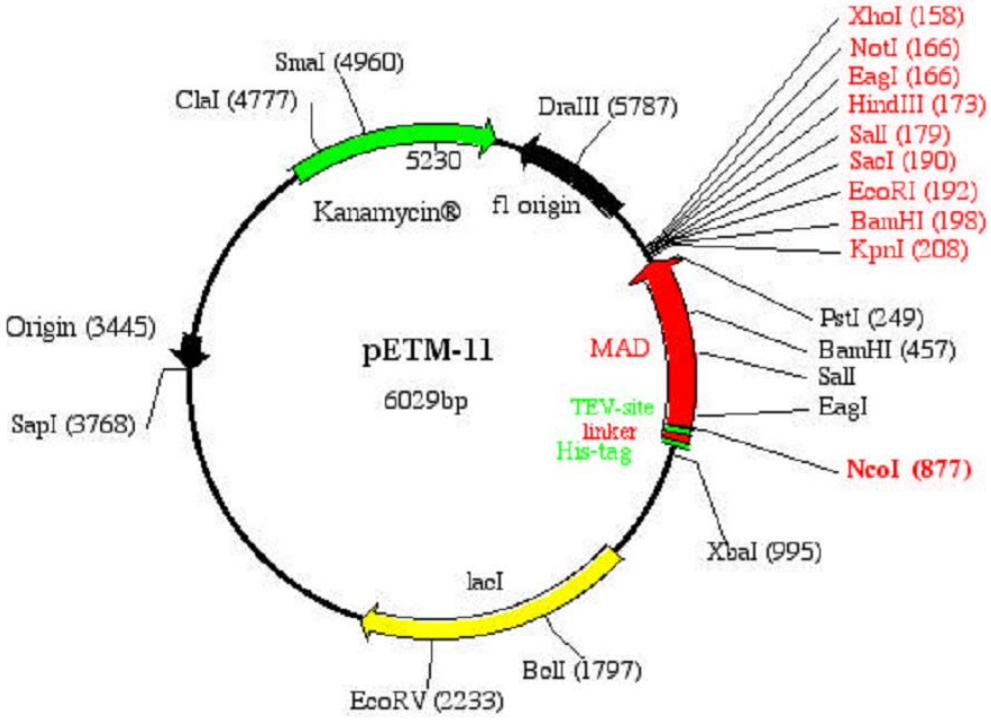

Figure 3. 1. Features of pETM-11 plasmid

3.3. Bacterial cultivation

The PAO-JP2 (pKD201-*lasI*) and PAO-JP2 (pKD-*rhlA*) strains were cultured on LB-agar plates (10 g L^{-1} tryptone; 5 g L^{-1} yeast extract; 10 g L^{-1} NaCl; 15 g L^{-1} agar) containing 300 µg mL^{-1} trimethoprim for 24 h at 37 °C in the incubator (Binder, Camarillo, CA, USA). A single colony was introduced into 10 mL LB medium containing 300 µg mL^{-1} trimethoprim (with the cap of the tube half-opened stabilized with autoclave tape) and grown overnight at 37 °C with shaking (140 rpm) on a rotary thermo-shaker (Gerhardt, Germany) as shown in **Figure 3.2**. *E. coli* BL21-pETM-11 strain was cultured on LB-agar plates (10 g L^{-1} tryptone; 5 g L^{-1} yeast extract; 10 g L^{-1} NaCl; 15 g L^{-1} agar) containing 50 µg mL^{-1} kanamycin for 24 h at 37 °C in the incubator (Binder, Camarillo, CA, USA). A starter culture was prepared by inoculating a single colony from the agar plate into 5 mL LB broth containing 50 µg mL^{-1} kanamycin (with the cap of the tube half-opened stabilized with autoclave tape) and incubated at 37 °C with shaking (140 rpm) on a rotary thermo-shaker (Gerhardt, Germany) for 18 h (15:00–9:00) (**Figure 3.3**). The respective LB-agar plates containing PAO-JP2 (pKD201-*lasI*), PAO-JP2 (pKD-*rhlA*), and BL21-pETM-11 strains were stored at 4 °C for future use, but not more than one month. The kanamycin stock (50 mg mL^{-1}) and trimethoprim stock (50 mg mL^{-1}) were prepared using double distilled water (DDW) and stored at –20 °C.

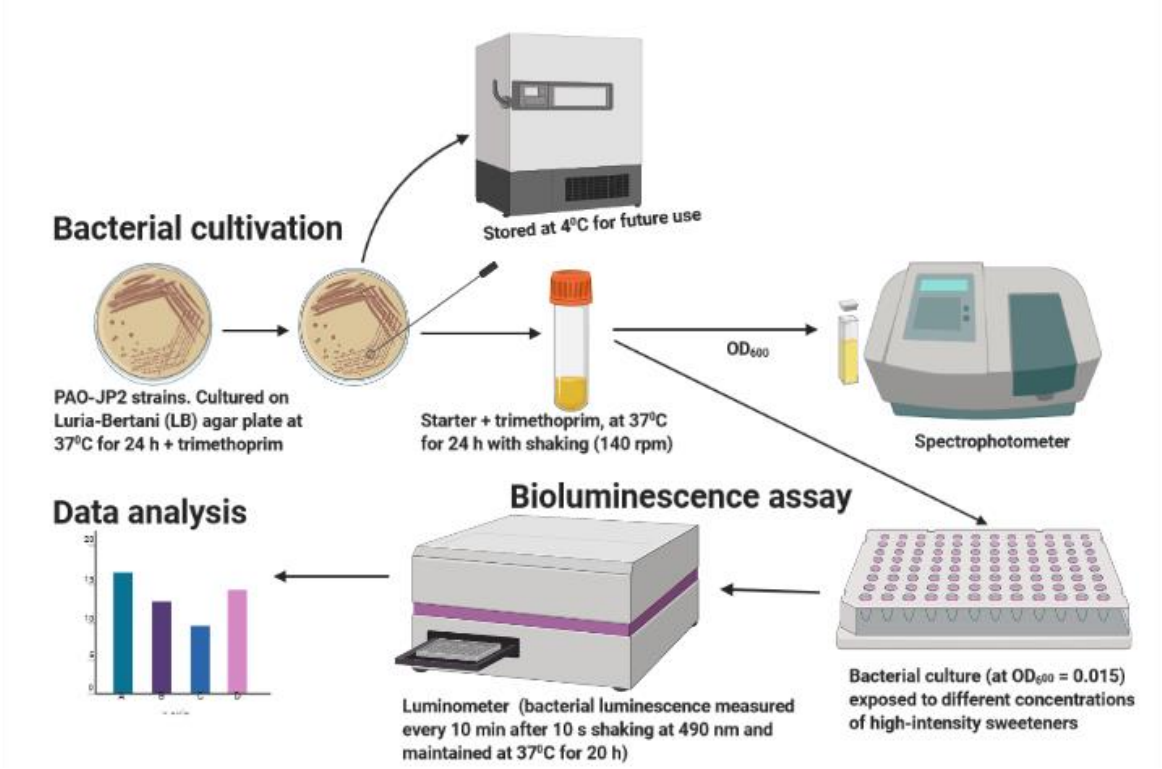

Figure 3. 2. Process of determining the anti-QS activity of HIS

3.4. Induction of bioluminescence

Different concentrations of 3-oxo-C12-HSL and C4-HSL were tested for the induction of PAO-JP2 (pKD201-*lasI*) and PAO-JP2 (pKD-*rhlA*) bioreporter strains, respectively. The induction of the bioluminescence was monitored using the Luminoskan Ascent Luminometer (Thermo Fisher Scientific) set at 37 °C and 490 nm. The measurement was done in a white opaque 96-well microtiter plate containing 90 µL of the bacterial culture and 10 µL 3-oxo-C12-HSL for PAO-JP2 (pKD201-*lasI*) or C4-HSL for PAO-JP2 (pKD-*rhlA*). The plate was read for 20 h at 10 min intervals (after a 10 s shaking in each case). The final concentration range of 3-oxo-C12-HSL was 0.1–1,000 nM while that of C4-HSL was 0.001–100 µM. As for the control, 90 µL bacterial cultures and 10 µL LB medium were used. Luminescence was expressed in relative light units (RLU).

3.5. Bioluminescence assay

The anti-QS activity of HIS was evaluated using PAO-JP2 (pKD201-*lasI*) and PAO-JP2 (pKD-*rhlA*) reporter strains monitored by Luminoskan Ascent Luminometer (Thermo Fisher Scientific) as shown in **Figure 3.2.** The measurement was done in a white opaque 96-well microtiter plate containing 10 µL of different concentrations of HIS, 80 µL of overnight bacterial cultures (diluted to OD_{600} = 0.015) and 10 µL of 3-oxo-C12-HSL (final concentration 100 nM) for PAO-JP2 (pKD201-*lasI*) or 10 µL of C4-HSL (final concentration 10µM) for PAO-JP2 (pKD-*rhlA*). The final concentrations of the HIS used were as follows: aspartame (1.36–0.085 mM), saccharin (2.72–0.17 mM), sucralose (25.2–0.575 mM), stevia extract (5–0.3125 mg mL^{-1}), stevioside (1–0.0125 mM), Reb A (0.31–0.019375 mM), steviol (0.52–0.0325 mM). The positive control contained 80 µL of the overnight bacterial culture (diluted to OD_{600} = 0.015), 10 µL of the native ligands (1 µM 3-oxo-C12-HSL or 100 µM C4-HSL), and 10 µL LB broth. The negative control contained 80 µL of the overnight bacterial culture (diluted to OD_{600} = 0.015) and 20 µL LB broth. The luminescence was expressed in RLU. The OD_{600} was determined by Ultrospec 2100 pro spectrophotometer (Amersham, Berks, England). The aspartame, saccharin, and sucralose were prepared in DDW. Stevia extract and stevioside were dissolved in DDW, while Reb A and steviol were dissolved in 17% (v/v) DMSO. Concentration ranges for the HIS used in this study were chosen based on the published scientific studies for each of the compounds (Bian, Chi, *et al.*, 2017; Bian, Tu, *et al.*, 2017; Bornemann *et al.*, 2018; Deniņa *et al.*, 2014; Mahalak *et al.*, 2019; Purkayastha *et al.*, 2016; Suez *et al.*, 2014; Q. Wang *et al.*, 2018) and calculated to reflect concentration ranges within the ADI established by FDA (FDA, 2018) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO, 2019).

3.6. Protein expression

Overnight starter culture of *E. coli* BL21-pETM-11 ($OD_{600} \approx 1$) was diluted 100 times with LB broth (3 mL starter in 300 mL LB broth). To the diluted starter culture, 300 µL kanamycin (50 mg mL^{-1}) was added to obtain a solution with a final concentration of 50 µg mL^{-1} kanamycin. The solution was incubated at 37 ºC for 2–2.5 h with agitation (180 rpm) till OD_{600} = 0.4 before adding the artificial sweeteners. The

addition of the artificial sweeteners was immediately followed by the introduction of 450 µL 3-oxo-C12-HSL (5 mM). The negative control contained 450 µL DMSO (final concentration 0.15%, v/v). The positive control contained 450 µL 3-oxo-C12-HSL (5 mM) only. All the cultures (tests and controls) were incubated at 20 ºC for 30 min with agitation (150 rpm) before inducing the expression of the protein by adding 120 µL IPTG (1M). The cultures remained at 20 ºC with agitation (150 rpm) till the next morning (12:00–9:00). The effect of the natural sweeteners was not evaluated due to insufficient samples.

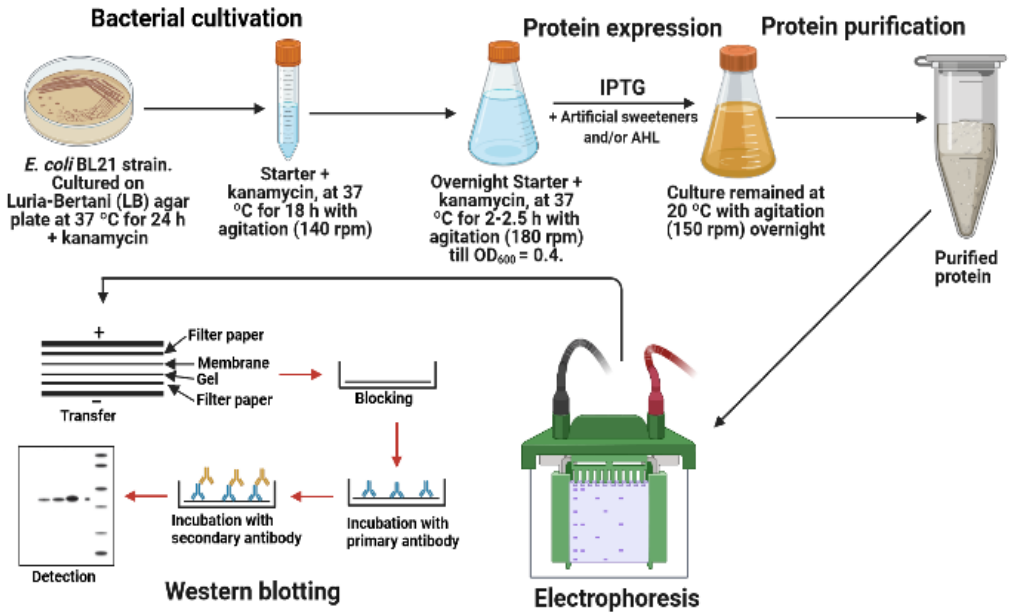

Figure 3. 3. Protein expression, purification, and monitoring.

3.7. Protein purification

After the expression of the protein, the cells' pellets were obtained by centrifugation at 6,000 rpm, 4 ºC for 15 min. The pellets were resuspended in lysis buffer (50 mM Na_2HPO_4, 300 mM NaCl, 10 mM imidazole, pH 8) containing 1 mg mL^{-1} lysozyme and 1 mM PMSF. To every 1g of pellets, 3 mL of the lysis buffer was added and peptized to form a colloidal solution. The colloidal solution was transferred into 15 mL sterile Eppendorf tubes, incubated on ice for 30 min, and sonicated for 40 s (4-s

interval) with 3-s pulses off at 30% amplitude. The lysate was centrifuged at 13,000 rpm, 4 ºC for 15 min. Leaving the cellular debris at the bottom of the tube, the soluble fraction was collected for purification using nickel-affinity chromatography.

To prepare the Ni-NTA slurry, 10 mL DDW was added to 1.5 mL beads and centrifuged at 1,500 rpm for 5 min. After centrifuging, the DDW was taken out gently leaving the concentrated nickel beads at the bottom of the tube. The soluble fraction obtained after centrifugation was added to the nickel beads and kept at 4 °C for 60 min with shaking at 30 rpm on a rotary shaker. For every 1 mL Ni-NTA, 4 mL lysate was required.

The chromatography columns were set up with each column appropriately marked. The protein-Ni-NTA mixture was loaded into a column and allowed until the blue beads got down to the bottom of the columns. Then, the bottom cap of the column was removed and the flow-through was collected (for SDS-PAGE analysis) followed by a twice-wash with 4 mL wash buffer (50 mM Na_2HPO_4, 300 mM NaCl, 20 mM imidazole, pH 8). The wash was also collected and stored for SDS-PAGE analysis. Next, 0.5 mL elution buffer (50 mM Na_2HPO_4, 300 mM NaCl, 250 mM imidazole, pH 8) was added to elude the protein from the columns into a tube marked E1. This was repeated for tubes marked E2 and E3. PMSF stock solution (200 mM) was prepared in ethanol and stored at –20 ºC.

3.8. SDS-PAGE analysis

Portions of the purified protein (120 µL each) were mixed with 30 µL of 5x sample buffer (Carl Roth, Germany) and heated at 95 ºC for 5 min before loading into the gels' wells. The gel preparation is as shown in **Table 3.1.** A 10–180 kDa molecular marker (Bio-Lab) was used. The electrophoresis was run at 100 V for 90 min. After the electrophoresis, the gels were stained using Instant Blue (Expedeon) for 15 min.

Table 3. 1 Polyacrylamide gel preparation (for two gels)

Components	**Separator gel (12%)**	**Stacking gel (4%)**
40% Acrylamide/bisacrylamide	6.0 mL	1.0 mL
1.5 M Tris-HCl, pH 8.8	5.0 mL	-
0.5 M Tris-HCl, pH 6.8	-	2.5 mL
Distilled Water	8.8 mL	6.4 mL
20% w/v SDS	0.10 mL	0.05 mL
10% w/v APS	0.15 mL	0.075 mL
TEMED	0.04 mL	0.02 mL
Total	20.09 mL	10.045 mL

3.9. Western blotting

The western blotting transfer was done by the semi-dry method according to standard protocol (Bio-Rad) (**Figure 3.3)**. The nitrocellulose membrane and transfer filter papers (six pieces) were equilibrated by soaking in the 1x transfer TG-Buffer. The 1x TG-transfer buffer was prepared by mixing 20 mL 10x TG-transfer buffer (Bio-Lab), 40 mL methanol (Bio-Lab), and 140 mL DDW. The transfer sandwich assembly was prepared in the following order: cassette electrode (Cathode-negative electrode, at the top), three pieces of filter papers, gel, nitrocellulose membrane, three pieces of filter papers, cassette electrode (anode-positive electrode, at the bottom). A 15 mL Eppendorf tube was used as an improvised roller to remove any air trapped between the layers. The transfer was performed at 25 V for 30 min.

At the end of the transfer, the membrane was blocked using 5% (w/v) BSA prepared in 1x TBST/TTBS solution (Bio-Lab). The blocking was done for 1 h at room temperature with shaking (30 rpm) on a shaker (New Brunswick Scientific, Edison NT-USA). After the blocking, the membrane was washed with 10 mL 1x TBST solution 3 times. The membrane was washed further with 10 mL 1x TBST solution three times, for 5 min with shaking (30 rpm) in each case. Next, the membrane was incubated with 10 mL blocking solution (5% w/v BSA) containing 3.3 µL primary antibody (Anti-mouse IgG; Cell Signaling Technology) for 16 h at 4 °C with shaking (30 rpm). After the incubation, the membrane was washed with 10 mL 1x TBST solution three times, and then further washed with 10 mL 1x TBST solution three times for 5 min with shaking (30 rpm) in each case. The used primary antibody solution was stored at 4 °C to be reused in the future, but not more than a month.

A freshly prepared 5% (w/v) BSA in 1x TSBT solution containing 5 µL secondary antibody (Cell Signaling Technology) was used to incubate the membrane (the one already incubated in primary antibody) for 1 h at room temperature with shaking (30 rpm). After the 1 h incubation, the membrane was washed with 10 mL 1x TBST solution three times, and then further washed with 10 mL 1x TBST solution three times for 5 min with shaking (30 rpm) in each case. Next, the membrane was incubated for 2 min with a chemiluminescent substrate solution (Cyanagen) at room temperature

with gentle agitation. The chemiluminescent substrate solution was prepared by mixing 1 mL of reagent 1 (luminol-enhancer solution) with 1 mL of reagent 2 (peroxide solution) so that the total solution per membrane was 2 mL. Finally, the membrane was imaged with Luminescent Image Analyser (DanyeBiotech). The Imager was set as follows: exposure type = increment, time interval = 10 s, and sensitivity/resolution = high resolution.

3.10. Statistical analysis

GraphPad Prism Software version 6.00 for Windows (La Jolla, CA, USA) was used to perform all statistical analyses. Student's *t*-test was used to compare all tests with the controls and calculations of the *p*-values. Each data point on the graphs represents the average of three different experimental readings to ensure the repeatability/reproducibility of the results. Values are expressed as mean ± standard deviation (SD).

Chapter four

4. RESULTS

4.1. Induction of bioluminescence

The maximum bioluminescence emission in PAO-JP2 (pKD201-*lasI*) and PAO-JP2 (pKD-*rhlA*) reporter strains (Bukelman *et al.,* 2009) were evaluated using different concentrations of 3-oxo-C12-HSL (final concentration from 0.1–1,000 nM) and C4-HSL (final concentration 0.001–100 μM) respectively. Except for the lowest concentrations, all concentrations tested induced significant bioluminescence emission in the reporter strains (**Figures 4.1 and 4.2**). Accordingly, the bioluminescence emissions with the highest statistical significance were induced at100 nM 3-oxo-C12-HSL (**Figures 4.1**) and 10 μM C4-HSL **(Figures 4.2**).

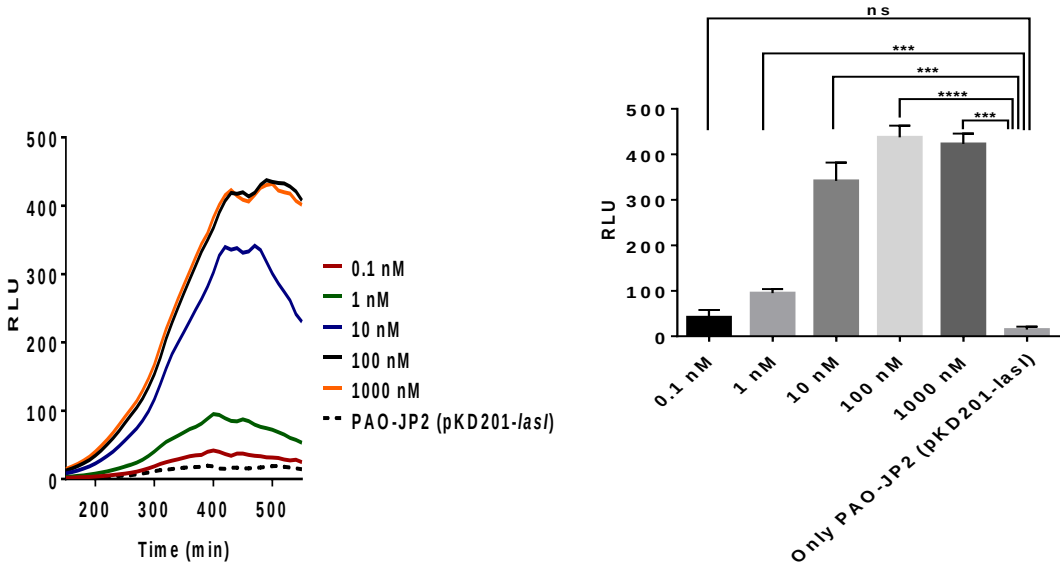

Figure 4. 1. Induction of bioluminescence in PAO-JP2 (pKD201-*lasI*) by 3-oxo-C12-HSL. ***Left panel***: bioluminescence emission of PAO-JP2 (pKD201-*lasI*) in the presence of different concentrations of 3-oxo-C12-HSL. ***Right panel*:** bioluminescence induction relative to the control, corresponding to the data in the left panel. All concentrations presented are the final concentrations. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. *** $p < 0.001$, **** $p < 0.0001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

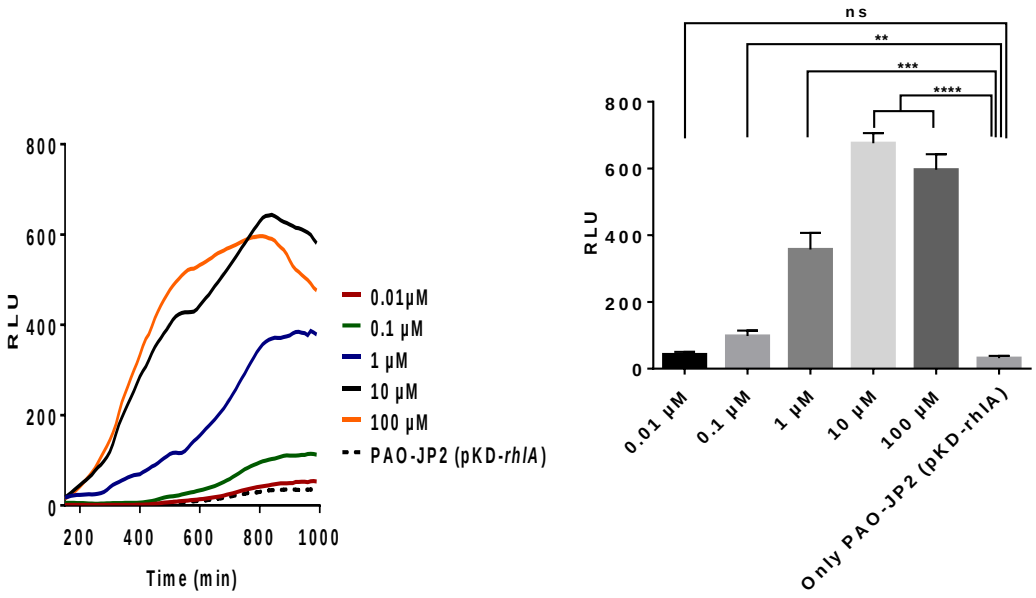

Figure 4. 2. Induction of bioluminescence in PAO-JP2 (pKD-*rhlA*) by C4-HSL. ***Left panel***: PAO-JP2 (pKD-*rhlA*) luminescence in the presence of different concentrations of C4-HSL. ***Right panel:*** bioluminescence induction relative to the control, corresponding to the data in the left panel. All concentrations presented are the final concentrations. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

4.2. Action of HIS on LasR-dependent QS

The anti-QS activity of the aspartame, saccharin and sucralose was assessed by monitoring the bioluminescence emission of PAO-JP2 (pKD201-*lasI*), an engineered *P. aeruginosa* strain carrying only a LasR-dependent QS (Bukelman *et al.*, 2009). In the absence of 3-oxo-C12-HSL, PAO-JP2 (pKD201-*lasI*) emit only residual low light, however, it produced intense light in the presence of the native ligand. The addition of the artificial sweeteners significantly decreased the light production of the reporter strain. Aspartame (**Figure 4.3**), saccharin **(Figure 4.4**), and sucralose (**Figure 4.5**) show significant inhibitory activity against the bioluminescence emission of PAO-JP2 (pKD201-*lasI*) at the highest concentration tested. In like manner, stevia extract (**Figure 4.6**) and stevioside (**Figure 4.7**) also show significant inhibitory effects on the bioluminescence emission of PAO-JP2 (pKD201-*lasI*) at the highest concentrations tested. As for Reb A (**Figure 4.8**) and steviol (**Figure 4.9**), all the concentrations tested indicated significant anti-QS activity.

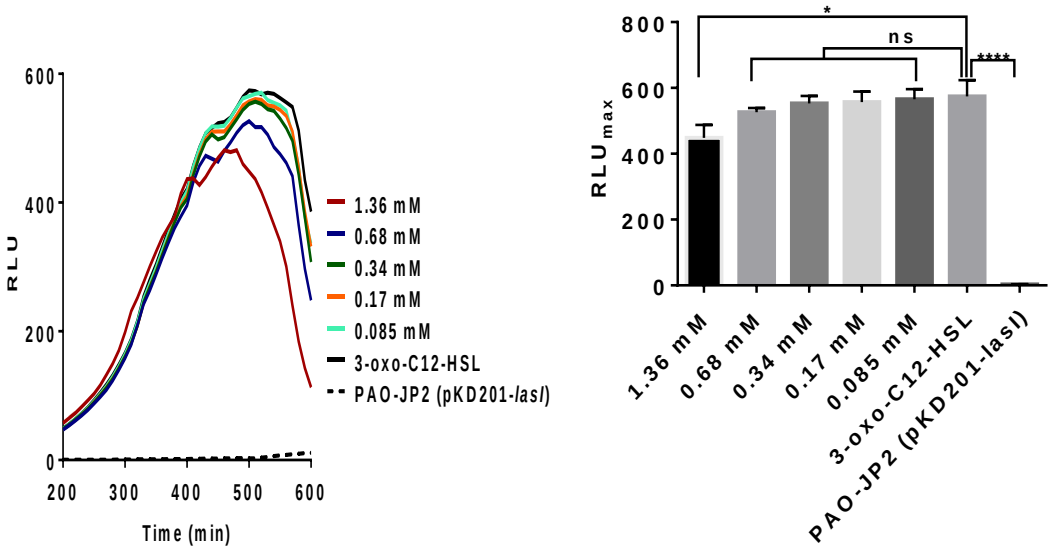

Figure 4. 3. Action of aspartame on PAO-JP2 (pKD201-*lasI*) QS. ***Left panel:*** bioluminescence emission of PAO-JP2 (pKD201-*lasI*) in the presence of aspartame. ***Right panel:*** decrease in bioluminescence emission relative to the control, corresponding to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of 3-oxo-C12-HSL was 100 nM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. * $p <$ 0.05, **** $p < 0.0001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

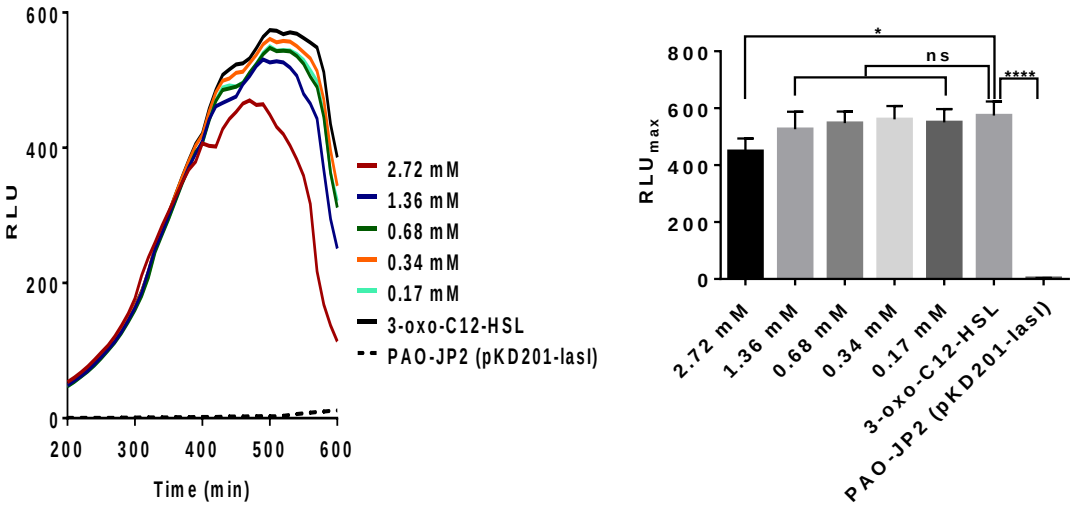

Figure 4. 4. Impact of saccharin on PAO-JP2 (pKD201-*lasI*) QS. ***Left panel:*** Inhibitory action of saccharin on the bioluminescence emission of PAO-JP2 (pKD201-*lasI*). ***Right panel:*** reduction in bioluminescence emission relative to the control, corresponding to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of 3-oxo-C12-HSL was 100 nM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. * $p < 0.05$, **** $p < 0.0001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

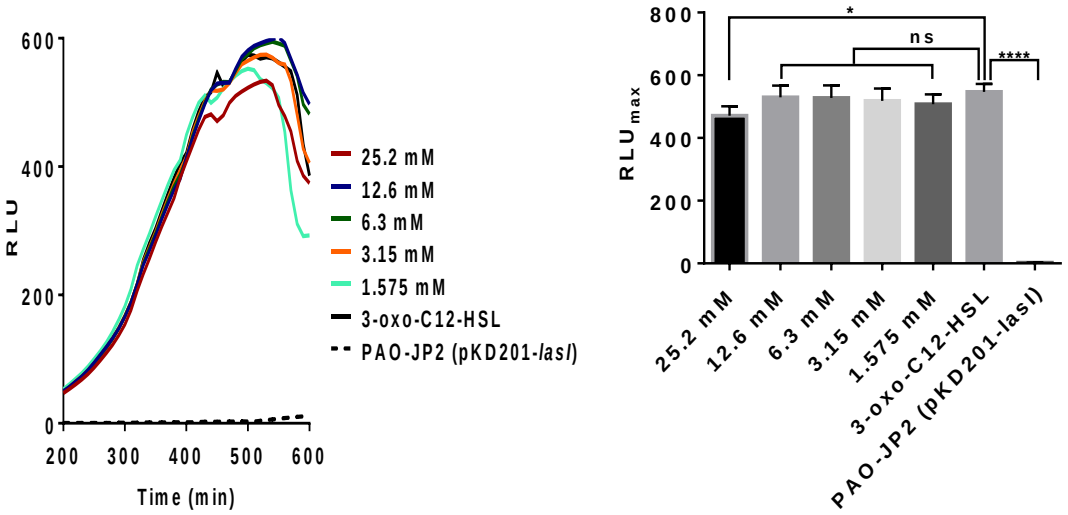

Figure 4. 5. Inhibitory activity of sucralose against PAO-JP2 (pKD201-*lasI*) QS. ***Left panel:*** response of PAO-JP2 (pKD201-*lasI*) reporter strain to sucralose treatment. ***Right panel:*** bioluminescence reduction relative to the control, corresponding to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of 3-oxo-C12-HSL was 100 nM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. * $p < 0.05$, **** $p < 0.0001$, *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

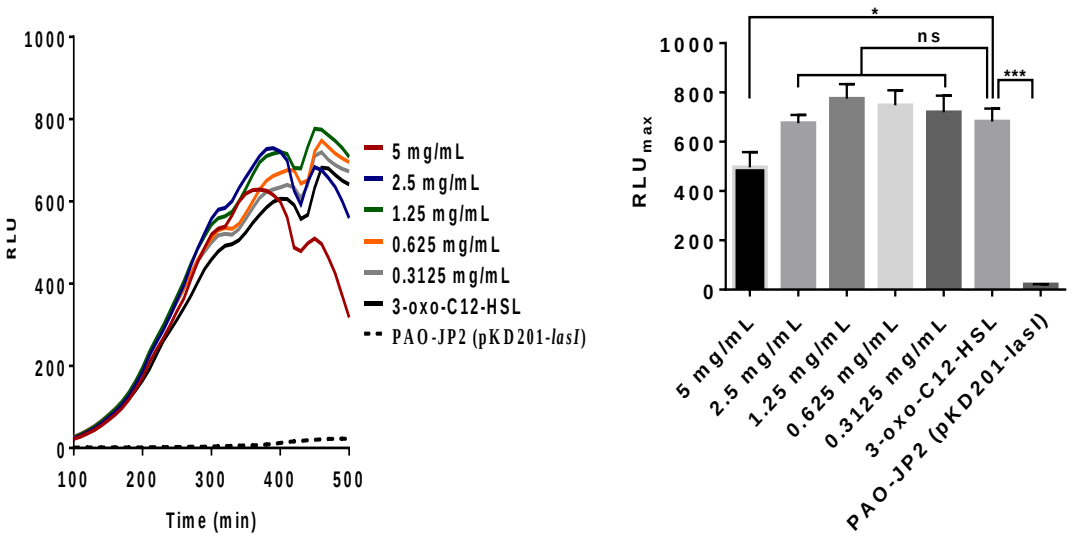

Figure 4. 6. Impact of stevia extract on PAO-JP2 (pKD201-*lasI*) bioluminescence emission. ***Left panel:*** inhibitory effect of stevia extract on the bioluminescence emission of PAO-JP2 (pKD201-*lasI*). ***Right panel:*** decrease in bioluminescence relative to the control, correlating to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of 3-oxo-C12-HSL was 100 nM. Luminescence of PAO-JP2 (pKD201-*lasI*) was expressed as RLU. The statistical method used was student's *t*-test. * $p < 0.05$, *** $p < 0.001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

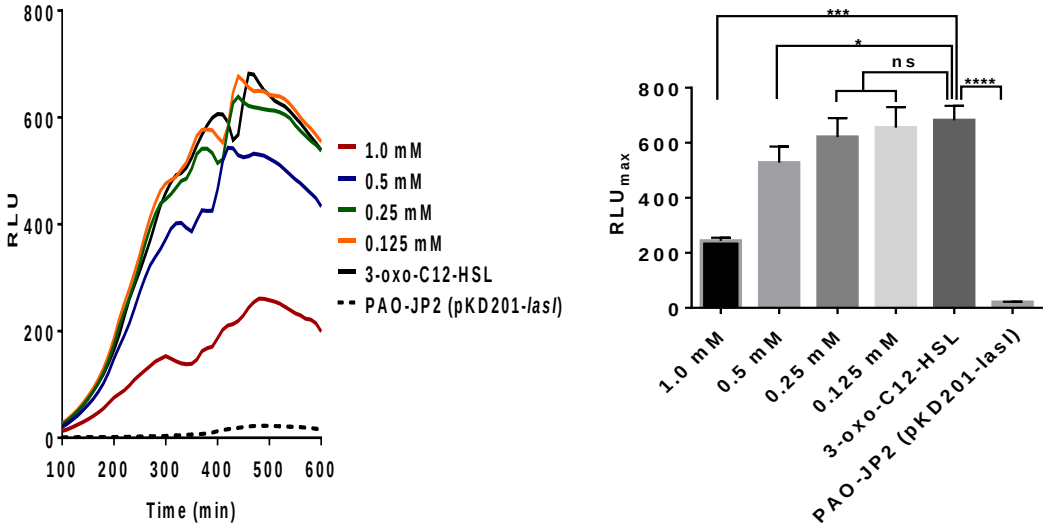

Figure 4. 7. Effects of stevioside on PAO-JP2 (pKD201-*lasI*) reporter strain. ***Left panel:*** bioluminescence emission of PAO-JP2 (pKD201-*lasI*) in the presence of stevioside. ***Right panel:*** decrease in bioluminescence relative to the control corresponding to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of 3-oxo-C12-HSL was 100 nM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

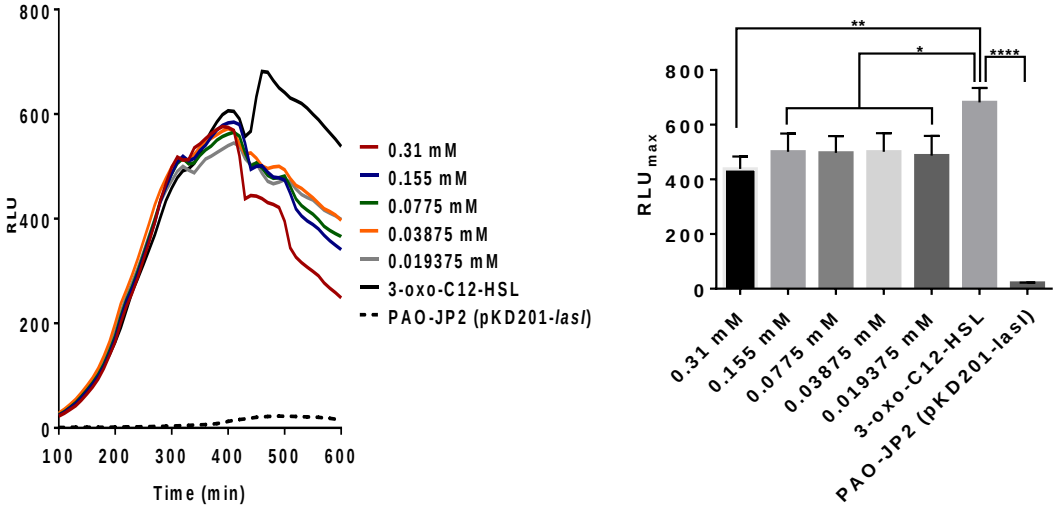

Figure 4. 8. The action of Reb A on PAO-JP2 (pKD201-*lasI*) reporter strain. ***Left panel:*** bioluminescence emission of PAO-JP2 (pKD201-*lasI*) in the presence of Reb A. ***Right panel:*** bioluminescence reduction relative to the control corresponding to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of 3-oxo-C12-HSL was 100 nM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

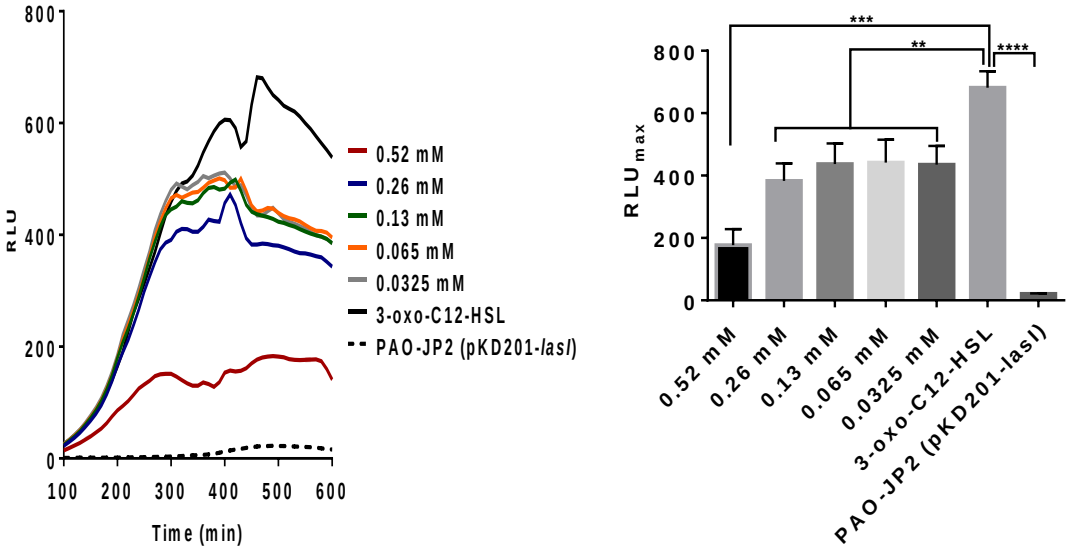

Figure 4. 9. Impact of steviol on PAO-JP2 (pKD201-*lasI*) reporter strain. ***Left panel***: response of PAO-JP2 (pKD201-*lasI*) after steviol treatment. ***Right panel***: bioluminescence reduction relative to the control, corresponding to the data in the right panel. All concentrations presented are the final concentrations. The final concentration of 3-oxo-C12-HSL was 100 nM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

4.3. Action of HIS on RhlR-dependent QS

To further assess the effect of aspartame, saccharin, and sucralose on AHL-mediated QS, PAO-JP2 (pKD-*rhlA*) reporter strain was used. While aspartame (**Figure 4.10)** and saccharin (**Figure 4.11)** appear unable to reduce the bioluminescence emission of PAO-JP2 (pKD-*rhlA*) within the concentrations tested, sucralose (**Figure 4.12**) show significant inhibitory activity at the highest concentration tested. Stevia extract (**Figure 4.13**) and stevioside (**Figure 4.14**) show significant inhibitory activity against PAO-JP2 (pKD-*rhlA*) bioluminescence emission at only the highest concentrations. Reb A (**Figure 4.15**) and steviol (**Figure 4.16**) show significant anti-QS activity at all the concentrations tested.

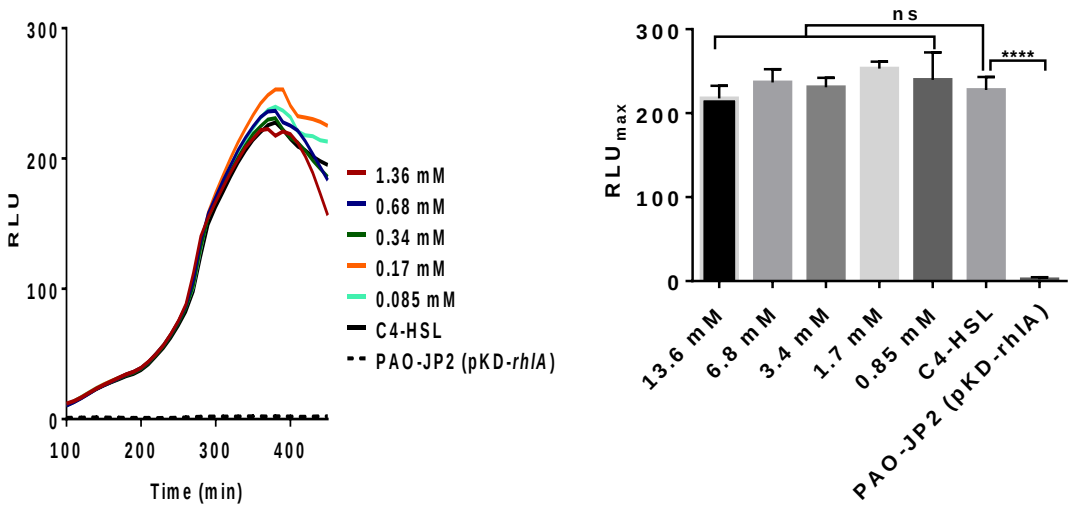

Figure 4. 10. Action of aspartame on PAO-JP2 (pKD-*rhlA*) QS. ***Left panel:*** bioluminescence emission of PAO-JP2 (pKD-*rhlA*) in the presence of aspartame. ***Right panel:*** decrease in bioluminescence emission relative to the control, corresponding to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of C4-HSL was 10 µM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. **** $p < 0.0001$, *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

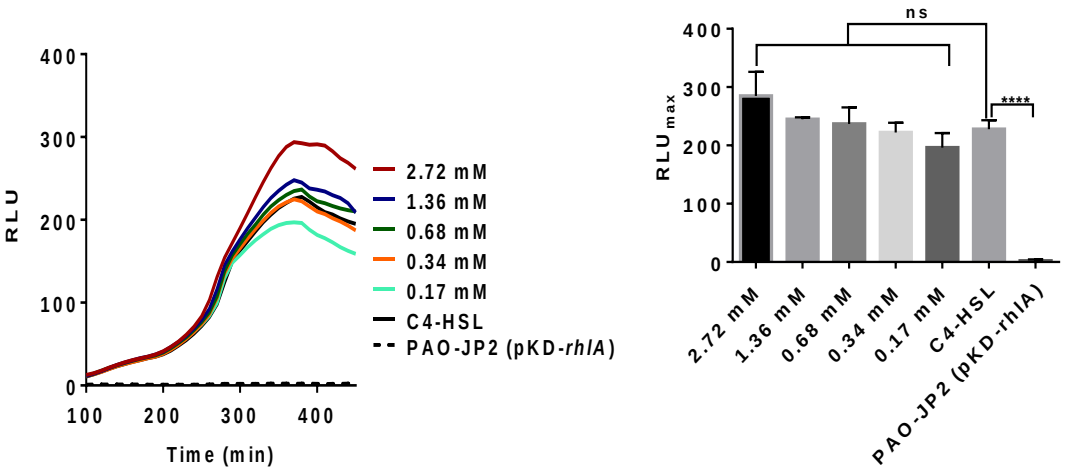

Figure 4. 11. Impact of saccharin on PAO-JP2 (pKD-*rhlA*) QS. ***Left panel:*** response of PAO-JP2 (pKD-*rhlA*) to saccharin treatment. ***Right panel:*** decrease in bioluminescence emission relative to the control, correlating to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of C4-HSL was 10 µM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. **** $p < 0.0001$, *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

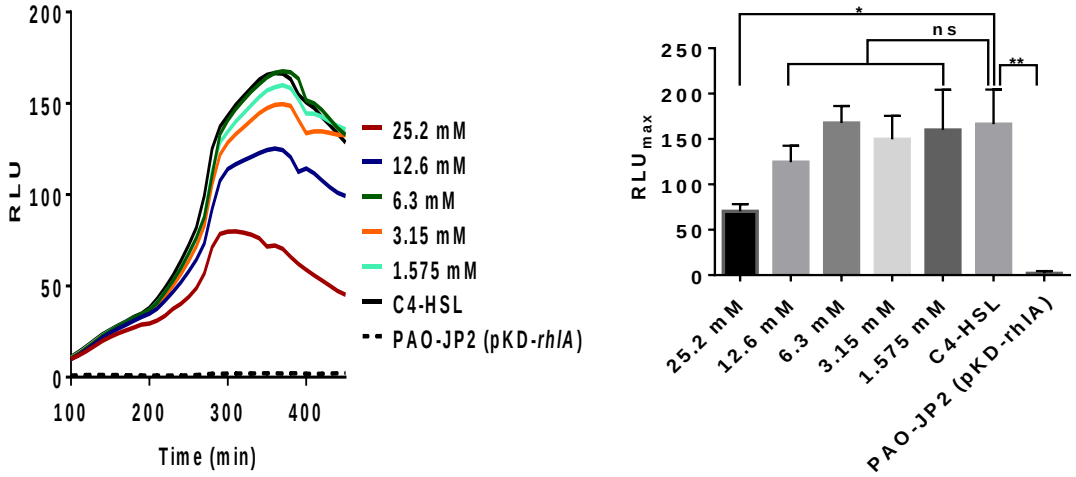

Figure 4. 12. Inhibitory activity of sucralose against PAO-JP2 (pKD-*rhlA*) QS. ***Left panel:*** bioluminescence emission of PAO-JP2 (pKD-*rhlA*) in the presence of sucralose. ***Right panel:*** bioluminescence reduction relative to the control, correlating to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of C4-HSL was 10 µM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

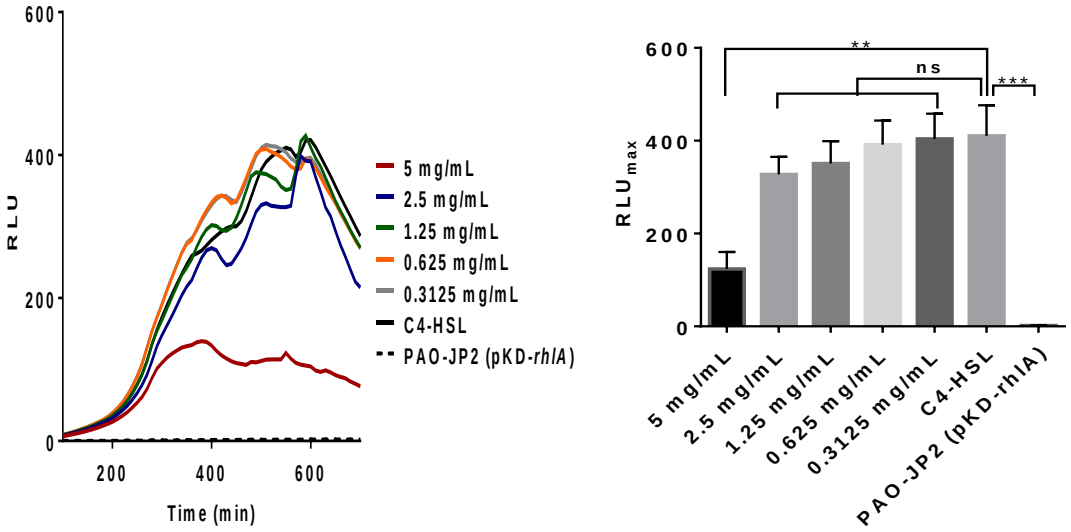

Figure 4. 13. Inhibitory effects of stevia extract on PAO-JP2 (pKD-*rhlA*) reports strain. ***Left panel:*** bioluminescence emission of PAO-JP2 (pKD-*rhlA*) in the presence of stevia extract. ***Right panel:*** reduction in bioluminescence emission relative to the control, correlating to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of C4-HSL was 10 µM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. ** $p < 0.01$, *** $p < 0.001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

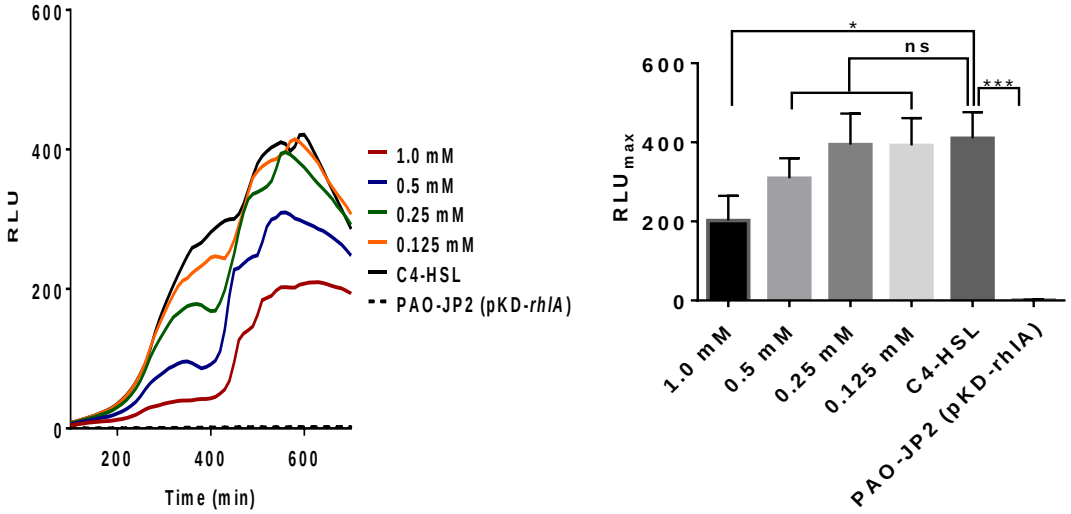

Figure 4. 14. The action of stevioside on PAO-JP2 (pKD-*rhlA*) reporter strain. ***Left panel:*** bioluminescence emission of PAO-JP2 (pKD-*rhlA*) in the presence of stevioside. ***Right panel:*** decrease in bioluminescence relative to the control, correlating to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of C4-HSL was 10 µM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. * $p < 0.05$, *** $p < 0.001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

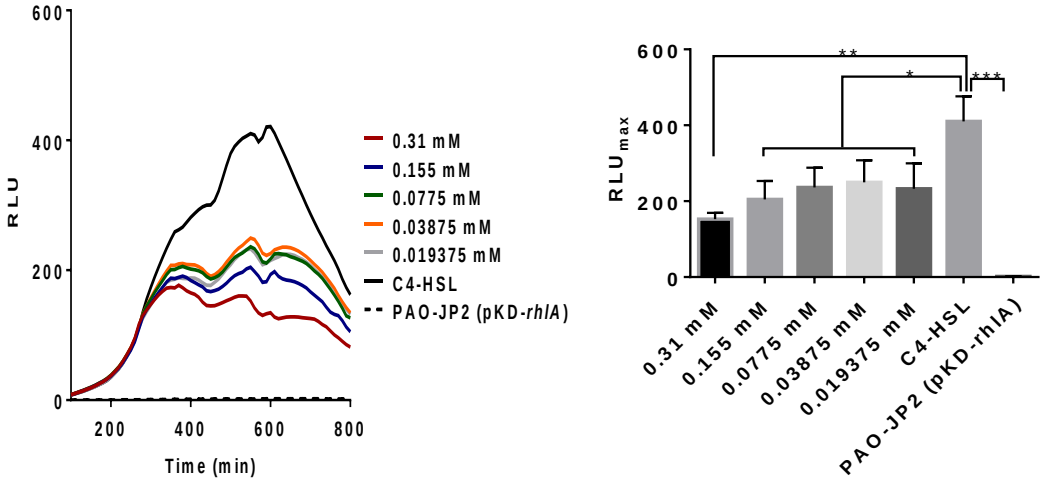

Figure 4. 15. The inhibitory impact of Reb A on PAO-JP2 (pKD-*rhlA*) reporter strain. ***Left panel:*** bioluminescence emission of PAO-JP2 (pKD-*rhlA*) in the presence of Reb A. ***Right panel:*** reduction in bioluminescence emission relative to the control, corresponding to the data in the left panel. All concentrations presented are the final concentrations. The final concentration of C4-HSL was 10 µM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

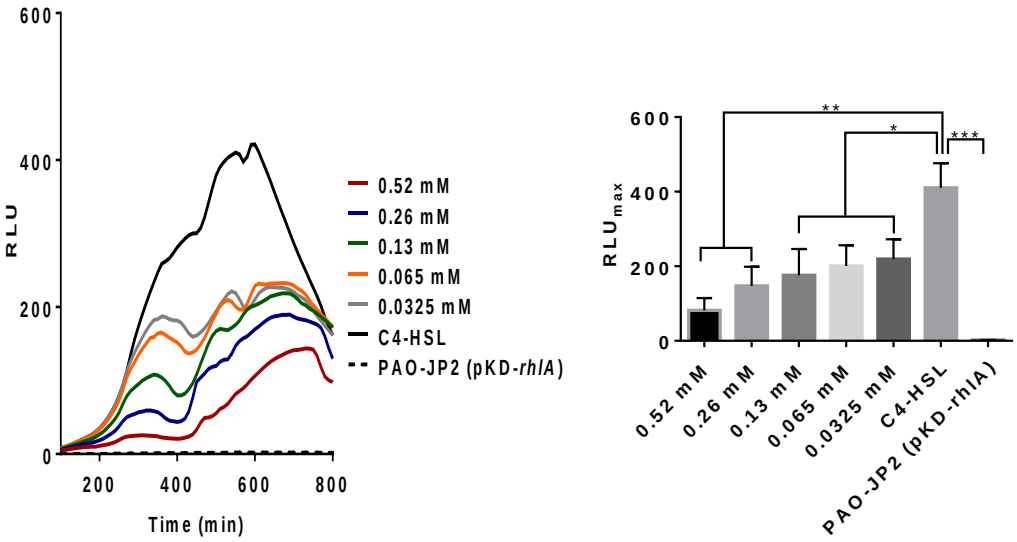

Figure 4. 16. Response of PAO-JP2 (pKD-*rhlA*) reporter strain to steviol treatment. ***Left panel:*** bioluminescence emission of PAO-JP2 (pKD-*rhlA*) in the presence of steviol. ***Right panel:*** bioluminescence reduction relative to the control, corresponding to the data in the left panel. The final concentration of C4-HSL was 10 µM. Luminescence was expressed as RLU. The statistical method used was student's *t*-test. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$, and *ns* not significant. Values represent mean ± SD, $n = 3$ (three experimental readings).

4.4. Effect of HIS on LasR solubility

LasR protein was expressed in *E. coli* BL21 (Amara *et al.*, 2009) in the presence of the 3-oxo-C12-HSL and/or the artificial sweeteners (aspartame, saccharin**,** and sucralose). After the expression of the LasR protein, it was purified using nickel-affinity chromatography. Six histidine amino acids (His_6) attached at the end of the LasR construct (Amara *et al.*, 2009, 2016) binds to nickel which has been linked to agarose bead via nitriloacetic acid (NTA). Using a low concentration of imidazole in phosphate buffer, the low affinity-bound proteins were first removed. Finally, a phosphate buffer containing a higher concentration of imidazole was used to elute the protein from the Ni-NTA beads. The purified protein was monitored by SDS–PAGE and western blotting. The negative control, containing only 0.15% (v/v) DMSO, showed no LasR protein in the supernatant. The positive control, containing 7.5 μM 3-oxo-C12-HSL, indicated significant expression of the protein. The addition of aspartame (**Figure 4.17**), saccharin (**Figure 4.18**), and sucralose (**Figure 4.19**) decreased the expression of LasR significantly in all the concentrations tested relative to the positive control, suggesting that the solubility of the protein was decreased.

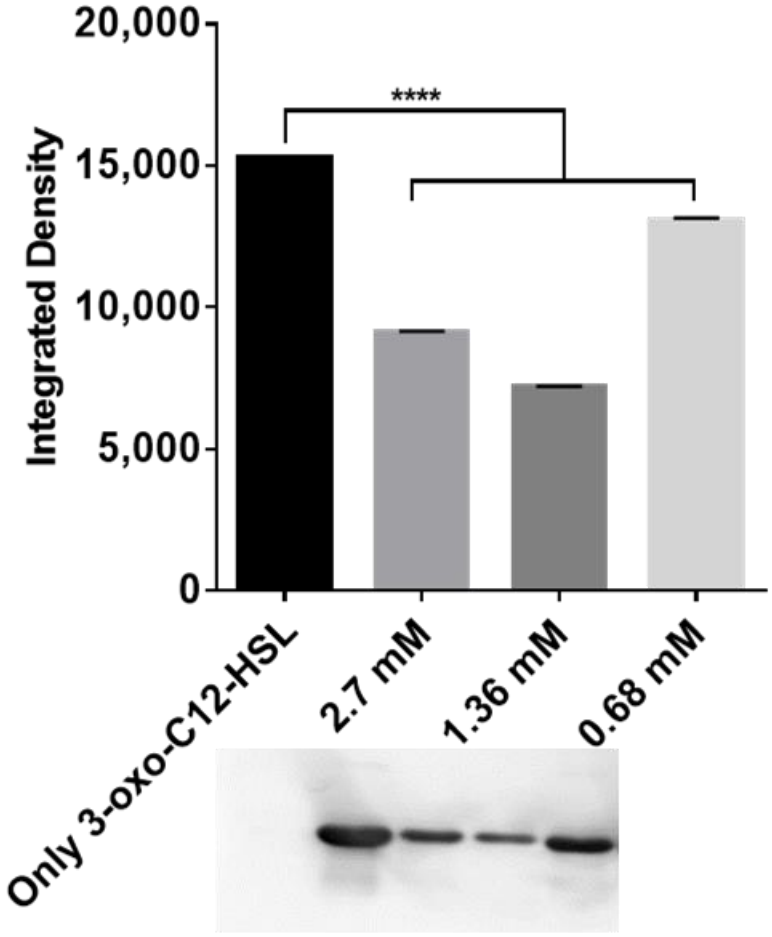

Figure 4. 17. The western blotting of LasR-LBD protein expressed in *E. coli* BL21 strain in presence of a different concentration of aspartame. All concentrations presented are final concentrations. The final concentration of 3-oxo-C12-HSL was 100 nM. Integrated density is the sum of the values of the pixels within a band, determined using ImageJ software (Schindelin *et al.*, 2012). The statistical method used was student's *t*-test. **** $p < 0.0001$. Values represent mean ± SD, $n = 3$.

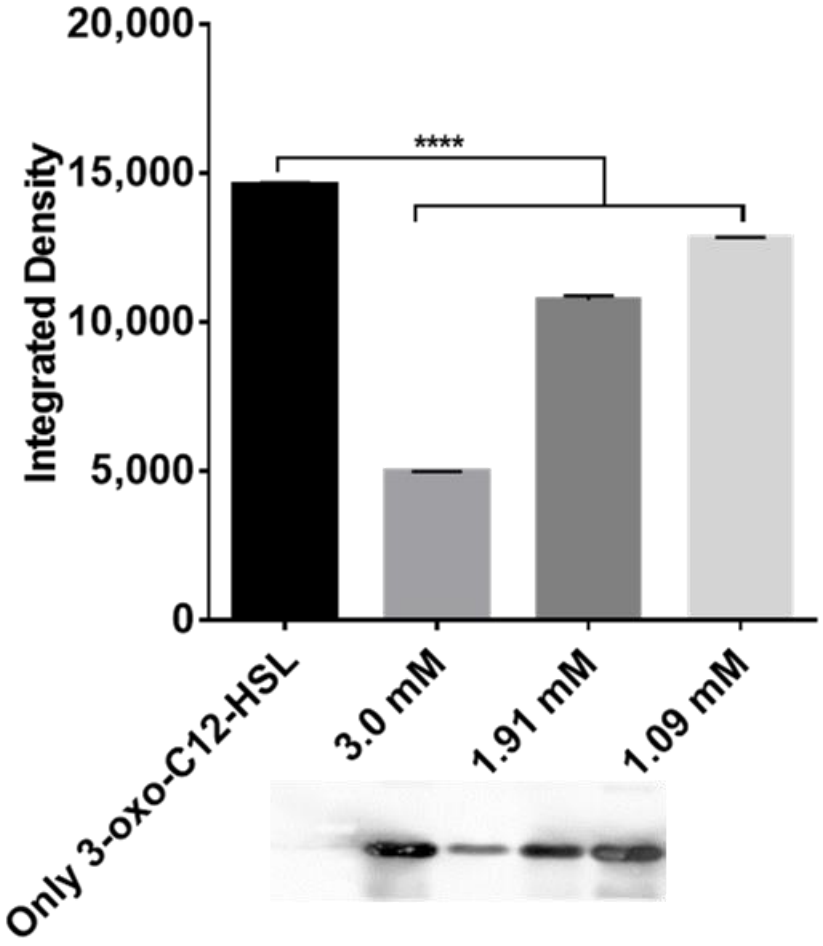

Figure 4. 18. The western blotting of LasR-LBD protein expressed in *E. coli* BL21 strain in the presence of different concentrations of saccharin. All concentrations presented are final concentrations. The final concentration of 3-oxo-C12-HSL was 100 nM. Integrated density is the sum of the values of the pixels within a band, determined using ImageJ software (Schindelin *et al.*, 2012). The statistical method used was student's *t*-test. **** $p < 0.0001$. Values represent mean ± SD, $n = 3$.

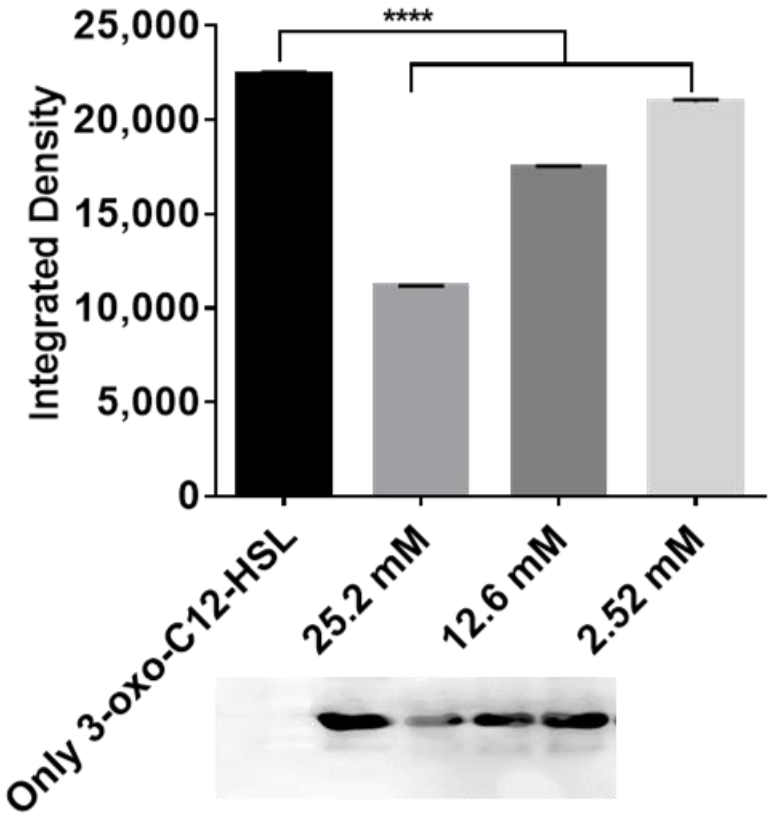

Figure 4. 19. The western blotting of LasR-LBD protein expressed in *E. coli* BL21 strain in the presence of different concentrations of sucralose. All concentrations presented are final concentrations. The final concentration of 3-oxo-C12-HSL was 100 nM. Integrated density is the sum of the values of the pixels within a band, determined using ImageJ software (Schindelin *et al.*, 2012). The statistical method used was student's *t*-test. **** $p < 0.0001$. Values represent mean ± SD, $n = 3$.

Chapter five

5. DISCUSSION

Toxicological evaluations of substances, in most cases, are undertaken using animal models and then human trials. These evaluations are carried out across different and wide ranges of exposure to understand the toxicity or potential toxicity of substances. Regarding the products already on the market, epidemiological studies and case reports provide evidence for toxicological manifestations of these products. In recent years, concerns regarding the metabolic effect of HIS have led to new evaluations of the data. This evaluation has been improved because of several advances in analytical methods including chromatographic techniques (Kokotou *et al*., 2012; Lange *et al*., 2012) that are used for the determination of the presence or concentration of HIS in samples. However, there are some important drawbacks associated with these methods including cost and time consumption. This, therefore, has placed a demand for simple, cheap, and fast methods to improve toxicological evaluations. Advances in genetic engineering including the development of recombinant bioluminescent bacteria, which produce light when in contact with a toxic substance, are being used as sensing models to represent systems of microbial complexity (Eltzov *et al*., 2008; Harpaz *et al*., 2018). Indeed, the use of these engineered microorganisms as bioreporters for monitoring toxic substances in food, biological specimens, water, soil, and air has gained recent attention due to the relatively simple, rapid, and cheap assay they provide (Van Der Meer & Belkin, 2010).

In this study, recombinant *P. aeruginosa* strains, PAO-JP2 (pKD201-*lasI*) and PAO-JP2 (pKD-*rhlA*), and *Escherichia coli* BL2, were used. The *P. aeruginosa* model was used because of its AHL-mediated pathways, employing the signaling molecules AHLs that have similarities in structure and function to 3-oxo-C12:2-HSL (the AHL directly linked to normobiosis, but not yet commercially available) (Aguanno *et al*., 2019, 2020; Cecilia Landman *et al*., 2013, 2015). *E. coli* is a member of the normal gut microbial community and an important barrier against enteropathogens (Hudault *et al*., 2001). The recombinant strain, *E. coli* BL21, is designed for expressing LasR

protein (Amara *et al*., 2009). Importantly, both *P. aeruginosa* and *E. coli* do not hydrolyse HIS (Buerge *et al*., 2009; Gardana *et al*., 2003; Lethco & Wallace, 1975; Roberts *et al*., 2000; Suez *et al*., 2014). PAO-JP2 (pKD201-*lasI*) and PAO-JP2 (pKD-*rhlA*) express LasR and RhlR respectively (Duan & Surette, 2007) that form complexes with 3-oxo-C12-HSL and C4-HSL (LasR:3-oxo-C12-HSL and RhlR:C4-HSL). The complexes form regulatory assemblies that drive the transcription of the target QS genes (Mukherjee and Bassler, 2019) including the *luxCDABE* gene cluster which serves as the transcriptional reporter (Craney *et al.,* 2007; Bukelman *et al.,* 2009). The maximum bioluminescence emissions of PAO-JP2 (pKD201-*lasI*) and PAO-JP2 (pKD-*rhlA*) were induced at 100 nM 3-oxo-C12-HSL (**Figure 4.1)** and 10 µM C4-HSL (**Figure 4.2)** respectively, which were significantly reduced by HIS. While aspartame (**Figure 4.3**), saccharin **(Figure 4.4**), sucralose (**Figure 4.5**), stevia extract (**Figure 4.6**), and stevioside (**Figure 4.7**) caused significant inhibitory action on the bioluminescence emission of PAO-JP2 (pKD201-*lasI*) at only the highest concentration tested, Reb A (**Figure 4.8**) and steviol (**Figure 4.9**) indicated significant anti-QS activity at all the concentrations tested. The effects of HIS on PAO-JP2 (pKD-*rhlA*) appear not to have a similar pattern as observed with PAO-JP2 (pKD201-*lasI*). Although aspartame (**Figure 4.10)** and saccharin (**Figure 4.11)** were ineffective in decreasing the bioluminescence emission of PAO-JP2 (pKD-*rhlA*), there were significant inhibitory actions by sucralose (**Figure 4.12**), stevia extract (**Figure 4.13**), and stevioside (**Figure 4.14**) at the highest concentrations of the agents. Reb A (**Figure 4.15**) and steviol (**Figure 4.16**) indicated significant anti-QS activity against PAO-JP2 (pKD-*rhlA*) at all the concentrations tested. These results suggest that the artificial sweeteners exert their inhibitory effect prominently via the LasR-mediated QS system, except sucralose that indicated an additional inhibitory impact on the RhlR-mediated QS system. On the other hand, all the components of the natural sweetener stevia exert significant inhibitory effects on both the LasR- and RhlR-mediated QS systems. HIS may have interfered with the LasR:3-oxo-C12-HSL and RhlR:C4-HSL binding to interrupt the transcription of QS target genes including the transcriptional reporter, *luxCDABE* gene cluster, in PAO-JP2 (pKD201-*lasI*) and PAO-JP2 (pKD-*rhlA*).

To further clarify the mechanism of action of HIS, LasR protein was expressed in *E. coli* BL21-pETM-11 strain (Amara *et al*., 2009, 2016) in the presence of aspartame,

sucralose, saccharin. The components of the natural sweetener stevia were not used here due to a lack of sufficient samples. Additionally, RhlR proved difficult to purify, possibly indicating why there is yet to be a crystal structure for the protein. Studies indicated that LasR and related proteins remain insoluble in the absence of the native ligand but fold and become soluble upon the addition of their native ligands (McCready *et al*., 2019; Paczkowski *et al*., 2017; Pinto & Winans, 2009; M. Schuster *et al*., 2004; Zhang *et al*., 2002; J. Zhu *et al*., 1998; J. Zhu & Winans, 2001). As expected, without any ligand except 0.15% DMSO, no LasR protein was expressed. But upon the addition of 3-oxo-C12-HSL, there was a significant expression of the LasR protein (**Figure 4.17, 4.18, and 4.19**). However, the protein expression was significantly decreased following the introduction of artificial sweeteners. Aspartame (**Figure 4.17**), saccharin (**Figure 4.18**), and sucralose (**Figure 4.19**) significantly decreased the expression of the protein relative to the control, suggesting a decrease in solubility of the protein. It has been suggested that LasR:3-oxo-C12-HSL binding is ultra-tight (M. Schuster *et al*., 2004) and important for the protein's folding, solubility, and stability (Bottomley *et al.*, 2007). It appears that the artificial sweeteners may have disrupted the LasR:3-oxo-C12-HSL binding to decrease the protein's solubility.

Since the AHL signaling molecules used in the present study has both structural and functional similarities to the molecule (3-oxo-C12:2-HSL) associated with normobiosis (Cecilia Landman *et al.*, 2015; Le Balc'h *et al*., 2017), the observations in this study are likely to reflect what may occur in the gut microbial community exposed to these HIS. 3-Oxo-C12:2-HSL was reported to protect the enterocytes and enhance the activities of Firmicutes (Aguanno *et al*., 2019, 2020; Cécilia Landman *et al*., 2018; Le Balc'h *et al*., 2017). *Faecalibacterium prausnitzii*, a member of the Firmicutes group, has been reported to exert anti-inflammatory activity through the activation of NF-κB and stimulation of IL-8 synthesis (Lopetuso *et al*., 2016). The important role of AHL signaling in normobiosis and protection of enterocytes' integrity suggest that alteration in the AHL-based communication network may have a far-reaching consequence. Current evidence shows that IBD active patients have significantly decreased levels of 3-oxo-C12:2-HSL molecules (16%) relative to the healthy individuals (65.4%) (Cécilia Landman *et al*., 2018). Additionally, IBD has been known to be associated with dysbiosis, characterized by a marked reduction in

specific taxonomic groups including Firmicutes and Bacteroidetes (Lane *et al*., 2017; Lopetuso *et al*., 2016). In healthy individuals, >90% of the gut microbial species belong to the Firmicutes and Bacteroidetes phyla (Lane *et al*., 2017). This data indicates that Firmicutes and Bacteroidetes are the main phyla of the gut microbial community, and the disruption of this composition in an event of considerable consumption of HIS may induce disease conditions. Although current data associates Firmicutes to 3-oxo-C12:2-HSL, there is no evidence yet showing Firmicutes are responsible for the synthesis of 3-oxo-C12:2-HSL molecules. Perhaps 3-oxo-C12:2-HSL could be better considered as a marker for normobiosis than directly being produced by Firmicutes(Cécilia Landman *et al*., 2018).

Chapter six

6. CONCLUSION

In the present study, the anti-QS activity of HIS was evaluated using a bioluminescence assay as well as biophysical protein characterization methods. The results show that aspartame and saccharin exert significant anti-QS action via the LasR-mediated system, and sucralose displayed anti-QS activity both through the LasR- and RhlR-dependent pathways. As for the natural sweeteners, all the components tested (stevia extract, stevioside, Reb A, and steviol) had significant anti-QS activity via both the LasR- and RhlR-mediated systems. The anti-QS activity of these HIS is proposed to be through the disruption of LasR:3-oxo-C12:2-HSL and RhlR:C4-HSL binding, thus interrupting the transcription of the QS target genes including transcriptional reporter, *luxCDABE* gene cluster, engineered into the bacterial models. The decrease in the solubility of the LasR protein expressed in presence of the artificial sweeteners supports the proposition that the agents' anti-QS activity could be via the interference of the binding between regulatory proteins and native ligands. The observations in the study, therefore, may explain the molecular events that might occur in the gut microbiota exposure to HIS. Since AHL-mediated bacterial communication has a role in normobiosis and the protection of enterocyte integrity, considerable consumption of HIS may likely have far-reaching consequences.

APPENDIX

APPENDIX 1

SIMILARITY REPORT

PhD Thesis

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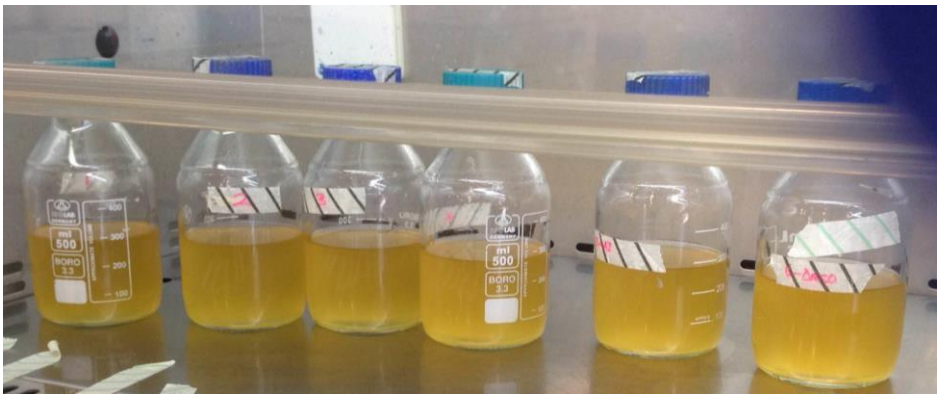
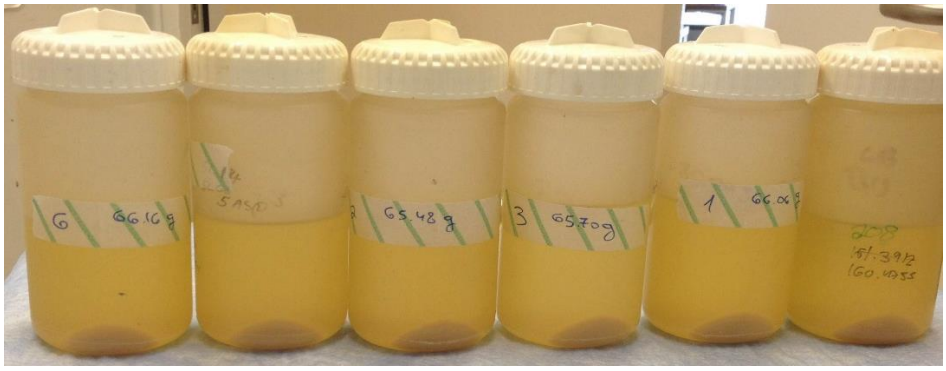
Appendix 2

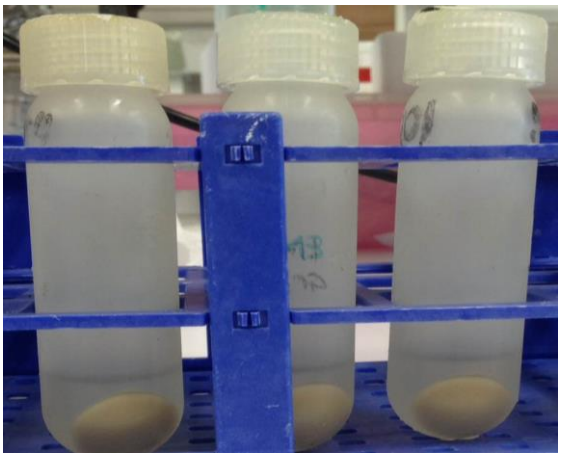
PROTEIN EXPRESSION



Starter culture of *E. coli* BL21-pETM-11



Cell pellets, obtained by centrifugation after IPGT-induced protein expression.

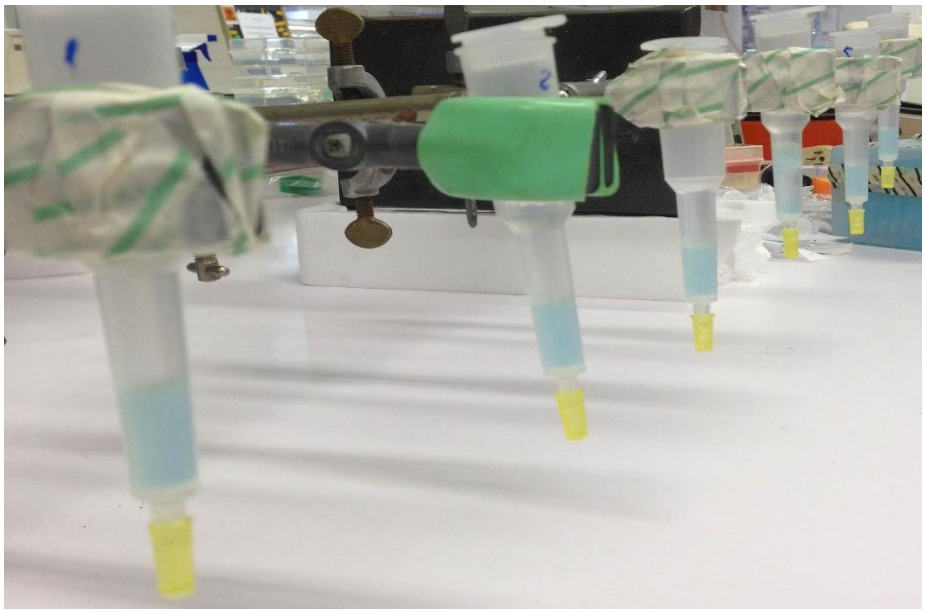


Soluble protein fraction at the top and cellular debris at the bottom of the tubes, obtained by centrifugation after cell lysis.

Appendix 3

PROTEIN PURIFICATION USING NICKEL-AFFINITY CHROMATOGRAPHY

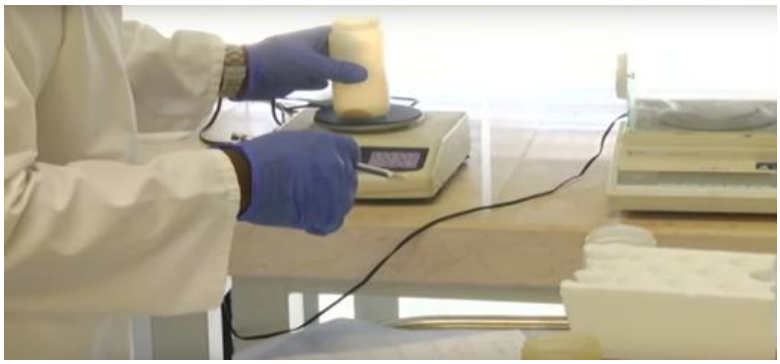
Improvised chromatography column holders



Nickel-affinity chromatography columns set up. Ni-NTA slurry is shown at the bottom of the columns in light blue.

Appendix 4

Observing an experiment



Weighing cell pellets

Incubation of cell pellets (suspended in lysis buffer) on ice before sonication

Appendix 5

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Anti-Quorum Sensing Activity of Stevia Extract, Stevioside, Rebaudioside A and Their Aglycon Steviol

by Victor Markus [1,2], Orr Share [2], Kerem Teralı [1], Nazmi Ozer [3], Robert S. Marks [2,4], Ariel Kushmaro [2,4,*] and Karina Golberg [2,*]

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Open Access Article

Assessing the Molecular Targets and Mode of Action of Furanone C-30 on *Pseudomonas aeruginosa* Quorum Sensing

by Victor Markus [1,2,†], Karina Golberg [2,†], Kerem Teralı [1], Nazmi Ozer [3], Esti Kramarsky-Winter [2], Robert S. Marks [2,4] and Ariel Kushmaro [2,4,*]

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Academic Editors: Ana Paula Duarte, Ângelo Luís and Eugenia Gallardo

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Appendix 6

CURICULUM VITAE

Personal Information

Surname and name: Markus Victor.
Date of Birth: 20th October 1985.
Nationality: Nigeria.
Marital status: Married.
Mobile: +905338630410.
Email: Official- victor.markus@neu.edu.tr.

Education

B.Sc. 2007-2012

Kaduna State University, Biochemistry.
Project Supervisor: Professor M.S ABDULSALAMI, PhD.
Project Title: "Effect of processing on the anti-nutritional factors in the seed kernel of *Thevetia peruviana."*

M.Sc. 2016-2017

Near East University, Medical Biochemistry.
Thesis Advisor/Supervisor: Professor Nazmi ÖZER, PhD
Thesis Title: "Human Placental Glutathione Transferase P1-1 (*hp*GSTP1-1): Inhibitory Activity and Molecular Docking Studies of Deltamethrin."

Res. Internship 2019-2020

Ben-Gurion University of the Negev, Biotechnology Engineering.
Advisors/Supervisors: Professor Ariel KUSHMARO, and Professor Robert S. MARKS
Research: Mode of action and molecular targets of quorum sensing inhibitors.

Ph.D. 2017-2021

Near East University, Medical Biochemistry.
Advisor/Supervisor**:** Associate Professor Kerem TERALI, PhD.
Dissertation Title: "Anti-Quorum Sensing Activity of High-Intensity Sweeteners."

Employment History

2016-present	Assistant, Department of Medical Biochemistry, Faculty of Medicine, Near East University, Cyprus.
2012-2013	Assistant, Department of Biochemistry, University of Uyo, Nigeria
2011-2012	Assistant, Dr. Timothy Bulus Animal Research Farm, Kaduna State University, Nigeria.
2011-2012	Biology and Chemistry Teacher, Emotan College Kaduna, Nigeria
2010-2011	Chemistry Laboratory Analyst of Quality Control Unit, ECWA Central Pharmacy, Nigeria.

Educational Activities

1. Teaching biochemistry to undergraduate

i. Molecular Biology and Genetics (MBG) students, Near East University, Nicosia Cyprus (2018-present): Introduction to biochemistry, Chemistry of Carbohydrates, Chemistry of Proteins, Chemistry of Nucleic Acid, Chemistry of Lipids, Biochemical Energetics, Enzymes, Carbohydrate Metabolism and Cellular Respiration.

ii. Medical students, Near East University, Nicosia Cyprus (2016-present): Biochemistry laboratory

2. Assisting in the teaching of the following courses

a. Research Techniques in Biochemistry – M.Sc. Course, 2017-2019 (Course taught by Professor Nazmi ÖZER, Near East University).

b. Biochemistry Laboratory – Undergraduate (University of Uyo, Nigeria, 2012-2013).

3. Teaching in Secondary School
Biology and Chemistry Teacher **(**Emotan College Kaduna, 2011-2012).

4. Position in academic administration
2019-present Member, Faculty of Medicine Web Portal Team, Near East University.

Memberships of Professional/Scientific Societies

2013-2014 Biotechnology Society of Nigeria (BSN).

Awards, Honors, Scholarships

2019 PhD Sandwich scholarship (Ginsburg Ingerman Overseas Students Program) Ben-Gurion University of the Negev, by the Israeli Council of Higher Education (ICHE).
2017 Near East University Full PhD Scholarship Award, Cyprus.
2014 Kaduna State Government of Nigeria Overseas Scholarship Award, Nigeria (MSc.).
2011 Faculty of Science Best Student Award, Kaduna State University, Nigeria, (BSc., 2012).
2010 Kaduna State University Senate Award of Excellent, Nigeria (BSc., first to final year).
2008 Shell Nigeria University Scholarship Award, Nigeria (BSc.).
2008 Kaduna State Government of Nigeria Scholarship Award, Nigeria (BSc. first to final year).

Peer review experience

Journal of Pharmaceutical Research International

Publications

A. Refereed Articles in Scientific Journals

1. **Markus, V**.; Golberg, K.; Teralı, K.; Ozer, N.; Kramarsky-Winter, E.; S. Marks, R.; Kushmaro, A. (2021). Assessing the Molecular Targets and Mode

of Action of Furanone C-30 on *Pseudomonas aeruginosa* Quorum Sensing. *Molecules*, *26*, 1620. https://doi.org/10.3390/molecules26061620.

2. **Markus, V.**, Share, O., Teralı, K., Ozer, N., Marks, R.S., Kushmaro, A., Golberg, K. (2020). Anti-Quorum Sensing Activity of Stevia Extract, Stevioside, Rebaudioside A, and Their Aglycon Steviol. *Molecules, 25,* 5480. https://doi.org/10.3390/molecules25225480.
3. Luka Tambaya, Magdalene Victor and **Victor Markus.** (2020). Level of Awareness of the Havoc and Consequences of Academic Dishonesty among College of Education Students in Nigeria. *American Journal of Educational Research*, 8(3),168-172. https://doi.org/10.12691/education-8-3-7.
4. **Markus, V.,** Teralı, K., Dalmizrak, O., Ozer, N., (2018). Assessment of the inhibitory activity of the pyrethroid pesticide deltamethrin against human placental glutathione transferase P1-1: A combined kinetic and docking study. *Environmental Toxicology and Pharmacology,* 61, 18–23. https://doi.org/10.1016/j.etap.2018.05.013.
5. Agada, E.M., **Markus, V.,** Bwankwot, T.M., Mohammed, I.D (2018). Low-Cost Multi-nutrient Blocks Produced from Locally Sourced Ingredients for Small Agro-Pastoral Farmers in the Sahel Zone of West Africa. *Journal of Biology, Agriculture, and Healthcare.* 8 (22), 30-34.
6. **Markus Victor**, Paul Abraham Abbey, Yahaya Joseph, Zakka Jonathan, Yatai Kenneth Bobai, Oladeji Maria (2016). An Underexploited Tropical Plant with Promising Economic Value and the Window of Opportunities for Researchers: *Cnidoscolus aconitifolius*. *American Journal of Food Science and Nutrition Research*, 3(6), 177-187.
7. Zakka Jonathan, **Markus Victor,** Paul Abraham, Tanta Emmanuel, and Jonathan Bege **(**2015). Biosurfactants Production Potential Of Native Hydrocarbon-Degrading Bacteria Strains in Refinery Effluent Collected From Kaduna Refinery and Petrochemical Company Nigeria**.** *International journal of innovation sciences and research,* 4 (9), 449-452.
8. Jonathan Bege, **Markus Victor,** Kure Mock Samuel, and Jamilu Sani. (2015). Some Hepatic Function indices in *Trypanosoma brucei brucei* Rats Treated with Aqueous Extract of *Mitrcarpus scaber*. *Internation Journal of Chemical and Biological Sciences*, 1(12): 21-26.
9. Jonathan Bege, **Markus Victor** and Gaiya Daniel (2015). Investigating Lipase Activity in Ungerminated *Colocynthis Citrullus lanatus* (Egusi Melon) Seeds. *Scientific Research Journal*, 3(2): 35-38.
10. **Markus, V.,** Abdulsalami, M.S., Mustapha, M., Igwilo I. and Gnimintakpa, J. (2014). Extractability of *Thevetia peruviana* Glycoside using Various Organic Solvents. *Journal of Biology, Agriculture and Healthcare*, 4 (17): 143-147.
11. **Markus, V.,** Abdulsalami, M.S., Mustapha, M. and Abdulgamiyu, A. (2014). Effect of Processing on the *Thevetia peruviana* Glycoside. *The Bioscientist*, 2 (1): 30-36

B. Published Conference Abstracts

Markus, V., Abdulsalami, M.S., Mustapha, M. and Abdulgamiyu, A. (2014). Comparative Studies of *Thevetia peruviana* Glycoside Extractability of Seven Organic Solvents. Biotechnology Society of Nigeria (BSN) 27th National Conference, Kaduna Nigeria.

C. Books:

Academic

Mohammed Sani Abdulsalami and **Victor Markus (**2016). Effect of Processing on the Anti-Nutrients in *Thevetia peruviana* seed. Lambert Academic Publishing: Germany ISBN: 978-3-659-82959-8.

Inspirational

Markus, V. (2014). Breaking the Shackles of Failure, Proven Principles and Strategies for Academic Success, Impact Empire: Nigeria ISBN: 978-978-52965-8-7.

Professional Training/ Conferences Attended

1. 1st International Scientific Conference on Biotechnology & Genetics Developments and Future Challenges (BGIC) organized by Academy of Sciences of Albania, Albania, 2021.
2. 1st International Conference on Water Environmental Protection and Sustainable Development (WEPSD) organized by Academy of Sciences of Albania, Albania -2021.
3. 2nd International Conference on "Water Problems in the Mediterranean Countries," Near East University North Cyprus, 2019.
4. Experimental Animal Model Course: from Gene to Function" organized by the Research Centre of Experimental Health Sciences, Near East University North Cyprus, 2019.
5. Bioinformatics Fall School: Applications in Molecular Basics and Clinical Services, organized by the Research Centre of Experimental Health Sciences, Near East University North Cyprus, 2018.
6. 9th National and 2nd International Congress of Hydatidology, organized by Turkish Association of Hydatidology in collaboration with Near East University, 2018.
7. Seminar on "The effects of microenvironment on pluripotency and differentiation," organized by Department of Medical Genetics, Faculty of Medicine, Near East University, Nicosia, Cyprus, 2016.
8. Seminar on "Development of an autoimmune-mediated strategy for bladder cancer vaccination in mice," organized by Department of Medical Genetics, Faculty of Medicine, Near East University, Nicosia, Cyprus, 2016.
9. 27th Biotechnology Conference on Biotechnology: A Critical Tool for Achieving Food Security, Affordable Healthcare Delivery and rapid Industrialization, organized by Biotechnology Society of Nigeria (BSN), 2014.
10. 32nd Biochemistry and Molecular Biology Conference on Biochemistry and Molecular Biology: Tools for achieving Millennium Development Goals (MDGs), organized by Nigerian Society of Biochemistry and Molecular Biology (NSBMB), 2012.
11. Entrepreneurship Education Workshop organized by International Council on Economic Education (ICEE) Altadena, California, 2011.
12. Entrepreneurial Capacity Building Summit, organized by Choice Concept Network Group (CCNG), Nigeria, 2007.

Analytical Software Skills

IBM-SPSS, PSPP, Jamovi, AMOS, ImageJ, GraphPad, Microsoft office, MedChem Designer.

Laboratory Techniques and Instrumentation

In addition to the basic laboratory skills, I am good with the following techniques/instrumentations: Spectrophotometric techniques, Chromatographic techniques, Electrophoresis techniques, Western Blotting, Bacteria culturing techniques, Bioluminescence assay.

Strength

Trustworthy, reliable, meticulous, sound mind, vision, courage, integrity, discipline, diligent, flexibility, versatility, proactive, cross-culturally exposed, open to learning and submissive to superiors, able to work under pressure and as part of a team, proficient in written and oral English, good public relations, computer literate, and able to adapt easily to a new environment.

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