Discovery of the Gene Cluster in *Bacillus velezensis* AP183 Responsible for Bacillusin A Biosynthesis, Evaluation of the Use of B. velezensis AP183 in Preventing *Staphylococcus aureus* Infection, and Identification of Bacteriocins From a Soil Metagenome

by

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Abstract

The spread of multidrug resistance among pathogenic bacteria is increasing worldwide. One of the multidrug resistance pathogens is methicillin-resistant *S. aureus* (MRSA) which poses a significant threat to health and causes severe infections in risk populations such as immunocompromised people. There is also alarming emergence of antibiotic resistant foodborne pathogens, however, there are a limited number of new antibiotics found in the last few decades. Therefore, it is essential to discover new antibiotics or antimicrobials to treat MRSA as well as other food borne pathogens. In this study both culture-dependent and culture-independent approaches were used to discover antibiotics or antimicrobials against methicillin-resistant clinical isolates of *Staphylococcus aureus* (MRSA) and other food borne pathogens. Rhizosphere-derived *B. velezensis* AP183 was previously discovered to produce bacillusin A, a novel and potent macrodiolide antibiotic capable of inhibiting methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium.* Genome sequencing of *B. velezensis* AP183 followed by predicting biosynthetic gene clusters (BGCs) revealed an approximately 70-Kb type I polyketide synthase (PKS) cluster. This type 1 PKS BGC was predicted to synthesize bacillusin A in *B. velezensis* AP183 because bacillusin A is a macrocyclic polyene compound generally synthesized by type 1 PKS. This study characterized the bacillusin A BGC in *B. velezensis* AP183. An in-frame gene knockout method was used to delete the 44 Kb region, predicted to encode the type I modular PKS responsible for bacillusin A biosynthesis. The mutant did not produce bacillusin A as revealed by a loss of activity as well as lost the anti-MRSA activity and by LC-MS. A CRISPR/Cas9 based method was used to capture and integrate the 70 kb predicted BGC into *B. subtilis* 168 and successful expression of bacillusin A was observed by LC-MS analysis as well as anti-MRSA activity.

A previous study found that B. *velezensis* AP183 showed potent inhibition of *S. aureus* proliferation and bioluminescence in a mouse cutaneous wound (P = 0.0198). This study conducted the experiments to evaluate the ability of *B. velezensis* AP183 to prevent *S. aureus* biofilm formation on a tracheostomy tube substrate. *B. velezensis* AP183 could form a biofilm on a tracheostomy tube inner cannula substrate, and that this biofilm was antagonistic to *S. aureus* colonization. *B. velezensis* AP183 was also observed to inhibit the growth of *S. aureus* isolates originated from bovine mastitis cases. To evaluate the inflammatory response of mammary tissue to intramammary inoculation with *B. velezensis* AP183, we used high dose and low dose inocula in dairy cows. At the high dose, a significant increase in somatic cell count (SCC) and clinical mastitis was observed at all post-inoculation time points ($P < 0.01$), which resolved quickly compared to *S. aureus*-induced mastitis; in contrast, the lower dose of *B. velezensis* AP183 resulted in a slight increase of SCC and no clinical mastitis. In groups treated with *B. velezensis* AP183, SCC and abundance of *S. aureus* decreased with significant reductions in *S. aureus* after three days post-inoculation with AP183 (P = 0.04). A milk microbiome analysis revealed significant reductions in *S. aureus* relative abundance in the AP183-treated group by eight days post-inoculation ($P = 0.02$). These data indicate that *B. velezensis* AP183 can inhibit *S. aureus* biofilm formation and its proliferation in murine and bovine disease models.

A metagenomic approach was used to discover novel bacteriocins encoded by soil microorganisms sampled from a long-term agricultural rotation (Cullars Rotation) at Auburn University. A previous study from our lab generated a soil metagenomic library containing 19,200 clones in a bacterial artificial chromosome vector with an average insert size of 110kb. The contig sequences from this library were used to screen bacteriocins using BAGEL3/4 and antiSMASH bioinformatics pipelines. In silico screening of soil metagenome identified a total of 136 different

types of bacteriocins that contained class I, class II and class III bacteriocins. Few classes I and class III bacteriocins were selected for further study. For the expression of class III bacteriocin, the selected clones were identified from the cryopreserved metagenomic library, amplified by PCR and subcloned and expressed using the Expresso Rhamnose SUMO Cloning and Expression system. For class I bacteriocin expression, the structural gene were amplified first and subcloned in the Expresso Rhamnose SUMO Cloning and Expression system. Later the plasmid construct was transformed into corresponding metagenomic clones containing the whole BGC and expressed. The expression of bacteriocin was confirmed by SDS-PAGE analysis. The expressed proteins of class III bacteriocin were partly soluble as we observed significant amount of protein in the pellet on SDS PAGE gel, it could be due to the aggregation of the expressed protein. No antimicrobial activity was observed against the indicator food borne organisms used in this study. For both class of bacteriocin, we observed successful expression of the bacteriocin but did not observe the expected modification in the lanthipeptides which accompanied by loss of molecular weight due to dehydration step in post translational modification. We did not observe any antimicrobial activity of any of the putative expressed bacteriocin against the selected food borne pathogens.

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List of abbreviation

Methicillin Resistant *S. aureus* (MRSA)

Biosynthetic gene clusters (BGCs)

Polyketide synthase (PKS)

Somatic cell count (SCC)

Antimicrobial resistance (AMR)

Task Force on Antimicrobial Resistance (TATFAR)

ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter*)

Multidrug resistant (MDR)

WHO (World Health Organization)

SCCmec (Staphylococcal cassette chromosome mec)

Vancomycin intermediate *S. aureus* (VISA)

Vancomycin-resistant *S. aureus* (VRSA)

Non-ribosomal peptide synthase (NRPS)

Genomic island (GI)

Dehydroalanine (Dha)

Dehydrobutyrine (Dhb)

Post-translational modifications (PTMs)

ATP-binding cassette (ABC)

Multiple Sequence Alignment (MSA)

BAC (Bacterial Artificial Chromosome)

Chapter I

Introduction and Review of Literature

1 Introduction

Over the last few decades isolation of antibiotic resistant bacteria has been increased in humans and animals. This global pandemic of antibiotic resistant bacteria is the result of overuse and misuse of antibiotics in both human and veterinary medicine (Jim O'Neill, 2016). According to the World Health Organization (WHO), antimicrobial resistance (AMR) is a global crisis as it spreads so rapidly and becomes the greatest current threat to global health. Despite this fact, the consumption of antibiotics is rising (Holmes et al., 2016; Laxminarayan et al., 2016; Mendelson et al., 2016; J O'Neill, 2016). Antibiotic sales across 71 countries rose by 36% during 2000–2010 among them the top five countries are Brazil, China, India, Russia, South Africa which accounted for 76% of the increase (Van Boeckel et al., 2014). India is the major producer of antibiotics with a fragile health system with the highest rates of global AMR (Laxminarayan et al., 2016; Patel et al., 2015; Van Boeckel et al., 2015). According to WHO, 2019, Approximately 700,000 people die due to global antimicrobial resistance in every year. By 2050, the predicted global deaths will rise exponentially above 10 million per year due to AMR (Renwick, Simpkin, & Mossialos, 2016). The WHO classifies antibiotics based on their importance to human medicine such as important, highly important, critically important and the highest priority is classified as critically important (WHO, 2017). Some antibiotics are required to treat very serious infection that are not responsive to other antibiotic. These antibiotics are categorized as the highest priority critically important antibiotics as loss of effectiveness due to resistance would have devastating impact on human health. The third/fourth generation cephalosporins, fluoroquinolones, glycopeptides and macrolides are those critically important antibiotics.

Effective strategies are necessary to fight the rising threat of AMR. According to the Transatlantic Task Force on Antimicrobial Resistance (TATFAR) three critical tasks can effectively fight AMR (Control & Prevention, 2014). First, appropriate use of antibiotics in medical and veterinary contexts, secondly control and prevention of drug resistant infections and finally preserving existing antibiotics and improving the development pipeline for new antibiotics, alternative therapies and diagnostic devices (Renwick et al., 2016). There was an innovation gap between 1962 to 2000 since no major classes of antibiotics were introduced at that time. Although a few novel antibacterial classes, linezolid, daptomycin, and the topical agent retapamulin were introduced between 2000 to 2007, their classes were reported before that period. No successful discovery of new antibiotic classes was made after 1987. This is called a discovery void. All the discoveries of antibiotics happened by empirical screening such as fermentation product screening or chemical screening to inhibit the bacterial growth. So, innovation was lacking in these discoveries. The discovery void was also due to the fact that big pharma withdrew their research in the development of new antibiotics though there was continuing need for new antibacterial to combat the rise of resistant organisms (Renwick et al., 2016).

The lagging antibiotic development pipeline was observed from 2000 to 2012. At that period only five novel classes of antibiotic were commercially available. The antibiotics were oxazolidinones, lipopetides, pleuromutilins, tiacumicins and diarylquinolines, though none of them works against Gram negative bacteria (Butler, Blaskovich, & Cooper, 2013). Many of the Gram-negative bacteria are deadly and there was clear demand for new class of antibiotics as they are readily showed resistance to antibacterial drugs (Butler et al., 2013). The “ESKAPE” pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter*), a leading cause of hospital acquired

disease are mostly multidrug resistant isolates (Boucher et al., 2009). So, the development of novel antimicrobial agents is very important to combat these pathogens.

2 Review of Literature

2.1 Antimicrobial resistance and food safety

Food safety is very important for health, economic and social perspectives. In food animal, the continuous use of antibiotics in therapeutically, prophylactically, and sub-therapeutically for growth promotion and disease prevention has been observed. It develops high frequency of resistant foodborne pathogens which can ultimately be transmitted to humans *via* the food chain. Spreading of antibiotic-resistant bacteria, for example methicillin-resistant *Staphylococcus aureus* (MRSA), multi- drug resistant *Salmonella* or *Escherichia coli* (Couric, 2010) has been observed in this way. It would be a big problem for vulnerable people such as immune compromised people or children or elderly people if foodborne bacterial infections are resistant to usually recommended antibiotic therapies. It complicates the treatment procedure and increases the illness duration, mortality, hospitalization, and cost.

2.2 The causes of antibiotic resistance

There are different reasons for antibiotic resistance. Gene mutation, drug inactivation and decreased accumulation due to decreased permeability or increased efflux or both can be the reasons (Lin et al., 2015). Mobile genetic elements for example plasmids, transposons and integrins promote spreading of antimicrobial resistance by horizontal transfer of resistance determinants and are responsible for the development of several multi-drug resistance pathogens.

2.3 Antibacterial resistance in food borne pathogens

In food agriculture, antibiotics are used for growth promotion and prevention purposes which can increases the antibiotic resistance (Landers, Cohen, Wittum, & Larson, 2012).

Salmonella causes foodborne illness and significant health and economic burdens globally. There are approximately 93.8 million illnesses and almost 155,000 deaths each year worldwide due to *Salmonella* infection (Eng et al., 2015). Most non typhoidal *Salmonella* infections are related to food sources. Serious health problems due to antibiotic resistant *Salmonella* infection has been increasing. *Salmonella* species have shown antibiotic resistance to many antibiotics such as tetracyclines, kanamycin, sulfonamides, chloramphenicol, streptomycin, cephalosporins and penicillins (Olsen et al., 2004). Most of them are resistant to fluoroquinolones which is alarming as these antibiotics such as ciprofloxacin, cephalosporins are commonly used to treat a severe case of gastroenteritis (Acheson & Hohmann, 2001; Angulo & Mølbak, 2005; Chen et al., 2007; Hur, Jawale, & Lee, 2012). Multidrug resistant *Salmonella* Heidelberg isolates are resistance to ampicillin, amoxicillin-clavulanic acid, ceftiofur and cephalothin (Alcaine, Warnick, & Wiedmann, 2007). Thermotolerant *Campylobacter* is another food borne pathogen that causes public health problem. Some of the strains of this foodborne pathogen developed resistant to macrolides, quinolones, chloramphenicol, ampicillin, tetracycline, lincosamides, aminoglycosides and other tylosin, β-lactams and cotrimoxazole (Alfredson & Korolik, 2007; Koluman & Dikici, 2013). *Vibrio* genus is especially described as shrimp pathogen (Flegel, 2012) and it occurs naturally in marine, estuarine, and freshwater environments (Johnson, 2013). The use of antibiotic as preventive measure in aquaculture increases the risk of transferring resistant genes to human pathogens and animals by the selection of resistant bacteria (Cabello, 2006). *V. vulnificus* and *V. parahaemolyticus* displayed multiple-antibiotic resistance against ampicillin, penicillin, and

tetracycline globally. It is a major concern in seafood and aquatic environments. One of the reasons for extensive use of antibiotics in animal feed is decreasing the manufacturing cost and enhancing the growth rate and feed efficiency of the animals. Thus, reducing the mortality of animal. According to WHO, amount of antibiotics used in animal is almost twice that used by human (WHO, 2012). In 2010, the global use of antimicrobials in food animals was 63151 tons with an anticipated increase of 67% by 2030 (Van Boeckel et al., 2015). In 1951, first streptomycin resistance of coliform bacteria was observed in turkeys and in the late 1950s tetracycline resistance was observed in chickens fed with that antibiotics (Starr & Reynolds, 1951).

2.4 *Staphylococcus aureus*

S. aureus is often an opportunistic pathogen (Lowy, 2003; Weems Jr, 2001). It is one of the most intractable pathogens due to its multidrug resistance characteristics. It develops resistance against all the antibiotics that have been developed since 1940s. Alexander Fleming discovered penicillin in 1928 by observing the exquisite susceptibility of *S. aureus* to Penicillin (Sengupta, Chattopadhyay, & Grossart, 2013) . But by the mid-1940s, introduction of penicillin resistant *S. aureus* created a significant problem in hospital and community by a plasmid encoded penicillinase that hydrolyzes the beta-lactam ring of penicillin essential for its antimicrobial activity (Barber & Rozwadowska-Dowzenko, 1948; Kirby, 1944). By the early 1950s and 1960s, those penicillin-resistant strains became pandemic (Rountree & FREEMAN, 1955). In 1961, methicillin resistant *S. aureus* strain was reported (Barber, 1961). This strain encoded a specific low affinity penicillin binding protein, PBP 2a which conferred the antibiotic resistance instead of drug inactivation. This broad beta-lactam methicillin resistance *S. aureus* showed resistance to penicillin, cephalosporins, and carbapenems. In the mid-1970s e of new MRSA strain emerged containing novel *SCCmec* types II and III (MRSA-II and III) (Crossley et al., 1979). It caused worldwide pandemic of MRSA

in hospitals and healthcare facilities. The extensive use of vancomycin for treatment infections led to emergence of vancomycin intermediate *S. aureus* (VISA) strains. In the mid-to-late 1990s, wave 4 began by marking the emergence of MRSA strains in the community. In 2002 Vancomycin-resistant *S. aureus* (VRSA) strains were first identified.

2.5 Discovery of novel antibiotics using a culture-based approach

In 1940s to 1950s the novel antimicrobials were identified from screening soil samples. Now, that screening approach is not appropriate using the traditional low-throughput culture-based method to find out novel classes of antibiotics. Within the last few decades, in 2003 only a novel class of antibiotics daptomycin was identified and marketed. It was identified by randomly screening actinomycetes from soil using culture based technique (Baltz, 2007). Thus, high-throughput screening is required to find new antibiotics in culture-based approach. Genome mining is one of the most promising and powerful methods for the discovery of novel compounds with biological activity. By analyzing the sequence of bacterial genomes, it was found that there are cryptic biosynthetic gene clusters not previously reported. Numerous polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) gene clusters (Bode & Müller, 2005; Gross & Loper, 2009) were discovered later by genome mining. For example, by sequence analysis, 20 gene clusters were identified for secondary metabolism in *Streptomyces coelicolor* though only 4 of them reported for this organism (Bode & Müller, 2005). Bioinformatic prediction and screening (Gross & Loper, 2009) is one of the ways to predict the active compound produced by the biosynthetic pathway based on the different domains of the PKS or NRPS. Ravu et al found in a study that AP183 produced bacillusin A has strong activity against methicillin-resistant *S. aureus* (Ravu et al., 2015).

2.6 *Bacillus* spp.

The endospore-forming rod-shaped *Bacillus* Spp. are ubiquitous in nature and can be found in different environments such as soil, water, air, rhizosphere of plants, animal's gastrointestinal tract, and many other extreme environments (Connor et al., 2010; Felske et al., 2004; López-Sánchez et al., 2009). One of the important characteristics of *Bacillus spp*., is that they secrete diverse secondary metabolites, including antibiotics, antifungals, and anticancer substances. Some of these secondary metabolites are lipopeptides, polyketides, and siderophores (Borriss et al., 2011; X. Chen et al., 2009; Velusamy & Gnanamanickam, 2008). These metabolites act as plant growth-promoting substances (Chowdhury et al., 2013; Idris, Iglesias, Talon, & Borriss, 2007) because these metabolites can compete with the pathogen and affect the rhizosphere (Velusamy & Gnanamanickam, 2008). *Bacillus* spp. devote a significant portion of the genome to encode secondary metabolites, for example, *B. subtilis* uses 4 to 5%, *B. velezensis* FZB42 utilize 8% of the genome, *B. velezensis* SQR9 dedicates more than 10% of its genome that encode secondary metabolites such as bacteriocins, antimicrobial peptides, lipopeptides, polyketides, siderophores surfactin, bacillomycin D, fengycin, bacillibactin, bacilysin, macrolactin, difficidin and bacillaene (Chen, A. Koumoutsi, R. Scholz, K. Schneider, et al., 2009; Guo et al., 2014; Stein, 2005).

2.6.1 *B. velezensis* SQR9

B. velezensis SQR9, a biofertilizer and biocontrol agent, was isolated from the rhizosphere of cucumber plants (Cao et al., 2012; Cao et al., 2011; L. Chen et al., 2011). As a root colonizer, this strain has many beneficial effects on plants, such as promoting growth, suppressing other soil-borne pathogens, inducing plant resistance (Wu et al., 2018; Xu et al., 2014) and enhancing plant tolerance to abiotic stress (Wu et al., 2018; Xu et al., 2014). This strain produces biofilm on the

root surface, which is an essential factor for efficient root colonization. Some other factors are involved in the successful biofilm formation, such as lipopeptide compounds bacillomycin D produced by the *Bacillus amyloliquefaciens* SQR9 involves in biofilm formation (Xu et al., 2013). A recent study published that *B. velezensis* SQR9 produced the novel bioactive long-chain fatty acid bacillunoic acid by genomic island GI3 through a hybrid FAS-PKS pathway and these bacillunoic acids had activity against closely related *Bacillus* spp. and *S. aureus*. Like SQR9, *B. velezensis* AP183 has a shorter GI of 69.8 Kb and 82.5% homology to the GI3 of SQR9 and responsible for producing bacillusin A. (Manuscript in preparation).

2.6.2 *B. velezensis* AP183

B. velezensis AP183, a rhizosphere derived bacteria was found to produce a potent macrodiolide antibiotic bacillusin A, which inhibited MRSA and vancomycin-resistant enterocci (Ravu et al., 2015). A recent study showed that *B. velezensis* AP183 can produce biofilm on tracheostomy tube inner cannula and significantly reduce *S. aureus* colonization (Afroj et al., 2021). Also, *B. velezensis* AP183 inhibited *S. aureus* proliferation in a mouse wound model. This *Bacillus* strain has inhibitory activity against many mastitis-derived *S. aureus* and reduced the relative abundance of *Staphylococcus* in the bovine mastitis model (Afroj et al., 2021).

2.7 Antimicrobial compounds from *Bacillus*

2.7.1 Lipopeptides

There are different lipopeptides biosurfactants, such as surfactin, iturin A 7, and Fengycin 8. Surfactin is a powerful biosurfactant that acts as a detergent (Carrillo, Teruel, Aranda, & Ortiz, 2003) and is used for antibacterial, antiviral, anti-adhesive, and anti-inflammatory uses (Kim, Ryu, Kim, & Chi, 2010). Many *Bacillus* strains include *B. subtilis*, *B. pumilus*, *B. licheniformis* (Tendulkar et al., 2007) and *B. amyloliquefaciens* strains (Wulff et al., 2002) produces different

types of lipopeptides as their secondary metabolites. These metabolites help the producer *Bacillus* strains to survive in the natural environment in different ways, such as binding to heavy metals, quorum sensing, motility, biofilm formation and plays a role in bacterial pathogenesis.

2.7.2 Polyketides

Polyketides (PKs) are produced from a mega enzyme Polyketide Synthase (PKS) that play important role in drug discovery. PKs belong to a structurally diverse family of secondary metabolites produced in microorganisms and plants by the mega enzymes polyketide synthases (Hertweck, 2009). The secondary metabolites PKS include many bioactive compounds with various biological activities such as antibacterial, antifungal, immunosuppressive, antitumor, and many other activities (Hertweck, 2009)

There are three core PKS domains which mainly responsible for chain elongation of polyketide. The domains are ketosynthase (KS), an acyltransferase (AT), and an acyl carrier protein (ACP) (Hertweck, 2009). These domains have a specific function, such as KS domains responsible for Claisen-type chain elongation, ACP domains responsible for binding to the acyl intermediates during elongation processes. Acyl intermediates and nascent product molecules are covalently tethered during the elongation and modification steps, which are required to produce the final compound. AT domains of PKS, deliver a malonyl or related unit to ACP domains (Jenner et al., 2018). Besides these essential domains, PKS also has some additional domains that contribute to the modifying the polyketides. The inactive apo ACP domains are converted into active holo-ACPs by post-translational modification with the 49-phosphopantetheine prosthetic group and carry a CoA-derived free thiol group. Then an acyl-CoA building block is transferred to the ACP domain, and with the free thiol group, a thioester bond is formed. KS domain of PKS

catalyzed the decarboxylative Claisen like condensations reaction to extend the chain in polyketide (Musiol & Weber, 2012).

According to the characteristics of the products, polyketides are divided into three structural classes. Type I PKS is modular PKSs due to their defined modular organization. Each of the modules contains a set of distinct domains responsible for one cycle of polyketide chain elongation. Type I PKS is often further subdivided into two major groups: the iterative type I PKS and non-iterative type I PKS (Cox & Simpson, 2009; Hertweck, 2009; Smith & Tsai, 2007). An iterative PKS (iPKS) is usually a single-module protein consisting of a single set of functional domains. Although this PKS contains only a single module, a fungal iPKS can conduct multiple rounds of chain extension and β-keto processing. The varied reduction level of the β-keto during each round of elongations is the most intriguing feature of this group of PKSs(Crawford & Townsend, 2010; Kennedy et al., 1999). These iPKSs are confined mainly to fungal systems.
Non-iterative modular Type I PKS contains multiple modules, and each module includes a single set of domains such as acyl carrier protein (ACP), ketosynthase (KS), and acyltransferase (AT) domains, and some additional modification domains. After processing the starter unit malonyl CoA, ACP transfers the fully processed intermediate to the KS domain in the next module for further extension or to the thioesterase domain in the final module for hydrolysis or cyclization and later release of the PKS compound.

Type II PKSs produce aromatic polyketides by catalyzing iterative Claisen condensation reaction, and the starter unit is usually acetate (Waldman & Balskus, 2014). They mainly have aromatic rings in their structure (Hertweck, Luzhetskyy, Rebets, & Bechthold, 2007). They have antibacterial, anticancer, and antiviral activity (Fang, Guell, Church, & Pfeifer, 2018; Katz & Baltz, 2016).

Type III PKSs are simple KS homodimer containing a single active site on each for priming, extension, and cyclization reactions to occur (Shimizu, Ogata, & Goto, 2017). This type of PKS does not require phosphopantetheine residues or ACP. They use free CoA thioesters as substrates. This PKS produces a variety of compounds despite their simple structure, such as halcones, pyrones, acridones, phloroglucinols, stilbenes, and resorcinolic lipids (Harwood, Mouillon, Pohl, & Arnau, 2018).

2.8 Metagenomic approach

High rediscovery rates of traditional culture-based methods for natural product discovery make it less attractive to pharmaceutical companies. On the other hand, the culture independent methods which is called "metagenomic" approach extracted DNA directly from environmental samples and then heterologously expressed the DNA in an appropriate host (Aakvik et al., 2009; Blin et al., 2013; Brady, 2007; Chang & Brady, 2011). By using this method, it is possible to access large fractions of the earth's microbial biosynthetic diversity. Culture-based natural product discovery programs fail to access the vast majority of bacterial biosynthetic diversity present in the environment. Soil is both ubiquitous and rich in bacterial diversity, though only less than 1% of bacterial species are identified and 99 % of bacterial species are not able to cultivated in the laboratory from soil, and hence large numbers of natural product biosynthetic gene clusters are not expressed (Giovannoni, Britschgi, Moyer, & Field, 1990; Rappé & Giovannoni, 2003). To overcome this situation, DNA can be extracted directly from environmental samples to unravel the cryptic gene cluster (Milshteyn, Schneider, & Brady, 2014). A metagenomic library can be prepared by cloning and storing the genetic information in a host organism.

The metagenomic approach can be divided into two categories; 1. Functional screening and 2. Sequence based screening

2.8.1 Functional screening

The clones are screened for phylogenetic markers such as 16S rRNA or conserved genes (Rondon, Raffel, Goodman, & Handelsman, 1999) or expression of easily observable phenotypes color, HPLC peak, enzyme activity, or antibiotic production (Brady, Chao, & Clardy, 2002, 2004; Feng et al., 2011; Rondon et al., 2000; Rondon et al., 1999).

There are some limitations of functional metagenomic screening. The host has limited capability to express the clone successfully. The limitations of this screening are the size of the metagenome library and the scarcity of biosynthetic machinery in the host that is required for secondary metabolism. (Bentley et al., 2002; Craig, Chang, Kim, Obiajulu, & Brady, 2010; Ikeda et al., 2003; Vickers, 2017; Wexler & Johnston, 2010).

2.8.1.1 Selection of hosts for heterologous expression

A broad host range of shuttle vectors is essential for successful drug discovery using a metagenomic approach. The host needs to transcriptionally activate a diverse set of the gene cluster and produce the compound from the metagenome. If required, the host needs to be engineered for heterologous expression of products from the metagenome. Broad host range shuttle vectors are constructed in many studies for effective cloning of metagenomic DNA (Aakvik et al., 2009; Brady, 2007; Chang & Brady, 2011). Also, a host may not have all the necessary machinery for the transcription and translation of exogenous DNA and responsible for the silent PKS pathway (Stevens et al., 2013). So, careful selection of host is helpful for heterologous expression of the metagenome.

2.8.1.2 Improvement of library construction methods

Recovering and expressing the larger pathway such as the PKS pathway from environmental DNA, depending on the capture of complete gene clusters. Most of the efficient

metagenomic cloning methods capture only ~40 kb and are not suitable for the cloning of large BGC. Therefore, large insert size vectors such as cosmid, fosmid, or bacterial artificial chromosome (BAC) are required to clone the complete gene cluster successfully. Some other techniques can be used when construct the metagenomic library that can help successful transfer of metagenomic DNA into host. The methods are 1) the synchronous coefficient of drag alteration that effectively removes all inhibitors and purifies and concentrates the DNA (Vickers, 2017). 2) indirect DNA extraction through microbial cell separation (Liles et al., 2008; Vickers, 2017) 3) to remove environmental inhibitor, formamide can be used (Pel et al., 2009; Vickers, 2017).

2.8.1.3 Selective library enrichment by functional complementation

Many bacteria devote only 2% of the genome for the secondary metabolite gene clusters (Garcia, Fernández-Guerra, & Casamayor, 2011). So, it is difficult to capture the biosynthetic gene cluster from a complex metagenome as only small fraction of gene clusters can be screened, and most of the BGC cannot be identified. In this condition, selective enrichment of metagenome library for secondary metabolite machinery is a potential approach to overcome this situation. One example would complement a phosphopantetheine transferase (PPTase) deficient *E. coli* strain with phosphopantetheine transferase genes. This gene is required to function with? non-ribosomal peptide synthetase and polyketide synthase and bacterial growth at low iron conditions (Charlop-Powers, Banik, Owen, Craig, & Brady, 2013; Lambalot et al., 1996). So, a metagenome clone will complement a PPTase-deficient *E. coli* that will grow in low iron conditions.

2.8.2 Sequenced-based analysis

Sequence-based metagenome identifies target gene cluster using sequence analysis of metagenome. This approach is different from functional metagenome as it does not require heterologous expression to identify target gene cluster. At first, the sequence is analyzed for the

target gene cluster, and then the target gene cluster pathways can be expressed in a heterologous host to generate metabolites. In functional screening, redundant isolation of secondary metabolite was observed (Tulp & Bohlin, 2005). This problem can be overcome by sequence-based screening by identifying target metabolites by *insilico* screening, making the process more efficient (Chang & Brady, 2013)

2.9 Bacteriocin

Bacteriocin is produced by many bacterial and archaeal species and first described in *E coli* almost 100 years ago and named as colicin (Baquero & Moreno, 1984; Klaenhammer, 1988; Shand & Leyva, 2008). Bacteriocins are ribosomally synthesized antimicrobial peptides that have mostly bacteriostatic or bactericidal activity against closely related organisms (Hatakka & Saxelin, 2008) and rarely some of them have broad spectrum activity against unrelated organism (Cotter, Ross, & Hill, 2013). The Self synthesizing immunity proteins or efflux pumps or both are used by the bacteriocin producing bacteria to protect them from their own bacteriocin (Bastos, Coelho, & Santos, 2015).

2.9.1 Classification of Bacteriocins

Several classification schemes have been proposed for bacteriocins. According to Arnison, ((Arnison et al., 2013), there are three major classes of bacteriocin; class I is small post translationally modified peptides with molecular masses <5 kDa where dedicated enzymes in bacteriocin gene cluster encoded PTMs, class II is unmodified heat stable bacteriocin of 6–10 kDa and no post translational modification enzyme and class III bacteriocin is larger heat labile of > 10 kDa proteins

2.9.1.1 Class I bacteriocin lanthipeptide

These class I bacteriocins needs post translational modification that make this class of bacteriocin stable to high temperatures, extreme pHs or proteolytic enzymes. Class I is further subdivided into four classes based on the post translational modification enzymes specially dehydratase and cyclase (Arnison et al., 2013). Class I bacteriocin includes lanthipeptides, sactipeptides, circular peptides, linear azole(ine)-containing peptides (LAP) and lasso peptides. Lanthipeptide has unusual thioether-linked amino acids, lanthionine (Lan) and methyllanthionine (MeLan) (Arnison et al., 2013). Lanthipeptide at first synthesized as a precursor peptide that has a leader peptide and core peptide. To become active peptide, post-translational modifications is required to get the characteristics cyclic structure. Post translational modification enzyme dehydratase involve dehydration of serine (Ser) and threonine (Thr) residues to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively. Later thiol groups of cysteine (Cys) residues added onto Dha and Dhb to form thioether cross-links that produces the Lan and MeLan residues, respectively (Arnison et al., 2013).

2.9.1.2 Class II bacteriocin

Class II bacteriocins are small heat-resistant peptides that do not go through post translationally modification like class I bacteriocin except leader peptide cleaved from a double glycine site during the exportation of mature bacteriocin from the cell and some molecules contain disulphide bridges (Cotter, Hill, & Ross, 2005). Class II bacteriocins can be subdivided into four classes; class IIa, pediocin-like bacteriocins that is very active against *Listeria monocytogenes*, class IIb, two-peptide bacteriocins; class IIc, circular bacteriocins; and class IId, the miscellaneous group.

2.9.1.3 Class III bacteriocin

Class III bacteriocin is a heat-labile larger antimicrobial protein that has molecular weight > 10 kDa (Nilsen, Nes, & Holo, 2003). Class III bacteriocin colicin has three domains including receptor binding, translocation, and lethal domain (Riley, 1993). Some of the well characterized class III bacteriocins are Colicin from *E. coli*, helveticin J from *Lactobacillus helveticus* , enterolysin A from *Enterococcus faecalis* can damage the cell walls of target bacteria with a broad spectrum activity (Lazdunski, 1995; Nigutová, Serenčová, Piknová, Javorský, & Pristaš, 2008; Thompson, Collins, & Mercer, 1996). Linocin M18 produces from Brevibacterium linens is a class III bacteriocin that was isolated from red smear cheese (Valdés-Stauber & Scherer, 1994). This bacteriocin has activity against a wide range of Gram-positive bacteria such as *Listeria monocytogenes*, *B. cereus* and *S. aureus*.

2.10 Mode of Action

Bacteriocins work in two ways; bacteriostatic that inhibits cell growth and bacteriocidal that killed the bacteria with or withour cell lysis (Sabo, Vitolo, González, & de Souza Oliveira, 2014). The bacteriocin that inhibits Gram-positive bacteria target the cell envelope (Cotter et al., 2013). Some of the lantibiotics and class II bacteriocins target Lipid II, a membrane-anchored cell-wall precursor that is required for peptidoglycan biosynthesis (Breukink & de Kruijff, 2006). Other bacteriocins use Lipid II as a docking molecule and form a pore on the cell membrane (Cotter et al., 2013). Some other bacteriocins can degrade the DNA and inhibit gene expression and protein production (Vincent & Morero, 2009).

Chapter II

Identification of a biosynthetic gene cluster for a macrocyclic polyene compound bacillusin A in *B. velezensis* AP183

1 Abstract

Rhizosphere-derived *B. velezensis* AP183 was previously discovered to produce bacillusin A, a novel and potent macrodiolide antibiotic capable of inhibiting methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium*. Genome sequencing of *B. velezensis* AP183 followed by predicting biosynthetic gene clusters (BGCs) revealed an approximately 70-Kb type I polyketide synthase (PKS) cluster. This BGC matched to the 25% ORF to the neighbor gene cluster and was predicted to be responsible for novel Bacillusin A biosynthesis. This type I PKS BGC was predicted to synthesize Bacillusin A in AP183 because bacillusin A is a macrocyclic polyene compound generally synthesized by type I PKS. Also, a chemically related compound, bacillunoic acid previously reported from *B. velezensis* SQR9 is produced from a similar BGC present in SQR9. An in-frame gene knockout method was used to delete the 44 Kb region, predicted to encode the type I modular PKS responsible for bacillusin A biosynthesis. The mutant did not produce bacillusin A as revealed by LC-MS and loss of the anti-MRSA activity. A CRISPR/Cas9 based method was used to capture and integrate the 70 kb predicted BGC into *B. subtilis* 168. The recombinant BGC was expressed and observed by LC-MS analysis as well as anti-MRSA activity. Interestingly, we observed that heterologous production of bacillusin A, from *B. subtilis* 168 pBAC-B-*basA* was almost double that expressed by wild type *B. velezensis* AP183. In conclusion, this study characterized the BGC in *B. velezensis* AP183 responsible for novel antibiotic bacillusin A biosynthesis.

2 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) are resistant to penicillin-like antibiotics and other existing antibiotics such as vancomycin, daptomycin and linezolid (Caballero et al., 2015; Ghahremani, Jazani, & Sharifi, 2018; Kaur & Chate, 2015; Roch et al., 2017). Due to antibiotic resistance, MRSA poses a significant threat to health and causes severe infections in at-risk populations such as immunocompromised people. This strain can form a biofilm that is an important factor in chronic infections (Oyama et al., 2016). It is essential to discover new antibiotics to treat MRSA; however, there have been a limited number of new antibiotics found in the last few decades. Therefore, it is crucial to discover new anti-MRSA antibiotics to combat MRSA and other multidrug-resistant pathogens.

Members of the *Bacillus* genus are well known for synthesizing biologically active molecules that have inhibitory activity against pathogenic microbes (Hamdache, Lamarti, Aleu, & Collado, 2011; Stein, 2005). Several different types of polyketides, such as bacillaene, difficidin and macrolactin, are produced by these *Bacillus* biocontrol strains (Chen, Koumoutsi, Scholz, & Borriss, 2009). Rhizosphere-derived *B. velezensis* AP183 was found to produce bacillusin A, a novel and potent macrodiolide antibiotic capable of inhibiting MRSA and vancomycin-resistant *Enterococcus faecium* (Ravu et al., 2015). To understand the biosynthesis of bacillusin A, it is important to identify biosynthetic gene cluster from *B. velezensis* AP183 through genome mining (Medema et al., 2015). Genome mining of secondary-metabolite-producing microorganisms is an approach that has revealed potential pathways of various bioactive metabolites present in the organisms by analyzing and utilizing functional microbial genomes through bioinformatic analyses (Zerikly & Challis, 2009). After mining and identifying biosynthetic gene clusters (BGC) through bioinformatic analysis, it is possible to characterize novel BGC and determine the

metabolite expressed by that BGC. To determine the contribution of a particular BGC, it is essential to construct a mutant that is deficient in the production of that compound. A gene's function can also be confirmed by cloning of a particular BGC and heterologous expression of that specific gene and encoded metabolite (Fujii, 2009).

Many studies have observed that a single BGC can be responsible for producing many structurally related molecules that could have varied bioactivities (Fischbach & Clardy, 2007; Martinet et al., 2019; Yin et al., 2014; C. Zhang et al., 2018). A previous study reported that a BGC produced bacillunoic acid in *B. velezensis* SQR9 (Wang et al., 2019), but was not reported to produce bacillusin A. Based on the structures, these two compounds are structurally related, with four bacillunoic acids cyclized to make one bacillusin A. We hypothesized that a cyclase uniquely present in *B. velezensis* AP183 could be responsible for converting bacillunoic acid to bacillusin A. We analyzed the unmapped contigs of AP183 absent in SQR9 and annotated those to find any predicted cyclase in *B. velezensis* AP183. However, no gene was discovered in *B. velezensis* AP183 predicted to encode a cyclase responsible for converting bacillunoic acid to bacillusin A.

This study has three objectives; they are 1) to identify genes responsible for bacillusin A biosynthesis in *B. velezensis* AP183, 2) to confirm the function of predicted bacillusin A BGC by targeted gene knockout study and 3) heterologous production of bacillusin A BGC in *B. subtilis* 168. Here, we characterized a type I PKS BGC in *B. velezensis* AP183 isolated from soil rhizosphere, responsible for synthesizing bacillusin A, a macrocyclic polyene compound. In this study, by utilizing LC-MS analysis, we observed that SQR9 also produced bacillusin A for the first time. Therefore, the study suggested that a single BGC is responsible for both bacillusin A and bacillunoic acid.

3 Methods and materials

3.1 Strains and growth conditions

The bacterial strains used in this study were the Methicillin Resistant *Staphylococcus aureus* 30 (MRSA30), *B. velezensis* AP183, *B. velezensis* SQR9, and the other bacterial strains mentioned in Table 1. All *Bacillus, Staphylococcus* and *Escherichia coli cultures* were cryopreserved in TSB containing 20% glycerol and isolated colonies were obtained on TSA. To generate a MRSA30 culture, a single colony of MRSA30 was inoculated into 3 mL of TSB and cultivated at 37ºC for 16 h, then 50 µL of the bacterial culture was transferred to 3 mL TSB and incubated for another 3 h. To cultivate *B. velezensis* AP183, *B. velezensis* AP183 Δ*basA*, *B. velezensis* SQR9, an isolated colony was inoculated into 3 mL of TSB and incubated at 30°C for 24h with shaking. *B. subtilis* 168 and *B. subtilis* 168 pBAC-B-*basA* strains were cultivated at 37°C for 12 h with shaking, and then inducers were added and grew for another 24 h at the same condition for expression of bacillusin A. The *E. coli* (DH10B, 2925, JM110, DH5G, INV) strains were used for the transformation; most of them were incubated at 37°C for 16 h with shaking or followed the protocol from the manufacturers (Table 1).

3.2 Whole-genome sequencing and prediction/characterization of bacillusin A BGC

Genomic DNA of *B. velezensis* AP183 was first sequenced in a previous study (Nasrin, Hossain, & Liles, 2015). The whole-genome sequence of this *B. velezensis* AP183 was used in an antiSMASH 5.0 genome mining approach to identify a BGC that could be responsible for the biosynthesis of bacillusin A. Further gene prediction and annotation were performed using RAST annotation server, and BLASTx was performed to confirm the identity of each open reading frame of this predicted BGC for bacillusin A.

3.3 DNA isolation and manipulation

Primers used in this study are listed in Table 2. Genomic DNA from *B. velezensis* AP183 was isolated using Omega genomic DNA isolation kit and plasmid DNA from *E. coli* strains was isolated using Omega plasmid extraction kit according to the manufacturer protocol. To obtain homologous arms, overlapping PCR was performed using the appropriate primers. Q5 High-Fidelity DNA Polymerase, Restriction endonucleases (BamHI, SalI), Gibson Assembly Master Mix were got from NEB. Digestion of plasmid and ligation of overlapping PCR products to the plasmid were performed according to the supplier's instructions. Amplified PCR products were analyzed using 1% agarose gels, and the DNA from agarose gel was extracted and purified using QIAquick Gel Extraction kit (Qiagen).

3.4 Construction of deletion plasmid

Deletion plasmid was constructed based on the rule of in-frame deletion following the procedure of a published paper (Zhang et al., 2014). First, 1000 bp of upstream and downstream flanking DNA sequences of bacillusin A BGC were amplified using *B. velezensis* AP183 as a template. The 1000 bp upstream fragment was amplified using the primers *basA*UP-F and *basA*UP-R and the 1000 bp of the downstream fragment was amplified using primers *basA*DN-F and *basA*DN-R. Then, the up and downstream fragments were joined through overlapping PCR to obtain homologous arms. The resulting fragment was then ligated to similarly digested pKSV7. *basA*OUT-F and *basA*OUT-R primers designed from the sequence flanking the homologous arm were used to confirm the deletion by PCR and later confirmed by sequencing the PCR products.

3.5 Transformation of pKSV7-*BasA* into *B. velezensis* AP183 and selection of mutant

Transformation of pKSV7-*basA into B. velezensis* AP183 was performed following published papers with some modifications (Ferradini, Nicolia, Capomaccio, Veronesi, & Rosellini,

2011; Roh et al., 2009; Yi & Kuipers, 2017). One colony was picked and inoculated into BHIS medium and incubated at 30°C, using 200 rpm to obtain OD600 of 0.85. Once an OD600 of 0.85 had been achieved, 1% glycine was added to the culture and set for another 1 hour. The culture was transferred to a precooled 50 ml centrifuge tube and incubated on ice for 30 min. The culture was then centrifuged at 7500 rpm for 8 min at 4 °C. Cells were then washed four times with electroporation buffer. Next, the electrocompetent cells were collected by adding electroporation buffer to the pellet at 1/100 volume of the original culture. 100 µl of electro competent cells were then mixed with 250 ng of plasmid in a prechilled Eppendorf tube. After that, the mixed cells were transferred into pre-chilled 2 mm gap electroporation cuvette and kept for 2 min on ice. Electroporation was performed with the settings of 25 µF capacitance, 200 resistance, and voltage 2.1 KV. After pulse, immediately, 1 ml pre-warmed growth medium with mannitol was added to the cuvette and contents were transferred to a poly vinyl culture tube. The culture tube was incubated at 30 °C for 5 h at 200 rpm shaking and then transferred the electroporation in the Eppendorf tube. The electroporated sample was then centrifuged for 5 min at 8000 rpm and the supernatant was removed. Next, 100 µL of growth medium was added to the tube and the pellet was resuspended. The total volume of the resuspended pellet was plated on LB agar containing 4 µg/ml chloramphenicol. After 24 h positive transformants were selected and screened with PCR. Then, 100 µl of positive transformant culture was inoculated into LB with chloramphenicol and incubated for 24 h at 42 °C. This subculture was repeated 3 times and next diluted and plated on LB with chloramphenicol plate at 42 °C. The colonies were detected using *BasA*OUT-F and *BasA*OUT-R primers. Next, the colonies were grown at 42 °C without chloramphenicol three times and plated in LB without chloramphenicol at 42 °C. Each colony was picked and inoculated and grew on 96 deep well microplates in LB without chloramphenicol. The culture of microplates was

transferred to 96 deep well microplates in LB with chloramphenicol by replicating the microplate. If there was no growth in the wells for any colonies in the chloramphenicol microplates, the culture was picked from no chloramphenicol microplate wells and then possible mutants were detected with PCR using *basA*OUT-F and *basA*OUT-R primers, and the PCR products were sent to Eurofins Genomic LLC for sequencing to confirm the deletion.

3.6 CRISPR/Cas9 based method for bacillusin A BGC construction for heterologous expression

B. velezensis AP183 was cultured in liquid broth and the cells pelleted by centrifugation. The cells were lysed, and high-MW genomic DNA was isolated. The gDNA was restricted *in vitro* by Cas9 and two guide RNAs, targeting cut sites 370 bp upstream and 713 bp downstream of the BGC. The restricted fragment was then joined via isothermal assembly to pBAC-Bv2. pBAC-Bv2 is a *Bacillus* integration vector that is optimized for large, repetitive inserts. It contains a kanamycin resistance gene, two promoters (Phy-spank and PxylA) flanking the cloning site and integrates into the *amyE* locus of *Bacillus* spp. Successful cloning of the basA BGC was confirmed by colony PCR, restriction digest mapping, and Sanger sequencing of the junctions.

3.7 Complementation experiment

The purified *basA* BAC DNA was transformed into BAC-Optimized replicator v2.0 electrocompetent cells. The copy number was increased by adding arabinose, and the DNA was extracted using BAC DNA extraction kit. Extracted DNA was then transformed into different demethylated *E. coli* strains i.e., *E. coli* JM110 and *E. coli* 2925, *E. coli* INV, with no success. tRNA precipitation of BAC DNA was performed to get a high concentration of BAC DNA from methylated *E. coli* and later tried to transform to demethylated strains with no success. Electroporation was done at 2.5 KV. 25 μF and 300Ω for BAC DNA transformation.

Electrocompetent cells were prepared according to the procedure described by Jana Novakova et al,.2014 (Nováková, Izsáková, Grivalský, Ottmann, & Farkašovský, 2014).

3.8 Production of bacillusin A from culture

B. velezensis AP183, *B. velezensis* AP183 Δ*basA* were grown at 30°C for 2 days and then the culture was centrifuged at 10,000 × *g* for 10 min and filtered with 0.2 µm filter. *B. subtilis* 168 and *B. subtilis* 168 pBAC-B-*basA* cultures were grown at 37 °C for 12 h and then 1% xylose, 1 mM IPTG and 1% xylose plus 1 mM IPTG were added as inducers and grown for another 24 h. The culture was centrifuged at 10,000 × *g* for 10 min and filtered with 0.2 µm filter. The filtrate was used immediately against MRSA or stored at -20 °C for later use against MRSA or LC-MS analysis.

3.9 Heterologous expression of bacillusin A BGC

Purified BAC DNA was transformed to *B. subtilis* RM125 sfp+ and transformants were selected for kanamycin resistance. For each BGC, 3 colonies were cultured and assayed via colony PCR on the left and right side of the BGC. For every clone tested, amplicons of the correct size as well as stable kanamycin resistance of the clones suggested correct introduction of the BGCs to the expression strain.

3.10 Antimicrobial assay

The filtrates were tested against MRSA using both spot-on-lawn assay as well as 96 well assays.

3.10.1 Spot-on-lawn assay

A single colony of MRSA was inoculated in TSB and grown at 37°C for 24 h. Later, 50 µl of culture was inoculated into 3 ml of fresh TSB and grown for another 3 h. Then with a sterile cotton swab, the MRSA culture was spread into TSA plates evenly. Later, to test the anti-MRSA activity,

10 µl of each filtrate from tested cultures were added to the MRSA plate in triplicate and incubated for 16 h.

3.10.2 96 well bioassay

A single colony of MRSA was first inoculated into 2 ml of LB and incubated at 37 °C, 180 rpm overnight. Following overnight growth, the culture was diluted 100-fold in fresh LB and incubated at 37°C until OD reached 0.3 at 600 nm. Cells were then diluted 1000-fold in fresh LB and used for the assays. To test the antibacterial activity expressed by selected cultures, 100 µl of cell-free filtrates were added in triplicate wells in a 96-well plate. Wells containing supernatant were then mixed with 100 µl of diluted log-phase MRSA. Additionally, wells containing a sterile growth medium were used as negative controls. After incubation at 37 ºC overnight with shaking at 180 rpm for 16 h, the optical density at 600 nm was quantified for each well using a Microplate Reader. The percent inhibition of pathogen growth was calculated for each supernatant after normalizing to the OD_{600} values for the negative controls.

3.11 LC-MS analysis

3.11.1 Sample and Standard Preparation

Exposure to light was minimized during all sample and standard handling steps. The TSB and samples (50 µL) were mixed with ice cold acetone (200 µL) vortexed for 30 sec frozen for 5 min, centrifuged for 5 min, and decanted. The liquid was evaporated for 4 h in a Thermo Savant DNA 120 speed vac concentrator (Waltham, MA USA) without any heating. The TSB and samples were reconstituted in 50 µL of 50% water and 50% acetonitrile, vortexed, and was analyzed via LC-MS. The bacillusin A standard was diluted in the reconstituted TSB blanks to create 5 solutions of differing concentrations.

3.11.2 LC-MS analysis

Analysis was performed on a Vanquish UHPLC system (Thermo Fisher, USA) coupled with a quadrupole orbitrap mass spectrometer (Orbitrap Exploris 120, Thermo) with electrospray ionization (H-ESI) in negative mode using Xcalibur software (V4.4.16.14). Injection of 5 µL of the sample or standard was made on a C18 column (ACQUITY UPLC® BEH C18, 1.7 µm, 2.1 × 50 mm, Waters) with a 200 µL/min flow rate of mobile phase solution A (freshly prepared 2 mM ammonium acetate in water) and solution B (acetonitrile) beginning at 35% B, held for 1 minute followed by a linear ramp to 50% B in 9 min, then to 95% B at 11 min, held 2 min, and back to 35%B with 6 min of re-equilibration. The MS scan range was 250-1500 m/z with resolution of 60,000, standard AGC target, 70% RF lens, automatic maximum injection time, with EASY-IC on. The spray voltage was 2500 V, ion transfer tube temperature was 320 °C, and the vaporizer temperature was 275 °C. A tSIM scan was also used with a 1.6 m/z isolation window, resolution of 30,000 with EASY-IC on and focus on 597.3069 m/z with z=2.

4.0 Results

4.1 Characterization of Biosynthetic Gene Clusters responsible for bacillusin A biosynthesis

A whole-genome sequencing and genome mining approach was performed to identify the biosynthetic gene cluster (BGC) present in the *B. velezensis* AP183 chromosome, which could be responsible for producing the bacillusin A compound. The antiSMASH 5.0 program analyzed the whole genome sequence and revealed that there were 12 BGC regions which produced different types of compounds. Most of the BGCs were predicted to produce polyketide and non-ribosome peptide-derived secondary metabolites (Table 1). Six BGCs have genes that encode type I, II or III PKS systems, including a type I modular PKS system. Some of them are hybrid BGCs

containing genes that code for more than one type of compound. One BGC possesses genes predicted to produce a PKS like compound, and two terpene BGCs were identified also. One of the BGCs was for saccharide, and two BGCs contained non-ribosomal peptide synthetase (NRPS) or non-ribosomal peptide synthetase (NRPS) like proteins.

All the polyketide BGCs showed 100% similarity to the ORF of the known BGCs except region 2. Region 2 has an approximately ~70 kb type I PKS biosynthetic gene cluster (BGC), which showed only 25% similarity to the ORF of its closest neighbor gene cluster (Figure 1, Table 3). It contained many domains (63 enzymatic domains) of ACP, KS, AT, DH, KR, and one domain of TE and 13 modules in total (Table 4). Annotation of GI3 in AP83 revealed 24 ORF. A published paper revealed that *B. velezensis* SQR9 which is closely related to AP183, produced bacillunoic acid from a BGC with 82.5% homology to the BGC located at region 2 of the AP183 genome. In contrast to AP183, antiSMASH analysis showed that the BGC in SQR9 has 77 enzymatic domains, 17 modules, and 26 ORF (Figure 2). Based on the structures, these two compounds are structurally related, with four bacillunoic acids cyclized to produce one bacillusin A. We hypothesized that a cyclase uniquely present in *B. velezensis* AP183 could be responsible for converting bacillunoic acid to bacillusin A. To test this hypothesis, we analyzed the unmapped contigs of AP183 that were absent in SQR9 and annotated those to find any predicted cyclase in *B. velezensis* AP183.

4.2 Evidence disfavoring the involvement of putative cyclase responsible for conversion of bacillunoic acid to bacillusin A

To investigate the putative cyclase, unmapped contigs in *B. velezensis* AP183 were annotated, but no gene was discovered in *B. velezensis* AP183 predicted to encode a cyclase that could be responsible for that conversion of bacillunoic acid to bacillusin A. In addition, it was

found that SQR9 also produced bacillusin A (Figure 4) and had anti-MRSA activity, so it could be possible that the same BGC can produce more than one compound.

4.3 Deletion of 44 kb region from *basA* BGC from *B. velezensis* AP183 failed to produce bacillusin A and lost anti-MRSA activity

To confirm that the predicted *basA* BGC is responsible for bacillusin A production in *B. velezensis* AP183, a 44 kb region was deleted from this BGC, and mutant was generated. As expected, LC-MS analysis showed that the disrupted PKS BGC failed to produce bacillusin A, suggesting that this predicted type 1 PKS BGC is indeed responsible for the biosynthesis of bacillusin A in *B. velezensis* AP183 (Figure 3). Bioassay against MRSA showed that the mutant lost anti-MRSA activity completely.

4.4 Heterologous Expression of predicted BGC

The predicted bacillusin A BGC was integrated to the chromosome of *B. subtilis* 168 by CRISPR-Cas9 method. According to manufacturer instructions, both host *B. subtilis* 168 and *B. subtilis* 168 pBAC-B-*basA* strains were induced with the different inducers (Xylose, IPTG, xylose+IPTG and no inducer). LC-MS analysis showed that induced or uninduced *B. subtilis* 168 pBAC-B-*basA* produced bacillusin A, whereas the host strain *B. subtilis* 168 did not produce any bacillusin A. (Figure 6). Bioassay of the 50% diluted filtered supernatant of *B. subtilis* 168 *pbasA* against MRSA30 showed more than 90% inhibition of MRSA30 in 96 well bioassays (Figure 5). It seemed that all the induced and uninduced cultures of *B. subtilis* 168 pBAC-B-*basA* showed the same percent inhibition of MRSA.

The bacillusin A BGC is very large and the architecture of this BGC is such that not many critical genes may be activated by driving transcription from the borders. That could be a reason not observed difference between inducer states. Most importantly, we observed robust basal

expression. LC-MS analysis and anti-MRSA activity of heterologously expressed *B. subtilis* 168 pBAC-B-*basA* confirmed productions of bacillusin A.

5.0 Discussion

Polyketides are a diverse group of secondary metabolites which have potential pharmaceutical value because of their uses in many clinical drugs such as antibiotics, immunosuppressants, cytotoxins, and cholesterol lowering substances (Helfrich, Reiter, & Piel, 2014; Hertweck, 2009; Khosla, 1997; Staunton & Weissman, 2001). These polyketides are produced by the enzyme polyketide synthases (PKSs). Polyketides have a highly diverse structure that includes macrolides, enediynes, polyphenols, polyenes, and other types (Hertweck, 2009).

To identify the BGC responsible for the biosynthesis of bacillusin A, we searched for a type I PKS pathway in *B. velezensis* AP183 through a genome mining tool because bacillusin A, which is a macrocyclic polyene compound, is most likely synthesized by type I modular PKS pathway like many other studies (Low et al., 2018; Park, Nah, Kang, Choi, & Kim, 2021). As expected, with the genome mining tool, we identified a type I modular PKS BGC in *B. velezensis* AP183 that could be responsible for the biosynthesis of bacillusin A. Later, we inactivated the predicted BGC by deleting 44 kb region from this BGC and found that the mutant failed to produce bacillusin A in LC-MS analysis and lost anti-MRSA activity, confirmed that *basA* BGC encodes the necessary enzymes and proteins to make bacillusin A.

Later, we attempted to complement the mutant *B. velezensis* AP183 with pBAC-B-*basA* DNA. To do that, pBAC-B-*basA* DNA was first transformed into adenine and cytosine methylation deficient strains to prevent potential digestion by the restriction modification system of *B. velezensis* AP183. It was not possible to transform the large BGC construct into a demethylated strain of *E. coli* and ultimately no transformation was possible in mutant *B. velezensis* AP183.

As an alternative strategy, the pBAC-B-*basA* was cloned using a CRISPR/Cas9 method and integrated into *B. subtilis* 168 using a bacterial artificial chromosome vector and expressed with and without induction of transcription. We were successfully able to clone the pBAC-B-*basA* into *B. subtilis* 168 and LC-MS analysis found that host *B. subtilis* 168 was able to synthesize bacillusin A at a higher level of production (almost double what was observed for wild-type *B. velezensis* AP183) and the heterologously expressed pBAC-B-*basA* also showed anti-MRSA activity. The successful heterologous expression based on LC-MS and antibacterial activity confirms that *basA* BGC indeed responsible for biosynthesis of bacillusin A.

Based on the available literature, we hypothesized that a cyclase responsible for converting bacillunoic acid to bacillusin A was uniquely present in *B. velezensis* AP183 but absent in *B. velezensis* SQR9. However, when a comparative genomic analysis was conducted to identify any gene(s) uniquely present in *B. velezensis* AP183, no gene was discovered in *B. velezensis* AP183 that was predicted to encode a cyclase enzyme. Most importantly, LC-MS analysis indicated that *B. velezensis* SQR9 produced bacillusin A, which had not been previously reported. Interestingly, many studies revealed that a single BGC can produce more than one compound (Fischbach & Clardy, 2007; Martinet et al., 2019; Yin et al., 2014). These results supported the conclusion that the *basA* BGC is responsible for biosynthesis of both bacillusin A and bacillunoic acid.

In summary, the BGC responsible for biosynthesis of bacillusin A was determined to be *basA* which is responsible for biosynthesis of bacillunoic acids which are a precursor for biosynthesis of bacillusin A, both of which have antimicrobial activity against drug resistant MRSA. In addition to generating a knockout mutant that lacked bacillusin A biosynthesis in *B. velezensis* AP183, the entire large (~70 kb) *basA* BGC was cloned and expressed in *B. subtilis* 168 and was found to produce bacillusin A that had anti-MRSA activity.

Figures and Tables

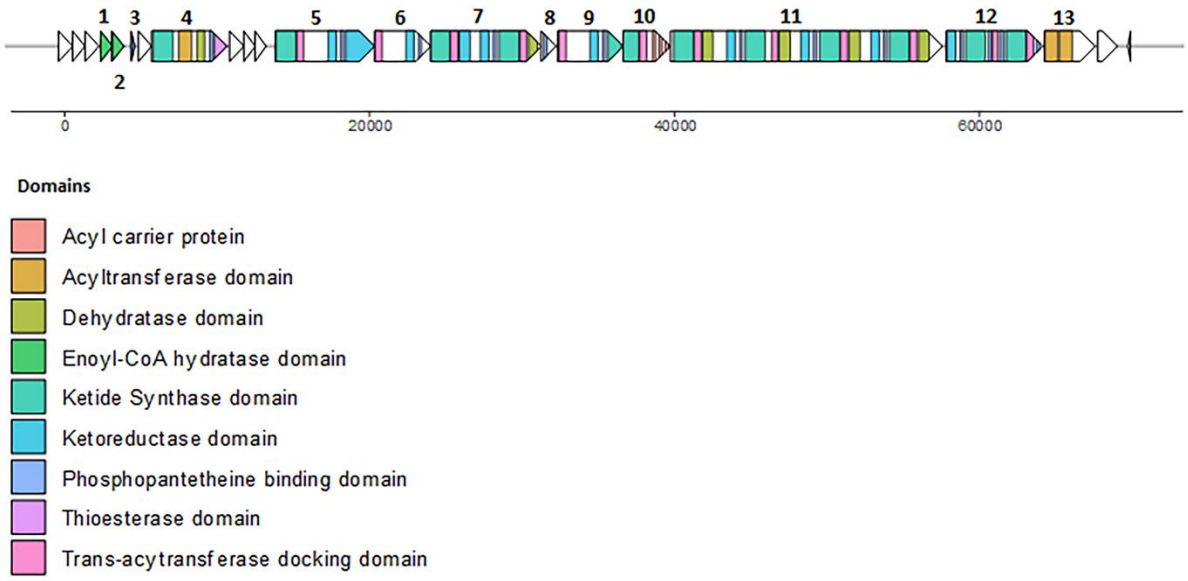

Figure 1. Predicted bacillusin A BGC of *B. velezensis* AP183.

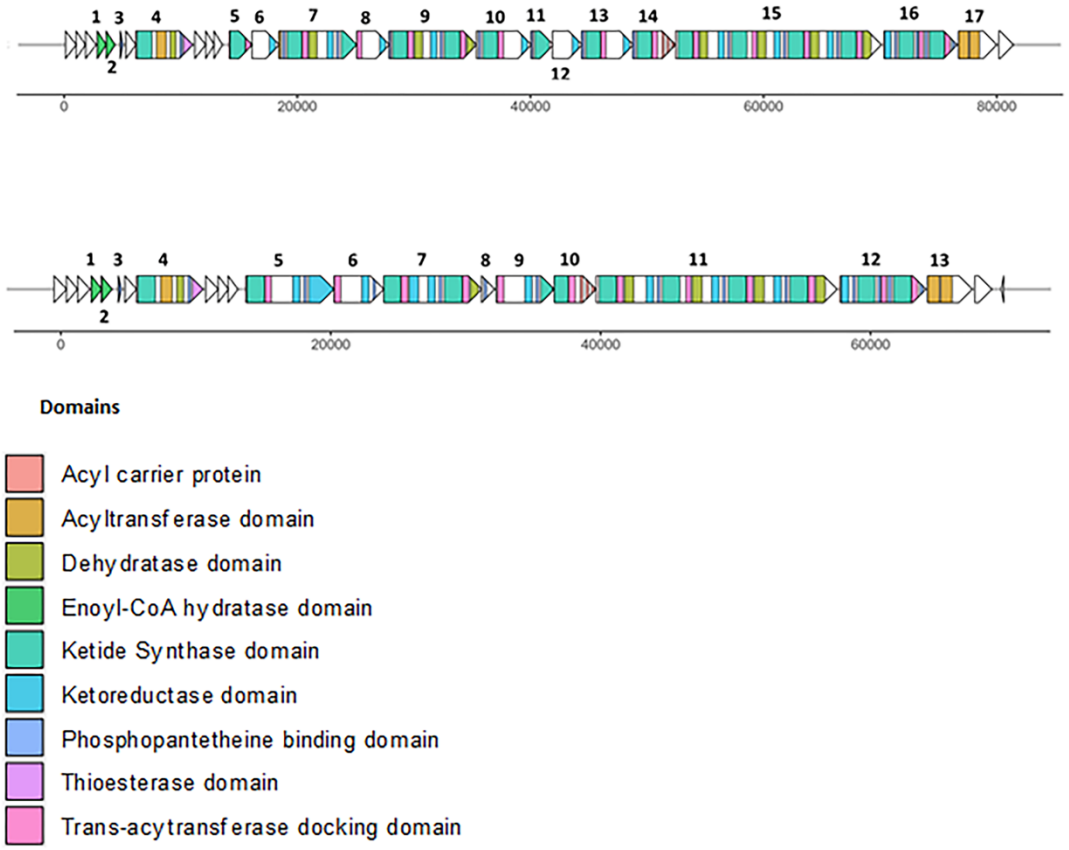

Figure 2. Predicted bacillusin A BGC of *B. velezensis* AP183 and *B. velezensis* SQR9.

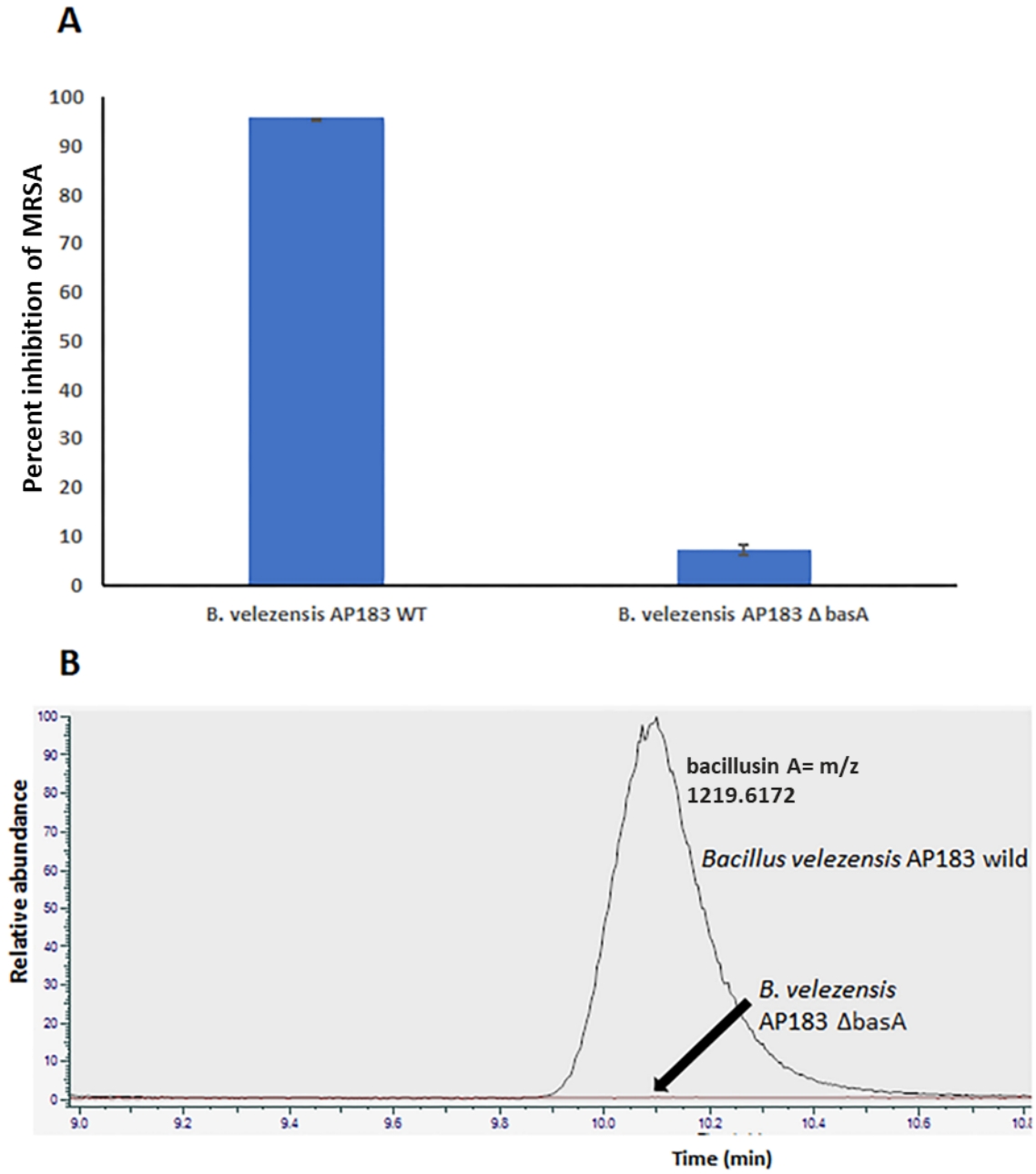

Figure 3. Antibacterial activity of *B. velezensis* AP183 wild type and mutant.

(A) antiMRSA activity of *B. velezensis* AP183 wild and *B. velezensis* AP183 Δ*basA* mutant **(B)** LC-MS chromatograms for the bacillusin A from culture supernatant of the wild-type *B. velezensis* AP183 and *B. velezensis* AP183 *ΔbasA* mutant.

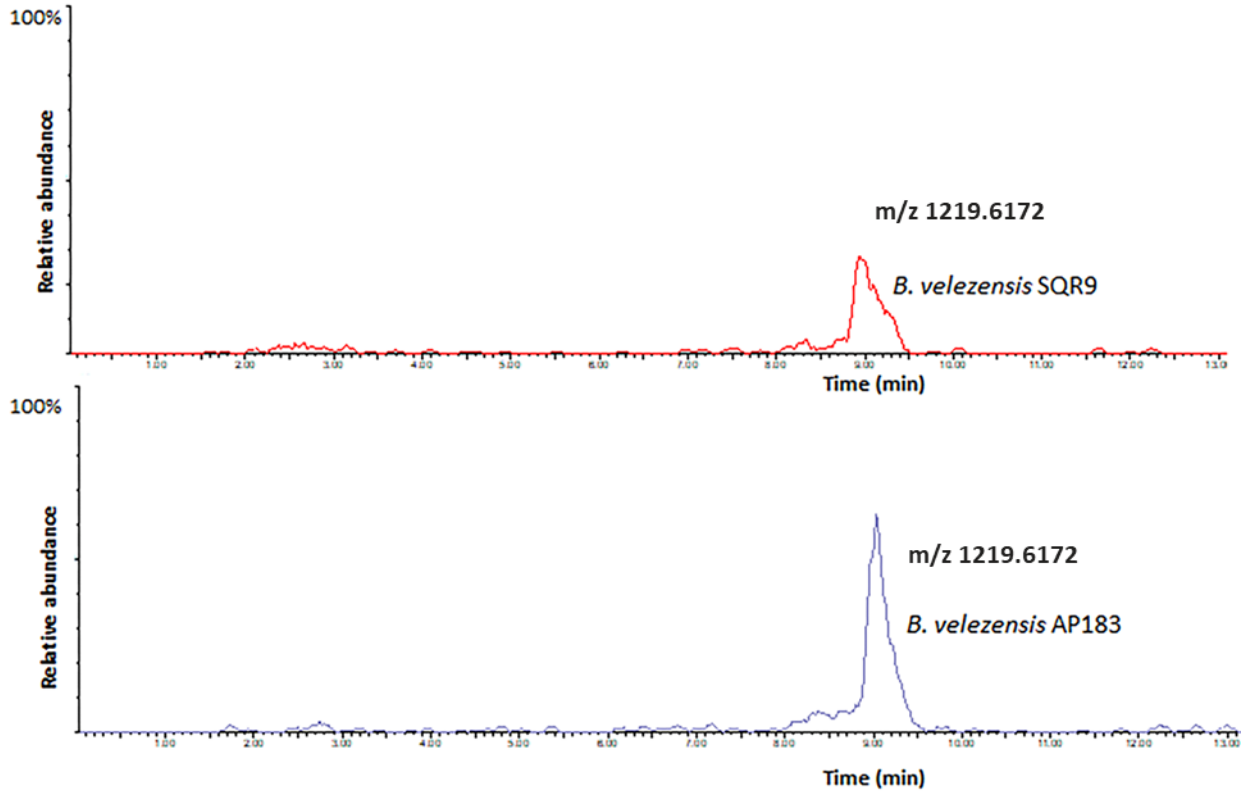

Figure 4. LC-MS chromatograms analysis LC-MS chromatograms of the culture supernatant from the wild-type *B. velezensis* AP183 and *B. velezensis* SQR9 for bacillusin A production.

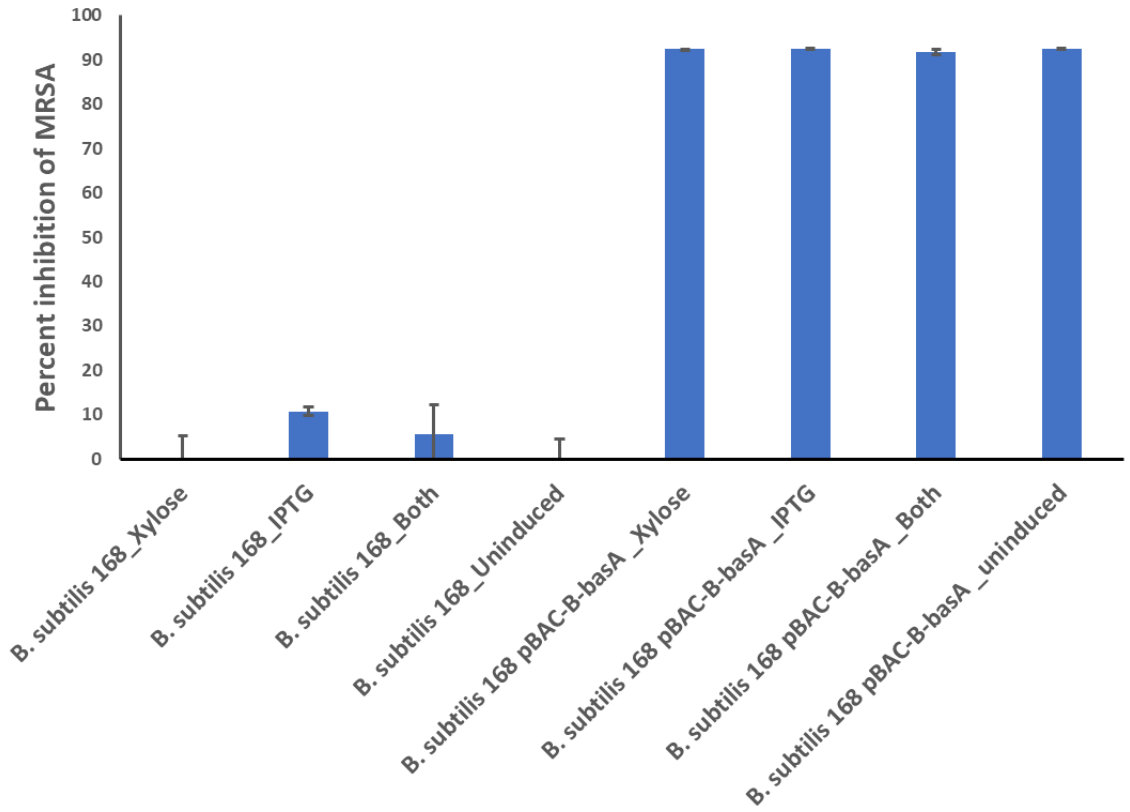

Figure 5. anti-MRSA activity of heterologously produced bacillusin A in *B. subtilis* 168 pBAC-B-*basA* using different inducers.

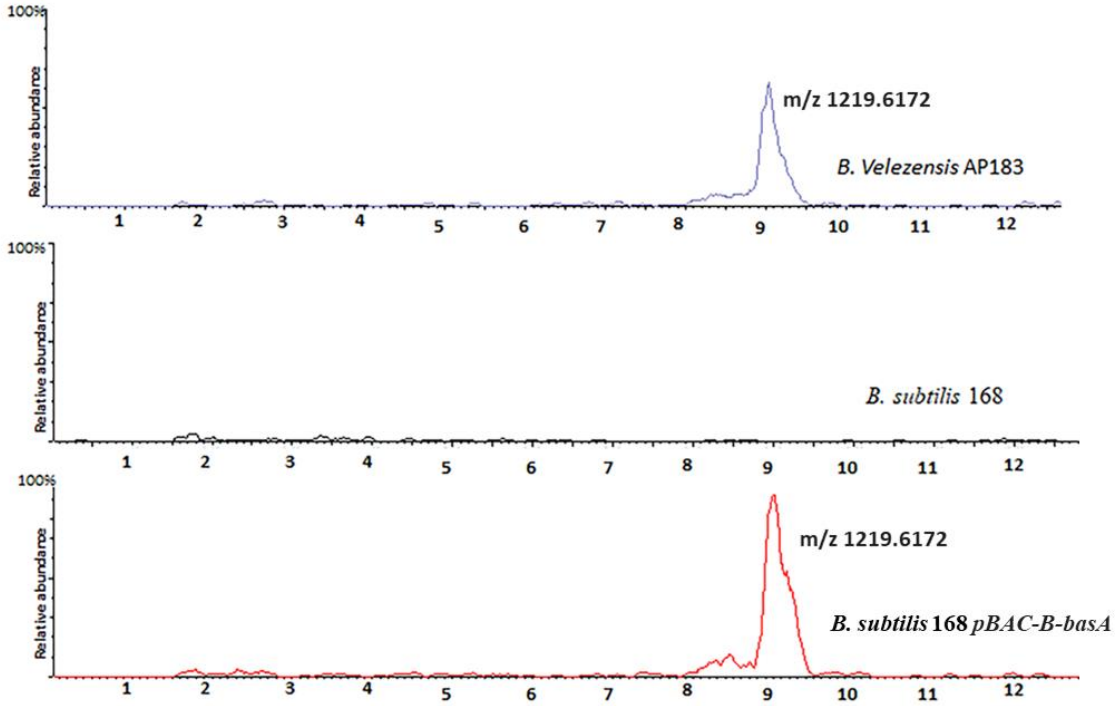

Figure 6. LC-MS chromatograms analysis of culture supernatant from the *B. velezensis* AP183 wild, *B. subtilis* 168, *B. subtilis* 168 pBAC-B-*basA* for bacillusin A production.

Table 1. Microbial strains used in this study.

Organism	Strain	Source or reference
***Bacillus* strains**		
B. velezensis AP183	Bacillusin A producing strain	This study
B. velezensis AP183 ΔbasA	AP183 carrying an in-frame deletion in basA BGC	This study
B. velezensis SQR9	Bacillunoic acid producing strain	Wang et al. 2019
B. subtilis 168	Host for basA BGC	This study
B. subtilis 168 pBAC-B-*basA*	Carrying bacillusin A BGC	This study
***Staphylococcus* strains**		
Methicillin Resistant *S. aureus 30*	Pathogenic indicator organism used in this study	EAMC*, Opelika, AL
***E. coli* strains**		
DH10B	Routine transformation	Thermo fisher
DH5G	Routine transformation	Thermo fisher
INV110	Used to demethylate the plasmid DNA before AP183 transformation	Invitrogen
JM110	Used to demethylate the plasmid DNA before AP183 transformation	Agilent Technologies
2925	Used to demethylate the plasmid DNA before AP183 transformation	NEB

*EAMC- East Alabama Medical Center.

Table 2. Primers used in *basA* BGC knock out experiment.

Primers	Sequence
*BasA*_UP-F	GACCGCATGTTTGGAACAAAATAAGTGCATGGAATGTGGAAGGATTG
*BasA*_UP-R	ATTTGTGTACAATATCTTACGATCCCTGAAGCCGCCAAGG
*BasA*_DN-F	TTCAGGGATCGTAAGATATTGTACACAAATGTTAATGTAG
*BasA*_DN-R	AGTGCCAAGCTTGCATGCCTGCAGGATTTTACGCATCATTTTTCGATGT
*BasA*_OUT-F	AGAACCGATTGGTATCATTGGTATG
*basA*OUT-R	TAAAATCATTCTGAATAACATCGGT
pku-F	GCAGAAGCATCATTCGGACG
pku-R	GCACAGATGCGTAAGGAGAA

Table 3. AntiSMASH-predicted BGCs for *B. velezensis* AP183.

BGC	From	To	Most similar gene cluster	Type
Region 1	307,984	372,843	surfactin (86% of genes show similarity),	NRPS
Region 2	656,902	759,161	aurantinin B / aurantinin C / aurantinin D (25% of genes show similarity)	T1PKS, T3PKS, transAT-PKS
Region 3	980,158	1,021,402	butirosin A / butirosin B (7% of genes show similarity)	PKS like
Region 4	1,107,586	1,124,757	No matches found.	Terpene
Region 5	1,409,768	1,496,157	macrolactin H (100% of genes show similarity)	TransAT PKS
Region 6	1,719,645	1,820,216	bacillaene (100% of genes show similarity)	NRPS, T3PKS, transAT-PKS
Region 7	1,896,942	2,031,159	Fengycin, plipastatin, bacillomycin D, mycosubtilin (100% of genes show similarity)	NRPS, betalactone,trans AT-PKS
Region 8	2,060,908	2,082,791	No matches found.	Terpene
Region 9	2,152,304	2,193,404	No matches found.	T3PKS
Region 10	2,363,822	2,457,616	difficidin (100% of genes show similarity)	Trans AT PKS
Region 11	3,093,444	3,145,236 nt.	bacillibactin	NRPS,RiPP-like
Region 12	3,669,471	3,710,889	bacilysin (100% of genes show similarity),	Other

Table 4: Predicted function of the genes from the bacillusin A BGC.

Gene	Protein length	Identity	Predicted function	Nearest neighbor	E value	Accession
1	774 nt	100%	Enoyl-CoA hydratase	enoyl-CoA hydratase/isomerase [*Bacillus* velezensis]	0	WP_043021024.1
2	795 nt	100%	Enoyl-CoA hydratase	enoyl-CoA hydratase/isomerase family protein [*Bacillus*]	0	WP_038457184
3	243 nt	100%	Acyl carrier protein	acyl carrier protein [*Bacillus* velezensis]	0	WP_043021042
4	4899 nt	100%	Polyketide synthase modules and related proteins	short-chain dehydrogenase [*Bacillus* velezensis]	0	WK45265.1
5	6471 nt	100%	Polyketide synthase modules and related proteins	SDR family NAD(P)-dependent oxidoreductase, partial [*Bacillus* velezensis]	0	WP_162533090.1
6	3666 nt	100%	Modular polyketide synthase	SDR family NAD(P)-dependent oxidoreductase, partial [*Bacillus* velezensis]	0	WP_162533092.1
7	7200 nt)	100%	Modular polyketide synthase	short-chain dehydrogenase/reductase SDR	0	WP_162533093.1

8	960 nt	100%	TransAT-PKS: PP-binding	hypothetical protein, partial [*Bacillus* velezensis]	0	WP_162533094.1
9	4266 nt	100%	Polyketide synthase modules and related proteins	SDR family NAD(P)-dependent oxidoreductase, partial [*Bacillus* velezensis]	0	WP_162533095.1
10	3081 nt	97.95%	Polyketide synthase modules and related proteins	SDR family NAD(P)-dependent oxidoreductase, partial [Bacillus velezensis]	0	AWK45275.1
11	17718 nt	100%	Modular polyketide synthase	SDR family NAD(P)-dependent oxidoreductase [*Bacillus* velezensis]	0	WP_052495564.1
12	6300 nt	100%	Modular polyketide synthase	KR domain-containing protein [*Bacillus* velezensis]	0	AWK45277.1
13	3294 nt	100%	Modular polyketide synthase	ACP S-malonyltransferase [*Bacillus* velezensis]	0	WP_043021002.1

Chapter III

Bacillus velezensis AP183 inhibits *Staphylococcus aureus* biofilm formation and proliferation in murine and bovine disease models

1 Abstract

The increasing frequency of *Staphylococcus aureus* antimicrobial resistance has spurred interest in identifying alternative therapeutants. We investigated the *S. aureus*-inhibitory capacity of *Bacillus velezensis* strains in mouse and bovine models. Among multiple *B. velezensis* strains that inhibited *S. aureus* growth *in vitro*, *B. velezensis* AP183 provided the most potent inhibition of *S. aureus* proliferation and bioluminescence in a mouse cutaneous wound ($P = 0.0198$). Histology revealed abundant Gram-positive cocci in control wounds that were reduced in *B. velezensis* AP183-treated tissues. Experiments were then conducted to evaluate the ability of *B. velezensis* AP183 to prevent *S. aureus* biofilm formation on a tracheostomy tube substrate. *B. velezensis* AP183 could form a biofilm on a tracheostomy tube inner cannula substrate, and that this biofilm was antagonistic to *S. aureus* colonization. *B. velezensis* AP183 was also observed to inhibit the growth of *S. aureus* isolates originated from bovine mastitis cases. To evaluate the inflammatory response of mammary tissue to intramammary inoculation with *B. velezensis* AP183, we used high dose and low dose inocula in dairy cows. At the high dose, a significant increase in somatic cell count (SCC) and clinical mastitis was observed at all post-inoculation time points ($P < 0.01$), which resolved quickly compared to *S. aureus*-induced mastitis; in contrast, the lower dose of *B. velezensis* AP183 resulted in a slight increase of SCC and no clinical mastitis. In a subsequent experiment, all mammary quarters in four cows were induced to have grade 1 clinical mastitis by intramammary inoculation of a *S. aureus* mastitis isolate; following mastitis induction, eight quarters were treated with *B. velezensis* AP183 and milk samples were collected from

pretreatment and post-treatment samples for nine days. In groups treated with *B. velezensis* AP183, SCC and abundance of *S. aureus* decreased with significant reductions in *S. aureus* after three days post-inoculation with AP183 (P = 0.04). A milk microbiome analysis revealed significant reductions in *S. aureus* relative abundance in the AP183-treated group by eight days post-inoculation (P = 0.02). These data indicate that *B. velezensis* AP183 can inhibit *S. aureus* biofilm formation and its proliferation in murine and bovine disease models.

2 Importance

S. aureus, an opportunistic pathogen that is commonly resistant to antibiotic treatment, is responsible for potentially life-threatening infections in human and veterinary medicine. In this study, we present for the first-time *in vivo* studies demonstrating the inhibition of *S. aureus* by *B. velezensis* AP183 in a mouse cutaneous wound model, and in a dairy cow mastitis model. Biofilms formed by *B. velezensis* AP183 on a tracheostomy tube inner cannula substrate were resistant to colonization by *S. aureus.* Furthermore, a low dose inoculum of *B. velezensis* AP183 inoculated into bovine mammary tissue did not induce clinical mastitis but did significantly reduce the *Staphylococcus* relative abundance within a milk microbiome. The results of this study provide support for the use of *B. velezensis* AP183 and/or its metabolites as an alternative to traditional antibiotics for the treatment of *S. aureus* infection in humans and animals.

3 Introduction

S. aureus is commonly responsible for cutaneous infections in human and veterinary medicine, with the rising prevalence of antibiotic resistant strains prompting the need for novel strategies to combat infection (Y. Guo, Song, Sun, Wang, & Wang, 2020). Damage to the

integument from injury, including surgical implants such as tracheostomy tubes (Cheikh, Barbosa, Caixêta, & Avelino, 2018), creates an opportunity for opportunistic pathogens like *S. aureus* to colonize and often precedes systemic infection (Ki & Rotstein, 2008). Asymptomatic carriage of *S. aureus* in humans is estimated to be approximately 20 percent, though the pathogen is common to a variety of acute and chronic skin pathologies including impetigo, cellulitis, furuncles, scalded skin syndrome, and mastitis (Delgado et al., 2011; Iwatsuki, Yamasaki, Morizane, & Oono, 2006). Cutaneous infections are burdensome in agriculture as well, with bovine mastitis alone resulting in an estimated annual cost exceeding $1.7 billion to the dairy industry and *S. aureus* as the causative pathogen in 20% of cases (Shim, Shanks, & Morin, 2004; Wells & Ott, 1998). Intramammary infection (IMI) resulting in mastitis poses significant financial and production losses in dairy cows and other dairy species, including decreased milk production, increased culling rates, and increased production costs due to treatment (Halasa, Huijps, Østerås, & Hogeveen, 2007; Heikkilä, Nousiainen, & Pyörälä, 2012; Leslie & Petersson-Wolfe, 2012; Mekibib, Furgasa, Abunna, Megersa, & Regassa, 2010; Ruegg, 2017). The primary causative agents of IMI are bacterial, including *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus uberis*, and *Escherichia coli* (Barkema, Green, Bradley, & Zadoks, 2009; Piessens et al., 2011; Supré et al., 2011). Coagulase-negative staphylococci are frequently associated with IMI (Piessens et al., 2011) while coagulase-positive *Staphylococcus* spp., such as *S. aureus*, can induce acute and chronic IMI that are often difficult to control (Almeida, Matthews, Cifrian, Guidry, & Oliver, 1996; Bouchard, Rault, Berkova, Le Loir, & Even, 2013; Contreras & Rodríguez, 2011; Roberson). Antibiotic use remains the most common treatment against mastitis (Barlow, 2011); however, the use of antibiotics may not only foster bacterial antimicrobial resistance, but can also cause contamination of milk and meat, potentially

contributing to dissemination of antibiotic resistance in the environment and in humans (Oliver & Murinda, 2012; Sharma et al., 2018).

The frequency of antibiotic resistance among *S. aureus* strains is of particular concern and may be the result of the acquisition of mobile genetic elements encoding antibiotic resistance (Davis et al., 2016). More than 80,000 severe human infections are caused every year in the United States from MRSA (Solomon & Oliver, 2014). Worldwide, this has become the most common infection due to drug-resistant pathogens, with reservoirs of drug-resistant *S. aureus* within hospitals and in livestock (Lakhundi & Zhang, 2018). Mounting concerns have recently led to updated guidance for antibiotic use in livestock by the Food and Drug Administration, including a federal government initiative to promote voluntary elimination of antibiotics in livestock for growth purposes (NSP, 2016). The rapidly growing threat of antibiotic resistant pathogens in humans and the impending restrictions on antibiotic use in livestock illustrate the need for innovative strategies for controlling the spread of antibiotic-resistant pathogens.

Probiotics may be an effective alternative to antibiotics to prevent or treat *S. aureus* infections. For example, using probiotic treatment against *S. aureus* infections includes the ability of the probiotic to effect pathogen inhibition by multiple mechanisms, including competitive exclusion (Hojjati, Behabahani, & Falah, 2020), direct antagonism via metabolites (Mohamed, Elmohamady, Abdelrahman, Amer, & Abdelhamid, 2020), and/or stimulation of the host immune response (Liu et al., 2020). The multifactorial action of probiotics has also been shown to preclude the development of pathogen resistance; for example, studies of intramammary infusion with different *Lactobacillus* spp. (*L. lactis, L. perolens, L. rhamnosus, L. brevis,* and *L. plantarum*) were shown to be effective agents in the prevention of and treatment of mastitis (Bouchard et al., 2013; Camperio et al., 2017; Frola et al., 2012; Gomes & Henriques, 2016; Mignacca et al., 2017). Other

results indicate that *Lactobacillus* strains are not effective in decreasing bovine mastitis and instead initiate an inflammatory response (Frola et al., 2013). So far, few studies have assessed the *in vivo* effects of probiotics in the mammary gland. Catozzi *et al*. investigated intramammary treatment with *L. rhamnosus* in water buffalo and observed transient pro-inflammatory activity and modification of the milk microbiota (Catozzi et al., 2019). Most of these studies did not evaluate efficacy in chronic clinical mastitis cases. Moreover, the inhibitory activities of these previously evaluated probiotic strains were not observed to be highly potent against major mastitis pathogens such as *S. aureus*. To develop an effective probiotic strategy against mastitis, identification of novel probiotics with potent antagonistic activity is crucial, and there is a benefit in using spore-forming species in the *B. subtilis* group, including the species *B. amyloliquefaciens* and *B. velezensis*, that have a long shelf-life, produce many bioactive metabolites and are known to be safely used in agriculture and aquaculture (Cook et al., 1995; Fan et al., 2018; Hossain et al., 2015; Kaspar, Neubauer, & Gimpel, 2019; Kloepper, Leong, Teintze, & Schroth, 1980; Ngalimat et al., 2021; Ongena & Jacques, 2008; Ran et al., 2012), but few strains have been examined for potential clinical applications (Geeraerts, Ducatelle, Haesebrouck, & Van Immerseel, 2015; Y. Wang et al., 2017).

Our previous research screened 177 spore-forming *Bacillus* spp. isolates that were primarily cultivated from plant rhizospheres for the ability to inhibit MRSA growth, from an extensive microbial collection containing thousands of unique isolates cultivated at Auburn University by the research group of Prof. Joseph Kloepper (Melnick, Bailey, & Backman, 2013). Among these MRSA-inhibiting isolates, rhizosphere-derived *B. velezensis* AP183 was found to produce bacillusin A, a novel and potent macrodiolide antibiotic capable of inhibiting methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus faecium* (Ravu et al., 2015). In addition

to bacillusin A, *B. velezensis* AP183 and many of these other *Bacillus* spp. strains are known to produce other metabolites with anti-bacterial (e.g. difficidin, surfactin, diketopiperazine, and macrolactin) and anti-fungal (e.g. iturin) activities (Ravu et al., 2015). Interestingly, bacillusin A was shown to be highly labile, suggesting that the *in vivo* effects of this strain would be localized and transient; however, while each of these *Bacillus* spp. strains produced secondary metabolite(s) under *in vitro* conditions that inhibited MRSA growth, none of these *Bacillus* spp. strains had been evaluated for their ability to prevent *S. aureus* infection under *in vivo* conditions. In this study, we began our investigation by testing five different *Bacillus* spp. strains for their respective ability to prevent *S. aureus* infection in a murine cutaneous wound model, as a first step toward investigation of the use of a specific *Bacillus* spp. strain for biocontrol of *S. aureus* infections in clinical and veterinary medicine.

4 Materials and Methods

4.1 Microorganisms and Growth Conditions - *Mouse Experiments*

A commercially available *S. aureus* Xen29 (PerkinElmer Inc., Waltham, MA) was used for *in vivo* cutaneous wound challenge studies **(Table 1)**. *S. aureus* Xen29 from glycerol stocks stored at -80°C were streaked for isolation onto Brain-Heart Infusion (BHI) (Research Products International Corp., Mt. Prospect, IL) agar plates containing 50 μg/mL of kanamycin monosulfate (Research Products International Corp., Mt. Prospect, IL) and incubated for 16 h at 37 °C. Following incubation, plates were imaged using a LAS-1000 luminescent image analyzer (FUJIFILM Corporation) to confirm bioluminescence. One colony was selected to inoculate a flask containing 50 mL of BHI containing 50 μg/mL of kanamycin monosulfate. The culture was incubated at 37 ^{o}C for 16 h, shaking at 225 RPM. The bacterial suspension was subjected to centrifugation at 2,200 × *g* for 15 min at room temp. The supernatant was discarded, and the pellet was resuspended in 20

mL of sterile-filtered 1× PBS, followed by centrifugation at 2,200 × *g* for 15 min at room temp. The supernatant was again discarded, and the pellet was suspended in 5 mL of sterile-filtered 1× PBS with 10% glycerol. Serial dilutions of the bacterial suspension were diluted into sterile-filtered PBS with 10% glycerol and the optical density was determined at 600 nm (OD_{600}). The concentration of bacterial suspensions was calculated using the Beer-Lambert Law, wherein the molar absorptivity constant for *S. aureus* was assumed to be 1.44×10^8 CFU/mL. The bacterial suspension was adjusted to a final concentration of approximately 1.0×10^8 CFU/mL.

Bacillus spp. strains were cryopreserved in Tryptic Soy Broth (TSB) containing 20% glycerol. Each strain was grown on Tryptic Soy Agar (TSA) overnight at 30 ºC to obtain isolated colonies. *Bacillus* spp. spores used for *in viv*o challenge were prepared by inoculating an isolated bacterial colony into a 20 mL culture tube containing 5.0 mL of TSB and incubated on a rotatory shaker at 200 RPM at 30 °C for five days, after which the vegetative cells were killed by heating at 80 ºC for 20 min, and the CFU/mL were determined by serial dilution and plating onto TSA. To prepare for injection, spores were dispensed into 1.0 mL aliquots with 10% glycerol.

4.2 *In Vivo* Cutaneous Wound Model

Experimental protocols were reviewed, approved, and performed under regulatory supervision of Auburn University's Institutional Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC), under IACUC protocols 2011-2006 and 2016-2988. For this study, 22 female C57BL/6J mice at 6-8 weeks old were anesthetized by 1-3% isoflurane gas and hair was shaven with electronic clippers and depilated using Veet® (Reckitt Benckiser Group, Berkshire, England). Treatment groups (n=3 mice per *Bacillus* strain) were injected subcutaneously between the shoulder blades with a 20 μL suspension comprised of 10 μL 1.0×10^8 CFU/mL *S. aureus*

Xen29 and 10 μL of 1×10^8 CFU/mL *B. velezensis* spores with metabolites (total of 1.0×10^6 CFUs) for strains AB01, AP143, AP183, AP191 and AP218. Internal controls were administered by subcutaneous injection of 1.0×10^6 CFUs of *S. aureus* Xen29 in a volume of 10 μL on the lower back. Untreated control mice (n=3) were given subcutaneous injections of 1.0×10^6 CFUs of *S. aureus* Xen29 between the shoulder blades and the lower back. In a subsequent experiment, a group of mice was administered subcutaneous injections of AP183 spores (1×10^6 CFU/mL) with associated metabolites between the shoulder blades and lower back and another group was injected subcutaneously with metabolites only between the shoulder blades and lower back of each animal. At the end point of the experiment at six days post-inoculation, all mice were sacrificed by CO_2 asphyxiation, followed by cervical dislocation, and wound samples were harvested for histological analysis and homogenized for CFU determination.

4.3 *In Vivo* Imaging Procedures

All *in vivo* imaging was performed using an IVIS Lumina XRMS (Caliper Life Sciences). Mice were anesthetized by 1-3% isoflurane gas and placed in the imaging chamber of the IVIS Lumina XRMS. Bioluminescence exposures were 5 min with medium binning, *f-2* aperture, and field of view D. All mice were imaged once per day for a period of 7 days. At the endpoint, mice were sacrificed according to IACUC protocol guidelines and wound sites were excised and stored in 1× PBS. Wound samples were split in half, with one half embedded for histological analysis and the other half homogenized for DNA isolation (see DNA isolation section below). to enable 16S rRNA gene amplicon sequencing and serial dilutions were plated to determine final CFU/mg of tissue.

4.4 Evaluation of AP183 Formulations for *In Vivo* Administration

To determine the optimal formulation for administration of AP183 to wound sites, three formulations were prepared and tested *in vivo* for inhibition of *S. aureus* proliferation. AP183 spores (1.0×10^6 CFUs), supernatant containing AP183 metabolites without spores, and AP183 spores with supernatant (1.0×10^6 CFUs) were co-administered with 1.0×10^6 CFUs *S. aureus* Xen29. Mice (n = 3 per group) in a volume of 10 μL were imaged for bioluminescence once a day for 6 days. At the endpoint, mice were sacrificed by CO_2 asphyxiation and cervical dislocation. Wound tissue was resected, homogenized, and diluted followed by plating on TSA and incubated for 18 h at 37 °C. *S. aureus* colonies were counted by bioluminescent imaging to determine CFU counts. Plates were incubated for 18 h at 37 °C and bioluminescence imaging was performed on each plate to allow counting of luminescent colonies and ultimately determine CFUs present in each wound. The other half of each wound was prepared for histological Gram and hematoxylin and eosin (H&E) staining.

4.5 Tissue Staining and Histological Analysis

Wound tissue from two mice from each treatment or control group was embedded in clear frozen section compound (VWR International, West Chester, PA) medium and fixed by submersing in a metal container with 2-methylbutane (Alfa Aesar, Haverhill, MA) on dry ice. Sectioning was performed using a Microm HM 525 (Thermo Fisher Scientific Inc.) with a section thickness of 0.6 μM per slice. Alternating slices were stained with either the following Gram staining or H&E staining procedures. Microscopy was performed using a Zeiss Axioskop with Plan-NEOFLUAR objective lenses and Nikon Sight DS-Fi2 digital camera.

Gram staining of slides was performed by submersion in crystal violet (EMD Millipore, Billerica, MA) for 15 sec, followed by gentle rinsing with tap water, submersion in Gram's Iodine

(EMD Millipore, Billerica, MA) for 15 sec, tap water rinse, submersion in tartrazine (EMD Millipore, Billerica, MA) solution for 15 sec, and a final tap water rinse. Slides rinsed with 95% alcohol and allowed to dry, then dripped with xylene (EMD Millipore, Billerica, MA) and allowed to dry before a coverslip with Cytoseal™ (Thermo Fisher Scientific Inc.) was placed over each slide.

H&E staining was performed by submersing slides in hematoxylin (EMD Millipore, Billerica, MA) and placed on a rocker for 15 min, then rinsed in tap water with gentle shaking for 15 min. Slides were submersed in 95% alcohol for 30 sec and submersed into eosin (VWR International, West Chester, PA) for 45 sec. Slides were then submerged into 95% alcohol for 1 min, followed by submersion in 100% alcohol for 1 min. Slides were then submersed in xylene for 1 min and allowed to air dry. A coverslip with Cytoseal™ (Thermo Fisher Scientific Inc.) was then placed over each slide prior to microscopic examination.

4.6 Microorganisms and Growth Conditions - *Biofilm Experiment*

A single colony of *B. velezensis* AP183 was inoculated into 3 mL of TSB and cultivated overnight at 37 ºC with shaking at 250 rpm. Small (~3 mm) sections of a Shiley™ tracheostomy tube inner cannula (Medtronic, Minneapolis, MN) was sterilized in 70% ethanol and dried prior to placing into a culture tube containing 3 mL of TSB. Then 100 µL of the overnight culture of *B. velezensis* AP183 was added to the tube and incubated for 72 h at 37 ºC. The supernatant was then removed, and planktonic cells were removed by washing the inner cannula sections three times in 1× phosphate-buffered saline. After washing the inner cannula sections, 100 µL of an overnight TSB culture of *S. aureus* NE1260F (Bose, Fey, & Bayles, 2013) was added to each tube and incubated at 37 ºC with shaking at 250 RPM for 24 h. The experiment was conducted in triplicate, and a negative control was included that only had 100 µL of *S. aureus* culture added to the inner cannula

sections, which were incubated at 37 ºC with shaking at 250 RPM for 24 h. The next day each inner cannula section was washed three times in 1× phosphate-buffered saline and DNA was extracted from each section (see DNA isolation section below).

4.7 Evaluation of carbon sources on *Bacillus velezensis* AP183 inhibition of MRSA colonization

In this experiment the competition between *B. velezensis* AP183 and *S. aureus* was evaluated when log-phase cultures were co-inoculated in a 96-well plate containing a defined M9 salts minimal medium containing different carbon sources. Two of the carbon sources (glucose and sucrose) could be utilized for growth by both cultures, whereas pectin could only be utilized by *B. velezensis* AP183 (Hassan et al., 2019). In this experiment a thin film of the carbon source being evaluated (pectin, glucose, or sucrose) was prepared in 96 well plates by evaporative drying from a saturated solution. Each solution was prepared by mixing the respective carbon source with sterilized DI water for 24 h under magnetic stirring. The required amount of solution was then pipetted into the plate wells and the plate dried at 30 °C for 24 h. The amount of carbon source added ranged from 1% to 20% (w/v) with four replicates per each concentration. *B. velezensis* AP183 and *S. aureus* NE1260F were inoculated into separate 2 mL TSB cultures and incubated overnight at 37 ºC with shaking at 250 rpm. The cultures were normalized to OD_{600} = 1.0 and then 1 mL was removed, subjected to centrifugation at 10,000 × *g* and resuspended in 1 mL of M9 minimal salts medium. The *B. velezensis* AP183 and *S. aureus* NE1260F cells were then diluted 1:100 into the same tube of M9 medium, and 20 μL of this stock was added to each well of the 96-well plate containing different carbon sources (for a total 1:1000 dilution, approximately 10^6 CFU of each culture in each well) with a total volume of 200 μL per well. The 96-well plate was incubated for 72 h at 37 ºC with shaking at 250 rpm and then each well was serially diluted and plated on TSA to determine

CFU/mL for each of the two cultures, which were discriminated based on their unique colony morphologies. Due to the expected inhibition of *S. aureus* viability by *B. velezensis* AP183 when grown on TSA at high densities, only plates with lower numbers of CFUs (<100) were selected for calculation of CFU/mL.

4.8 Microorganisms and Growth Conditions – *Dairy Experiments*

The bacterial strains used in this study included the mastitis isolate *S. aureus* RF122 and other bacterial isolates isolated from clinical mastitis cases (Table 1). All *Bacillus* and *Staphylococcus* cultures were cryopreserved in TSB containing 20% glycerol and isolated colonies obtained on TSA. The antimicrobial activity of *B. velezensis* AP183 against bacteria isolated from mastitis cases (**Table 1**) was assayed using a soft agar overlay technique. The assay was performed in triplicate and inhibitory activity was quantified by measuring the zone of inhibition in mm.

To generate a *S. aureus* RF122 culture for intramammary inoculation, a single colony was inoculated into 3 mL of TSB and cultivated at 37 ºC for 16 h followed by transfer of 1 mL of the bacterial culture to 100 mL of TSB and incubated overnight at 37 ºC with shaking. After overnight incubation, the culture was normalized to an OD_{600} of 0.5 and then diluted to an approximate concentration of 10^2 CFU/mL and 10 mL of this diluted culture was used for the intramammary inoculation of bovine quarters.

For cultivation of *B. velezensis* AP183, an isolated colony was inoculated into 3 mL of TSB and grown at 30 ºC for 18 h followed by transferring 1 mL of the bacterial culture to 100 mL of TSB. After overnight incubation at 30 ºC with shaking, the final approximate concentration of the bacterial suspension was 10^8 CFU/mL and 10 mL of this dose was used directly for the high dose *B. velezensis* AP183 treatment containing 10^9 CFU total. For the low dose treatment, the overnight culture was diluted to 10^2 CFU/mL and 10 ml was used for intramammary inoculation

that contained 10^3 CFU total. To produce spores of *B. velezensis* AP183, a TSB culture was used to inoculate 10 spore preparation agar plates using a cotton swab and incubated for 10 days at 30°C. The spores were harvested, and the spore suspension was heat treated at 80 °C for 20 min to kill vegetative cells. The concentration of the spore suspension was determined by serially diluting the spore suspension in sterile water and incubating in TSA plates for overnight at 30 °C. The spore suspension was diluted to 10^4 CFU/mL and were preserved at 4 °C until further use. On the experimental day (D_0), the final concentration of the spore suspension was adjusted to 10^2 CFU/mL with sterile water and used for intramammary inoculation.

4.9 Experimental design for intramammary challenge and sample collection

The experimental protocol was approved by Auburn University Institutional Animal Care & Use Committee (IACUC Protocol Number: 2017-3120). This study was conducted on four first-lactation Holstein dairy cows, age two years, at the Auburn University College of Veterinary Medicine. Animal health status was monitored clinically, and milk quality was monitored by bacteriological culture and somatic cell count analysis of individual quarter milk samples.

Prior to evaluating interactions between *B. velezensis* AP183 and *S. aureus* within mammary tissue, experiments were conducted to assess the impact of *B. velezensis* AP183 alone on mammary inflammatory responses.

In the first experiment a high dose inoculum of *B. velezensis* AP183 (10 mL of a 10^8 CFU/mL bacterial suspension) was used for an intramammary inoculation in four animals, with half of the 16 healthy mammary glands (a fore and a rear quarter from each cow) inoculated and the other uninoculated quarters considered as negative controls. Surface cleaning was performed with a dry paper towel and a commercial teat dip containing 1% povidone iodine disinfectant for 30 sec, followed by disinfecting the teat ends using isopropyl alcohol swabs, milk samples were

collected every day from one day prior to inoculation (D_1), inoculation day (D_0) and at the post inoculation days from one to fifteen (i.e. D_1 to D_{15}) in 15 mL conical tubes. Milk samples were immediately placed on ice and then transferred to the laboratory for analysis of microbiological counts on TSA, SCC, fat, protein, lactose, and milk solids-not-fat (SNF) content.

In a second experiment, a lower dose inoculum of *Bacillus* AP183 (10 mL of a 10^2 CFU/mL) was administered intramammary in six healthy bovines, as described above. Milk samples were collected on inoculation day (D_0) and at the post inoculation days from D_1 to D_4, for determination of *Bacillus* CFU counts, SCC, and other raw components. The procedure for quarters disinfection, milk collection, transportation and analysis were the same as described above for the higher dose study.

To investigate the impact of *B. velezensis* AP183 on the microbiology and mammary inflammatory response during *S. aureus*-induced clinical mastitis, 10 mL of a 10^2 CFU/mL *S. aureus* RF122 culture were administered intramammary in all quarters of four animals, one day prior to low dose *B. velezensis* inoculation (D_{-1}). At time D_0, half of the quarters were subjected to intramammary inoculation with 10 mL of a 10^2 CFU/mL *B. velezensis* AP183 suspension. Milk samples were collected every day starting the day prior to *S. aureus* inoculation (D_{-2}) and continuing every day to nine days post-inoculation (i.e. D_9). The sample handling protocol was the same procedure described above for milk sample collection, transport, and analysis. In addition, the milk samples collected from this *S. aureus*-induced mastitis experiment were aliquoted into microcentrifuge tubes and stored at -80^{o}C until processed for microbiome analysis for samples D_0, D_2, D_5, and D_8.

4.10 LC-MS analysis of milk samples

Milk samples from the high dose *B. velezensis* AP183 study were analyzed for bacillusin A using LC-MS at the Auburn University Mass Spectrometry Lab, using purified bacillusin A diluted into milk known to be without exposure to the macrodiolide. The milk samples and standards were mixed with ice cold acetone (100 µL milk and 400 µL acetone), vortexed for 30 sec, frozen for 15 min, subjected to centrifugation at 10,000 × *g* for 5 min, and decanted. Exposure to light was minimized during all sample and standard handling steps. The liquid was evaporated for 4 h in a Thermo Savant DNA 120 speed vac concentrator without any heating. The milk standards and samples were reconstituted in 50 µL of 70% water and 30% acetonitrile, vortexed, and incubated for 5 min in a water bath ultrasonicator, and subjected to centrifugation at 10,000 × *g* for 5 min. The supernatant was analyzed using an ultra-performance LC system (ACQUITY, Waters Corp., USA) coupled with a quadrupole time-of-flight mass spectrometer (Q-Tof Premier, Waters) with electrospray ionization (ESI) in negative mode using Masslynx software (V4.1). Injection of 5 µL of the sample or standard was made on a C18 column (ACQUITY UPLC® BEH C18, 1.7 µm, 1 × 50 mm, Waters) with a 200 µL/min flow rate of mobile phase solution A (0.1% formic acid in 95% water and 5% acetonitrile) and solution B (0.1% formic acid in 95% acetonitrile and 5% H_2O) beginning at 35% B, held for 1 min followed by a linear ramp to 50% B in 9 min, then to 100% B at 11 min, held 2 min, and back to 35%B with 6 min of re-equilibration. The MS spectral range was 120-1300 m/z with a scan time of 0.1 s and 0.05 interscan delay. The 597.3 ion was chosen for MSMS with maximum sensitivity, collision energy of 30 eV, LM resolution of 4.7, and 0.3s scan and 0.05s interscan delay. The capillary voltage was set at 2.8 kV, the sample cone voltage was 30 V, and the extraction cone was 4.0 V. The source and desolvation temperature were maintained at 95 and 400 °C, respectively, with the desolvation gas flow at 600 L/h. The lock mass

was used to correct instrument accuracy with a 0.1 µM solution of HP 1221 (Agilent part number G1969-85003).

4.11 Dairy cow clinical observations.

Cows were pastured with the Auburn University Large Animal Teaching Hospital dairy herd and fed the same ration as the remainder of the herd. Milking was carried out twice daily in a milking parlor. The California mastitis test was performed on each quarter at the time of morning milking and milk was collected for laboratory submission to quantify somatic cell counts, percent lactose, percent protein, percent fat, and percent SNF and bacterial culture. A daily physical examination was conducted immediately following milking each morning with the cows restrained in self-locking stanchions in the feeding area.

4.12 Bacterial and SCC counting and raw milk component analysis.

Milk samples were analyzed for the determination of SCC by flow cytometry utilizing a Somacount FCM (Bentley Instruments Chaska, Minnesota) and other milk components including lactose, protein, fat, SNF were analyzed using a FTS Fourier Transform Spectrometer (Bentley Instruments) at the Mid-South Dairy Records Laboratory (Springfield, Missouri). Microbiological enumeration was performed by culture plate count method by serial dilution and plating for CFUs on TSA as described above.

4.13 Genomic DNA isolation from Wound Homogenates, Biofilm samples and Milk Samples

Following endpoint procedures, wounds for each respective treatment or control group were excised using a sterile scalpel for homogenization and extraction of genomic DNA as described previously (Nasrin, 2015). In the case of biofilm formed on inner cannula sections, the sections were removed sterilely and placed into DNA isolation tubes. Genomic DNA was extracted from

wound homogenates and biofilm samples using an ultraclean microbial DNA isolation kit following the manufacturer's instruction (MO BIO laboratories Inc., Carlsbad, CA USA). For milk samples, genomic DNA was isolated using a milk bacterial DNA isolation kit (Norgen Biotek, Thorold, ON). Briefly, the milk samples were subjected to centrifugation at 20,000 × g for 2 min using a Microfuge 22R (Beckman Coulter Life Sciences, Indianapolis, IN). The pellet was resuspended and used for DNA isolation according to the manufacturer's protocol. The purity of the DNA from each sample was determined using a TECAN infinite M1000 PRO and the DNA samples were quantified using Qubit dsDNA HS Assay kit and read with a Qubit 2.0 fluorometer (Invitrogen, Life Technologies) according to manufacturer's instructions and stored at -20 ºC until further use.

4.14 Bacterial relative abundance determination by 16S rRNA gene amplicon sequencing.

Each genomic DNA sample was used as a template for 16S rRNA gene sequencing using the "universal bacteria" 16S rRNA gene bacterial primer set 515F/926R that targets the hypervariable V4 and V5 regions and a high-fidelity polymerase to generate amplicons for sequencing using a MiSeq sequencer (Illumina, San Diego, CA), with a 2 x 300 paired-end v2 sequencing kit and a 30% phiX control, at the University of Illinois at Chicago's Sequencing Core. Changes in the relative abundance of bacterial taxa were assessed using the Python-based QIIME bioinformatics pipeline v.1.9.1 at the family and genera phylogenetic levels (Kuczynski et al., 2011). The specific order of processing was as follows: library generation (files available upon request), barcode trimming with the script split_libraries.py, selection of operational taxonomic units (OTUs) with the script pick_otus.py, selection of a representative set with the script pick_rep_set.py, assignment of taxonomy with the script assign_taxonomy.py, generation of an OTU table with the script make_otu_table.py, summarizing taxa with the script summarize_taxa.py, testing evolutionary

distance by building an alignment with the script align_seqs.py, hard sequence filtration of the alignment using a Lane mask with the script filter_alignment.py, generation of a phylogenetic tree with FastTree with the script make_phylogeny.py, rarefaction analyses with the script multiple_rarefactions.py, assessment of alpha diversity with the script alpha_diversity.py, assessment of beta diversity with the script jackknifed_beta_diversity.py, and distance statistics were created with the script dissimilarity_mtx_stats.py. All scripts referenced above are available on the QIIME website and are also available upon request.

4.15 Statistical Analyses

The R Software Package (R Foundation for Statistical Computing, Vienna, Austria) and Microsoft Excel were used to perform the statistical analyses of microbiome data. Student's t-test, a one-way analysis of variance and paired t-tests were performed for significance determination (statistical significance required a P value < 0.05). Statistical analyses for other data were performed using Microsoft Excel. Microsoft Excel was used to determine standard deviation and statistical significance using two-tailed paired and unpaired t test (statistical significance required a P value < 0.05). Data composed of pre and post treatment samples within the same group (Treatment T_0 vs T_8) was analyzed using paired t test and to compare the effect of treatment on microbiota (Treatment vs Control at T_8), an unpaired t test was performed.

5 Results

5.1 Identification of the *B. velezensis* strains showing the best *in vivo* efficacy in a murine wound model in preventing *S. aureus* infection

The initial animal model consisted of creating four distinct wounds on the dorsal skin of the mouse and pipetting *S. aureus* Xen29 onto each wound, followed by application of *B. velezensis* strains to designated treatment wounds. This procedure proved inconsistent due to mice licking the

bacterial suspensions from the wounds (data not shown). This prompted modification of the protocol wherein bacterial cultures were injected subcutaneously into two regions of the dorsal skin. To ensure that treatment was applied in the same site as the infection, *B. velezensis* spores and *S. aureus* Xen29 were co-administered by mixing in equal parts immediately prior to injection. This procedure ensured that the numbers of injected cells remained consistent, and mouse communal grooming habits did not interfere with the results.

In the first disease challenge using co-inoculation subcutaneously, spores of five *B. velezensis* (strains AP143, AP191, AP183, AP218 and AB01) were selected for evaluation of their respective ability to inhibit *S. aureus* Xen29 colonization in a mouse cutaneous wound model. Each mouse in the treatment group was simultaneously challenged with *S. aureus* Xen29 in two independent cutaneous wounds on each mouse back. The *S. aureus* Xen29 suspension and/or spores were pipetted onto wounds of two mice per treatment group. The final concentrations of *S. aureus* Xen29 and *B. velezensis* spores per wound were approximately 1.0×10^7 CFU respectively. The results in these small number of animals indicated that co-administration of *S. aureus* Xen29 with spores of *B. velezensis* AP183 resulted in the greatest inhibition of *S. aureus* Xen29 growth and bioluminescence as compared to the other *B. velezensis* strains AP191, AP218, AB01 or AP143, with *B. velezensis* AP191 showing a moderate degree of *S. aureus* inhibition (data not shown).

5.2 Evaluating inhibition of *S. aureus* Xen29 infection by two *B. velezensis* strains

A subsequent *in vivo* challenge of mice (n=5 in each treatment and control group) with wounds treated with a *B. velezensis* AP183 culture resulted in a significant decrease ($P < 0.05$) of bioluminescence from *S. aureus* Xen29 (Fig. 1 A-B). Furthermore, we observed a significant reduction ($P < 0.05$) in the number of cultured CFUs in homogenates corresponding to AP183 and

supernatant treated wounds (Fig. 1C). Injection sites treated with *B. velezensis* AP191 spores and supernatant were less consistent, but still resulted in decreased bioluminescent signal overall as compared to control wounds. Bioluminescent signal in untreated control wounds peaked between days 2-3 with a substantial decrease in reported signal by the end of the study. However, one control mouse inoculated with *S. aureus* Xen29 but no *Bacillus* spores succumbed to systemic infection seven days post-inoculation despite a substantial decrease in bioluminescent signal from wound sites, suggesting that in this control mouse that the *S. aureus* Xen29 had spread beyond the wound site several days after it was subcutaneously inoculated. Because *B. velezensis* AP183 was observed to provide the greatest degree of *S. aureus* inhibition compared to that observed for *B. velezensis* AP191 and other strains, and there was greater knowledge concerning the bioactive metabolite (i.e. bacillusin A) expressed by this strain that was responsible for *S. aureus* growth inhibition, subsequent biofilm and *in vivo* experiments in dairy cows focused on this strain.

5.3 Histological Analysis

Histological Gram staining revealed an abundance of Gram-positive cocci on sections of excised control wounds as compared to wounds treated with AP183 spores and/or metabolites (Fig. 2 A-B). However, Gram-positive cocci were also observed in treated wounds. H&E-stained sections of excised tissue appear necrotic and inflamed in contrast to AP183 treated wounds (Fig. 2 C-D).

5.4 Competition between *B. velezensis* AP183 and *S. aureus* under *in vitro* conditions

The previous experiments in a mouse wound model indicated that *B. velezensis* AP183 provided the strongest inhibition of *S. aureus* proliferation and systemic infection, among the tested *B. velezensis* strains. In those experiments the *S. aureus* inoculation was performed with a bacterial suspension, and co-inoculated with a *B. velezensis* culture. Because *S. aureus* commonly forms biofilms on medical device substrates that can lead to systemic infections, an experiment was

conducted to evaluate if a *B. velezensis* AP183 biofilm formed on a tracheostomy tube inner cannula substrate could impact subsequent *S. aureus* colonization. Due to difficulties in assessing accurate CFU counts from a biofilm, DNA was isolated from biofilm samples in which *B. velezensis* AP183 had formed a biofilm prior to inoculation with *S. aureus* NE1260F or from biofilm formed by *S. aureus* NE1260F only. In the treatment group that was inoculated previously with *B. velezensis* AP183, a significant decrease was observed in *S. aureus* relative abundance on the inner cannula sections ($P = 0.01$) compared to control samples. In the treatment group the *S. aureus* relative abundance was 3.5%, whereas the relative abundance of *Bacillus* was 96.5% (Fig. 3A). In contrast, the control group was dominated by 99.4% *S. aureus* (Fig. 3A).

The previous experiment showed that a *B. velezensis* AP183 biofilm was able to resist colonization by *S. aureus*. A subsequent experiment was designed to evaluate the relative abundance of these two bacteria when co-inoculated into M9 minimal medium containing different carbon sources. Whereas both *B. velezensis* AP183 and *S. aureus* can grow in the presence of glucose or sucrose, only *B. velezensis* AP183 can use pectin as a sole carbon source. When these two cultures were inoculated into 96-well plates containing each of these carbon sources, it was observed that the relative abundance of *S. aureus* NE1260F increased when the concentration of glucose or sucrose reached very high levels, but no corresponding increase in *S. aureus* NE1260F relative abundance was observed when pectin was the sole carbon source and this difference was significant at a 10% (w/v) concentration of these carbon sources (Fig. 3B; $P < 0.05$). While no significant difference was observed when lactose was included as a sugar ($P = 0.09$) the mean relative abundance of *S. aureus* NE1260F in the presence of lactose remained less than 25% even at the highest lactose concentrations (Fig. 3B). This experiment revealed that the competition between these bacteria is influenced by the available growth substrate. Subsequent experiments

were conducted to evaluate the ability of *B. velezensis* AP183 to antagonize a *S. aureus* biofilm that was formed within a microfluidic device, which similarly showed a strong inhibitory effect of *B. velezensis* AP183 on *S. aureus* viability and resulted in biofilm disruption (data not shown, manuscript in preparation). Collectively, these experiments indicated that *B. velezensis* AP183 can compete with *S. aureus* under different environmental conditions, including in microbial biofilms on a medically relevant substrate, and that this competition is influenced by the growth substrate.

5.5 Antibacterial activity of *B. velezensis* AP183 against mastitis isolates

The antibacterial activity of *B. velezensis* AP183 against mastitis-inducing pathogens was evaluated by recording the respective zones of inhibition against each isolate. *B. velezensis* AP183 was observed to produce antibacterial metabolites that inhibited the growth of all eight *S. aureus* mastitis isolates, one *S. uberis* mastitis isolate, and one *B. cereus* mastitis isolate (Table S1). No inhibitory effects were observed for *B. velezensis* AP183 against a Gram-negative bacterial mastitis isolate, *K. pneumoniae* (Table S1).

5.6 *B. velezensis* AP183 at a high dose induces a short-lived mammary inflammatory response

To evaluate the ability of *B. velezensis* AP183 to induce an immune response within mammary tissue, and the timing for inflammation resolution, a high dose (approximately 10^9 CFU) of *B. velezensis* AP183 was inoculated intramammary into healthy bovine mammary glands to evaluate the *in vivo* effects compared to healthy quarters that served as a negative control. This high-dose inoculum induced a significant increase in SCC (approximately 7×10^6 cells/mL) by 24 h post-inoculation which was significantly higher than the SCC observed for the negative control quarters (at 24 h, $P = 1.5 \times 10^{-6}$; $P < 0.01$ for all post-inoculation time points) (Fig. 4). All cows developed localized clinical mastitis with visible abnormal mammary secretions from inoculated quarters

without any signs of systemic disease. All quarters infused with the challenge dose of *B. velezensis* AP183 showed visibly evident caseous material in the milk. The California mastitis test indicated an increase in somatic cells for the *Bacillus*-infused quarters and for some non-infused quarters. No signs of systemic infection or elevated temperature were noted in any of the infused cows. All clinical cases resolved within five days without antimicrobial treatment and CMT results regressed without any further observation of mastitis symptoms. The somatic cell counts decreased to a normal level ($<1.42 \times 10^3$ cells/mL) by 10 days post-inoculation. This result as well as results from previous studies (Frola et al., 2012; Klostermann et al., 2008) suggest that, while *B. velezensis* could induce significant inflammation, the inflammatory response resolved within a short period of time. This also suggested that inoculation of mammary tissue with a lower dose of *B. velezensis* AP183 would be less inflammatory. Further analysis of the milk obtained from this preliminary experiment indicated that milk from quarters receiving a high inoculum dose had greater protein and fat content compared with control quarters at all time points with significant differences observed at D_2 and D_6 for fat ($P < 0.05$ at both time points) and D_2 and D5 for protein ($P < 0.05$ at both time points). Interestingly, the lactose and SNF values were less in the treatment group with significant differences observed for lactose levels at Days 1 through 8 ($P < 0.05$ for each time point) and for SNF levels at D_1, D_4 and D_5 ($P < 0.05$ at each time point) (Fig. S1). The reduced lactose and SNF levels in the treatment group with high SCC in this study is in accordance with the study of Reis *et al*. (2013) but the high protein and fat content of milk from inoculated, high SCC quarters that were observed in this study did not agree with observations made in some other studies (Dos Reis, Barreiro, Mestieri, de Felício Porcionato, & dos Santos, 2013; Schultz, 1977).

5.7 bacillusin A was not detected in milk samples from the high inoculum experiment

The milk samples from quarters treated with the high dosage of *B. velezensis* AP183 at D_5 did not have detectable bacillusin A based on LC-MS analysis. The detection of purified bacillusin A indicated that the limit of detection sensitivity was 3 ppm. A possible reason for the poor sensitivity for bacillusin A detection could include its instability (Ravu et al., 2015), or that during extraction from milk samples the bacillusin A was degraded or inefficiently recovered. It is unknown whether *B. velezensis* AP183 germinates and expresses bacillusin A within mammary tissues. Further research to provide better recovery of intact bacillusin A from milk samples and to assess bacillusin A biosynthetic gene cluster expression would be helpful in assessing any antibiotic residues present in milk, especially when a lower spore inoculum is used.

5.8 Intramammary inoculation of *B. velezensis* AP183 at a low dose does not induce clinical mastitis

A subsequent experiment was conducted to determine the effect of a lower dosage of *B. velezensis* AP183 on the mammary inflammatory response. A lower dose inoculum of 10^3 CFU *B. velezensis* AP183 did not induce clinical mastitis in any of the inoculated cows. Milk sampled from *B. velezensis* AP183-inoculated quarters was visibly abnormal, and a slight increase of SCC was observed for those samples, although the difference was not significant. In this experiment, the mean SSC was higher in treatment group compared to the control from day D_1 to D_4; however, the SCC for milk from *B. velezensis* AP183-inoculated quarters was below the regulatory threshold of bulk-tank SCC which is 750,000 in the United States (Fig. S2A). The *Bacillus* CFU counts observed from inoculated quarters increased slightly at D_2 compared to control (Fig. S2B). In addition, protein concentration was increased in milk from inoculated quarters, while lactose concentration was reduced in the treatment group compared to control. No changes were observed

in fat in the treatment group compared to control, though SNF showed a different pattern with a reduction of SNF observed in the treatment group at D_1 while it increased the control group (Fig. S3). The significance of this brief change in SNF at D_1 in terms of milk quality is unknown. While the experiments evaluating the mammary inflammatory response at the two dosages of *B. velezensis* AP183 were performed with different animals, at different times and with different sample sizes, the procedures for inoculation, milk collection, testing and analyzing were the same for these experiments. A comparison of the mammary tissue inflammatory response at low vs. high dose of *B. velezensis* AP183 over the time course post-inoculation revealed significant differences in SCC between low dose and high dose at each treatment day from D_1 through D_4 (D_1, $P = 6.8 \times 10^{-6}$; D_2, $P = 0.0005$; D_3, $P = 0.04$; D_4, $P = 0.03$), in each case a vigorous inflammatory response was observed for the high dosage of *B. velezensis* AP183 whereas the low dosage never exceeded the regulatory threshold of bulk-tank SCC in the United States (Fig. 4).

5.9 Low doses of *B. velezensis* AP183 reduces *S. aureus* abundance

Based on the observation of a significant inflammatory response observed when *B. velezensis* AP183 was inoculated by intramammary infusion at a high dose, we sought to evaluate the ability of this strain to antagonize *S. aureus* within mammary tissues when inoculated at a lower dosage, post-induction of clinical mastitis. All quarters challenged with 10^3 CFU of *S. aureus* RF122 were observed to have increased SCC with clinical mastitis by 24 h post-inoculation, with decreasing SCC observed at later time points (Fig. 5A). Interestingly, after D_1 a greater SCC reduction was observed in the *B. velezensis* AP183 treatment group as compared to control (Fig. 5A), indicating that the inoculated *B. velezensis* AP183 reduced mastitis severity. At seven days post-inoculation with *B. velezensis* AP183, SCC levels were decreased to approximately day 0 levels. Furthermore, at D_3 *S. aureus* counts were significantly higher ($P = 0.04$) in control samples as compared to

samples treated with *B. velezensis* AP183 (Fig. 5B). The levels of fat, protein, lactose and milk solids-not-fat (MSNF) were similar between the control and treatment groups in this lower dose inoculum experiment (Fig. S4), with no significant differences observed between control and *B. velezensis* AP183-treated quarters ($P > 0.05$).

5.10 *B. velezensis* AP183 at a low dose induces shifts in the milk microbiome when inoculated

In the previously described study in which *S. aureus* RF122 was inoculated into all quarters, followed by a low dosage inoculum of *B. velezensis* AP183 into half of these quarters, the milk bacterial community composition was observed to have differences between the control (only *S aureus* RF122) and treatment (*S. aureus* RF122 and *B. velezensis* AP183) groups. The microbiota of milk from cows affected by mastitis was comprised mainly of four bacterial phyla, including taxa affiliated with the Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes (Fig. 6A). At D_0, the milk microbiota was dominated by members of the Firmicutes (mean relative abundance of 74.3% at D_0) followed by Proteobacteria taxa (mean relative abundance of 17.5% at D_0). Interestingly, the quarters treated with *B. velezensis* AP183 were observed to have a decreased Firmicutes relative abundance over time post-inoculation, changing from 70.4% at D_0 to 48.0% at D_2 to 33.1% at D_8 (Fig. 6A). A similar trend for Firmicutes relative abundance was observed for the control group which was observed to be 78.3% at D_0 to 31.9% at D_2 to 42.6% at D_8 (Fig. 6A). It is important to note that the increased Firmicutes relative abundance in the control group at the final time point D_8 was due at least in part to the higher relative abundance of the genus *Staphylococcus*. In contrast, the relative abundance of Proteobacteria taxa were observed to increase in both the control and *B. velezensis* AP183-treated samples at these same time points, with observations of Proteobacteria abundance for control samples changing from 14.6% at D_0 to

53.4 % at D_2 to 40.1% at D_8, and similarly the Proteobacteria relative abundance for the *B. velezensis* AP183 treated samples were at 20.4% at D_0 and increased to 41.1% at D_2 to 40.1% at D_8 at the final time point (Fig. 6A). The relative abundance of Actinobacteria and Bacteroidetes taxa were observed to be at comparable levels in control and treatment groups (Tables S2 and S3). In addition to phylum-level differences, differences in the relative abundance of bacterial genera were also observed in milk microbiome samples. Milk samples were dominated by *Staphylococcus, Achromobacter, Delftia, Pseudomonas, Agrobacterium, Acinetobacter, Hydrogenophaga, Corynebacterium* and *Bacillus* spp. (Fig. 6B). In the quarters treated with *B. velezensis* AP183, a significant decrease was observed in the relative abundance of the genus *Staphylococcus* in milk samples over time (D_0 vs D_8, $P = 0.003$; D_2 vs D_8, $P = 0.030$), with the treatment group showing *Staphylococcus* relative abundance changing from 61.4% at D_0 to 39.3% at D_2, and to 7.4% at D_8. In contrast, in the control group the *Staphylococcus* relative abundance also decreased significantly over time ($P = 0.002$) from 69.8% at D_0 to 20.2% at D_8, but no significant difference was observed from 22.3% at D_2 to 20.2% at D_8 (Tables S4 and S5). Most importantly, the results indicated that *B. velezensis* AP183 treatment had a significant effect in decreasing *Staphylococcus* relative abundance relative to the control group at D_8 ($P = 0.021$). Further, the relative abundance of the genus *Bacillus* was higher in the treatment group (2.9%) at D_8 compared to control group (1.5%) as would be expected, although this result was not significant. Besides *Staphylococcus*, the only other bacterial genus in which the relative abundance exhibited a significant difference between early time points (D_0 or D_2) and D_8 in control and treatment groups was the genus *Hydrogenophaga*, which in control samples went from 2.3% at D_0 to 1.4% at D_8, whereas in treatment samples the *Hydrogenophaga* relative abundance was observed to be at 0.4% at D_0 and was at 3.5% at D_8 (Tables S4, S5).

Shannon and Simpson diversity indices were not different between the control and treatment groups, but the sample diversity was slightly higher at day 12 in the treatment group based on species richness and chao 1 (Table 2), although no statistically significant differences were observed ($P > 0.05$). Interestingly, there were distinct differences between the control and treatment groups observed in the principal component analysis based on weighted Unifrac distances (Fig. 7). The treatment group clustered together except for two outliers. While the distribution of data in the control group was dispersed and there were apparent differences from the treatment samples. Principal component 1 explained a large percentage of the variation (86%) for *Staphylococcus* spp. that demonstrated a high correlation of the relative abundance of the *Staphylococcus* with the first principal component.

6 Discussion

The primary objective of this study was to identify specific probiotic bacteria with the capacity to inhibit *S. aureus* colonization under various conditions that are relevant to human and veterinary medicine. The key considerations used for evaluating candidate probiotic bacteria were safety, efficacy, and commercial potential (i.e. shelf-life). For these reasons, our efforts focused on identifying endospore-forming members of the genus *Bacillus* that have efficacy in preventing *S. aureus* infections, without any known potential for pathogenicity. These criteria narrowed our search to *B. velezensis* strains available in an Auburn University microbial culture collection that had shown the ability to produce bioactive metabolite(s) that inhibit MRSA growth. However, none of these strains had been evaluated under *in vivo* conditions for *S. aureus* antagonism. The preliminary studies using a mouse wound model revealed that *B. velezensis* AP183 was the most effective strain at inhibiting *S. aureus* growth and bioluminescence and was the most promising candidate for future investigation. The antibacterial activity of AP183 is presumed to be due

primarily to the production of secondary metabolites, in particular the macrodiolide antibiotic bacillusin A that was previously shown to exhibit strong *in vitro* bactericidal activity against MRSA and vancomycin-resistant enterocci (Ravu et al., 2015). While bacillusin A was found to be a potent inhibitor of several relevant pathogens, it was determined to be chemically unstable in organic solvents and prone to photoisomerization as indicated by LC-MS analysis (Ravu et al., 2015). The photoisomerization and chemical instability in organic solvents ultimately resulted in a low isolation yield of the compound, thereby making it unlikely that purified bacillusin A would be a viable candidate for use as an oral or injectable antibiotic in the clinical setting. However, the biochemical instability of this compound might be advantageous for topical treatment of infected wounds where rapid elimination of antibiotic residues may be preferred. For instance, prophylactic topical application on dressings for severe burn wounds, wherein exposed tissues are readily infected by *S. aureus* and other pathogens, often resulting in patient mortality (Avni, Levcovich, Ad-El, Leibovici, & Paul, 2010).

The observation that *B. velezensis* AP183 provided the best inhibition of *S. aureus* proliferation and bioluminescence in a mouse wound model could be due to a combination of factors, including the production of secondary metabolites, competitive inhibition for growth substrates and/or other mechanisms. Even in the absence of *B. velezensis* AP183, bioluminescent signal intensity was consistently observed to decrease in control wounds beginning around day four of infection. This decrease in bioluminescent signal may be due to many factors, but histological analysis suggests that dissemination to distance sites from systemic infection may have resulted in some cases. Gram-stained sections of a heart of one control mouse that succumbed to systemic infection revealed clusters of Gram-positive cocci in the aortic region of the heart (data not shown). This observation is consistent with the ability of *S. aureus* to disseminate from a

cutaneous wound and establish life-threatening systemic infections at distance sites. In the presence of *B. velezensis* AP183 and its metabolites, the *S. aureus*-derived bioluminescence was almost completely eradicated even at early time points. In contrast, the same degree of *S. aureus* inhibition within a mouse wound was not observed for *B. velezensis* AP191, which did show the capacity for *S. aureus* growth inhibition under *in vitro* conditions. The difference in *in vivo* efficacy between these two *B. velezensis* strains may be due to differences in the secondary metabolites expressed. The *S. aureus* inhibitory activity of *B. velezensis* AP191 observed under *in vitro* conditions has not been attributed to a specific metabolite, nor does this strain have the encoded capacity to produce bacillusin A (data not shown). Based on the data available from the mouse wound experiments and the previously described structure and function of bacillusin A, *B. velezensis* AP183 was selected for all subsequent experiments.

The competitive interactions between *B. velezensis* AP183 and *S. aureus* are expected to be influenced by many different factors. The expression and stability of bacillusin A in association with host tissues is hypothesized to be a key factor in this interaction, and future studies examining the role of bacillusin A in mediating *S. aureus* control *in vivo* will be important in our understanding of these interactions and the degree to which secondary metabolites are involved, relative to other mechanisms such as competitive exclusion and substrate utilization. A newly constructed genetic mutant of *B. velezensis* AP183 that lacks the biosynthetic gene cluster responsible for bacillusin A will be especially useful in evaluating these molecular interactions (manuscript in preparation). In addition to metabolite-mediated inhibition of *S. aureus*, the role of biofilm formation in these bacterial interactions was explored in this study. By first allowing *B. velezensis* AP183 to grow as a “pioneer organism” that formed a biofilm on sections of a tracheostomy tube inner cannula, these biofilm-colonized surfaces significantly reduced *S. aureus*

populations based on ribotype relative abundance data. Considering that *S. aureus* commonly forms biofilms on medical implants that can result in life-threatening infections (Oliveira et al., 2018), there could be prophylactic value to establishing a beneficial bacterial biofilm that would be recalcitrant to biofilm formation by opportunistic pathogens. The growth substrate available to these bacteria also appears to impact their competitive interactions, as a significant reduction in *S. aureus* relative abundance was also observed when co-inoculated with *B. velezensis* AP183 in the presence of pectin, as opposed to other sugars such as glucose or sucrose that both cultures could readily utilize as a carbon and energy source. In association with host tissues, there will certainly be a diversity of growth substrates and interactions with host cells, that contribute to the complex molecular interactions that impact microbial physiology and pathogenesis. Given this complexity, there is no substitute for evaluating these interactions under *in vivo* conditions, and it was important to evaluate the ability of *B. velezensis* AP183 to impact *S. aureus* proliferation and infection in an animal disease model of significant agricultural relevance.

This is the first study in which a *B. velezensis* isolate has been evaluated for the ability to prevent or to treat bovine mastitis. The *in vivo* efficacy observed for *B. velezensis* AP183 in inhibiting *S. aureus* in a mouse wound model, and the strong *in vitro* inhibitory activity observed for *B. velezensis* against all the mastitis-derived *S. aureus* isolates tested in this study suggested that *B. velezensis* AP183 may have the ability to inhibit a broad spectrum of Gram-positive bacterial taxa that can induce mastitis. Given that a complete *B. velezensis* AP183 genome sequence is available (Nasrin et al., 2015), and this strain is predicted to also encode the biosynthetic gene cluster for the antibiotic difficidin that has been shown to inhibit Gram-negative pathogens such as *Aeromonas hydrophila* and *Xanthomonas campestris* pv. vesicatoria (Hossain et al., 2015), we subsequently evaluated the activity of *B. velezensis* AP183 against *A. hydrophila*

ML09-119 (Tekedar et al., 2013). While this strain did not inhibit the growth of *K. pneumoniae,* we did observe growth inhibitory activity expressed by *B. velezensis* AP183 against *A. hydrophila* ML09-119 (data not shown), suggesting that difficidin and/or other metabolites produced by this strain are active against some Gram-negative bacteria as well. Collectively, these results indicate that *B. velezensis* AP183 actively produces bioactive secondary metabolites (i.e. bacillusin A, difficidin) that are active against diverse bacterial mastitis isolates and that *B. velezensis* AP183 should be further evaluated for its ability to reduce clinical mastitis.

Prior to evaluating interactions between *B. velezensis* AP183 and *S. aureus* in mammary tissues, it was important to determine the mammary tissue immune response to *B. velezensis* AP183. We observed that intramammary inoculation of *B. velezensis* AP183 at a high dosage induced a short-term increase of SCC with clinical mastitis that rapidly resolved by five days post-inoculation. The results obtained in this study are like the study of Mignacca et al. (2017), who observed a short-term increase in SCC after probiotic *L. lactis* subsp. Lactis LMG 7930 treatment and rapid decline of SCC to pre-treatment level seven days post inoculation. Further, Crispie *et al.* (2008) observed increased numbers of polymorphonuclear leucocytes at two days post-inoculation that decreased by five days post-inoculation. We also observed that low dose *B. velezensis* AP183 caused low grade sub clinical mastitis that increased SCC but below the threshold level of bulk tank SCC level for US. Importantly, when the lower dose of *B. velezensis* AP183 was administered after *S. aureus*-induced clinical mastitis, a significant decrease in *S. aureus* was observed by D_3 in the treatment group relative to the control group, and these results were also shown by decreased relative abundance of *Staphylococcus* from the 16S rRNA gene amplicon sequencing results as well. Collectively, these results demonstrated that the SCC and *Staphylococcus* count reduction

was more rapid in the bovine quarters exposed to *B. velezensis* AP183, indicating that *B. velezensis* AP183 had some efficacy in reducing the severity of staphylococcal-induced mastitis.

Our data showed that the most abundant bacteria detected in milk from bovine mastitis quarters were from the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroides, which were similar observations to previous studies (Catozzi et al., 2019; Lima et al., 2017; Taponen et al., 2019). Furthermore, a lower relative abundance was observed for bacterial taxa affiliated with the phyla Verrucomicrobia, Cyanobacteria, and Acidobacteria, which corresponds well with the results obtained from the study of Catozzi et al. (Catozzi et al., 2019). In this study, differences were found primarily in the relative abundances of bacterial taxa affiliated with the phyla Firmicutes and Proteobacteria in milk between the treatment and control groups.

The differences of the relative abundance of the top microbiota of milk between treatment and control at D_8 were observed in *Staphylococcus*, *Bacillus,* and *Alicyclobacillus* taxa. The major finding of this study was the *Staphylococcus* relative abundance was significantly decreased in the treatment group relative to the control group at D_8, indicating that *B. velezensis* AP183 inoculation was effective in reducing the relative abundance of *S. aureus* in mastitis quarters. Interestingly, the relative abundance of *Bacillus* was slightly higher in the treatment group at D_8 in this study which supports a negative correlation between the relative abundance of *Bacillus* and *Staphylococcus*. The only other genus in which a significant change was observed in the milk microbiome was an increase in the relative abundance of the genus *Alicyclobacillus* from D_0 to D_8 in the treatment group. This soil borne genus is usually associated with the spoilage of fruit juices (Bevilacqua et al., 2015; Groenewald, Gouws, & Witthuhn, 2009). This may indicate that the growth substrates used by *Alicyclobacillus* spp. are increased in their availability after inoculation with *B. velezensis* AP183 (Laurence, Hatzis, & Brash, 2014; Salter et al., 2014; Weiss et al., 2014).

In conclusion for the bovine mastitis experiments, this study observed that intramammary inoculation of *B. velezensis* AP183 at a relatively low dose in a bovine model of *S. aureus*-induced mastitis reduced the relative abundance of *Staphylococcus* and had minor effects on the rest of the milk-associated microbiota. Despite the small number of cows available for use in this study, significant differences were observed that support additional research to better understand how the use of a beneficial microorganism can antagonize the bacteria such as *S. aureus* that can induce chronic mastitis. Future studies can contribute to understanding the molecular interactions that impact *Bacillus* and *Staphylococcus* interactions with each other, the host immune response and the ways in which an understanding of these interactions can best be applied to reduce the prevalence of mastitis in dairy animals, and in other applications in human and veterinary medicine.

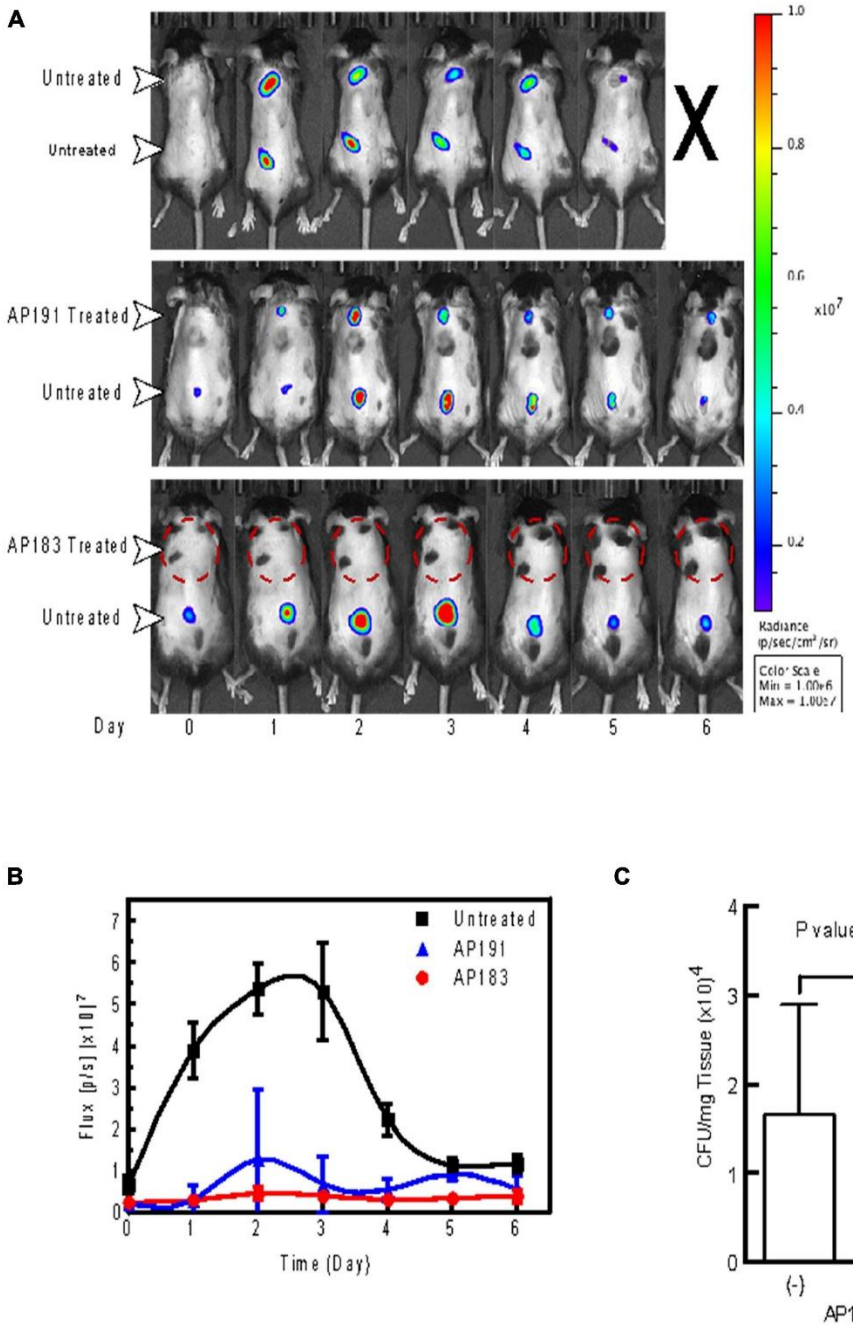

Figure 1. In vivo evaluation of *B. velezensis* strains AP183 and AP191 inhibition of *S. aureus*. In vivo evaluation of *B. velezensis* strains AP183 and AP191 inhibition of *S. aureus* Xen29 in a cutaneous wound model. Mice were given subcutaneous injections of 1×10^6 CFU/mL of *S. aureus* Xen29 between the shoulder blades and on the lower back. (A) Upper panel, untreated mouse that

succumbed to systemic infection 5 days after initial cutaneous wound challenge. Center panel, mouse treated with *B. velezensis* AP191 culture on upper wound and untreated in the lower wound. Lower panel, mouse treated with *B. velezensis* AP183 culture on upper wound and untreated in the lower wound. (B) Bioluminescent flux (photons/second) of *S. aureus* Xen29 in AP183/AP191 treated wounds and untreated controls over time. (C) CFU determined for AP183 treated and untreated wounds from excised homogenates plated on TSA. **(This data and figure were generated by Shamima Nasrin and Andrew D. Brannen)**

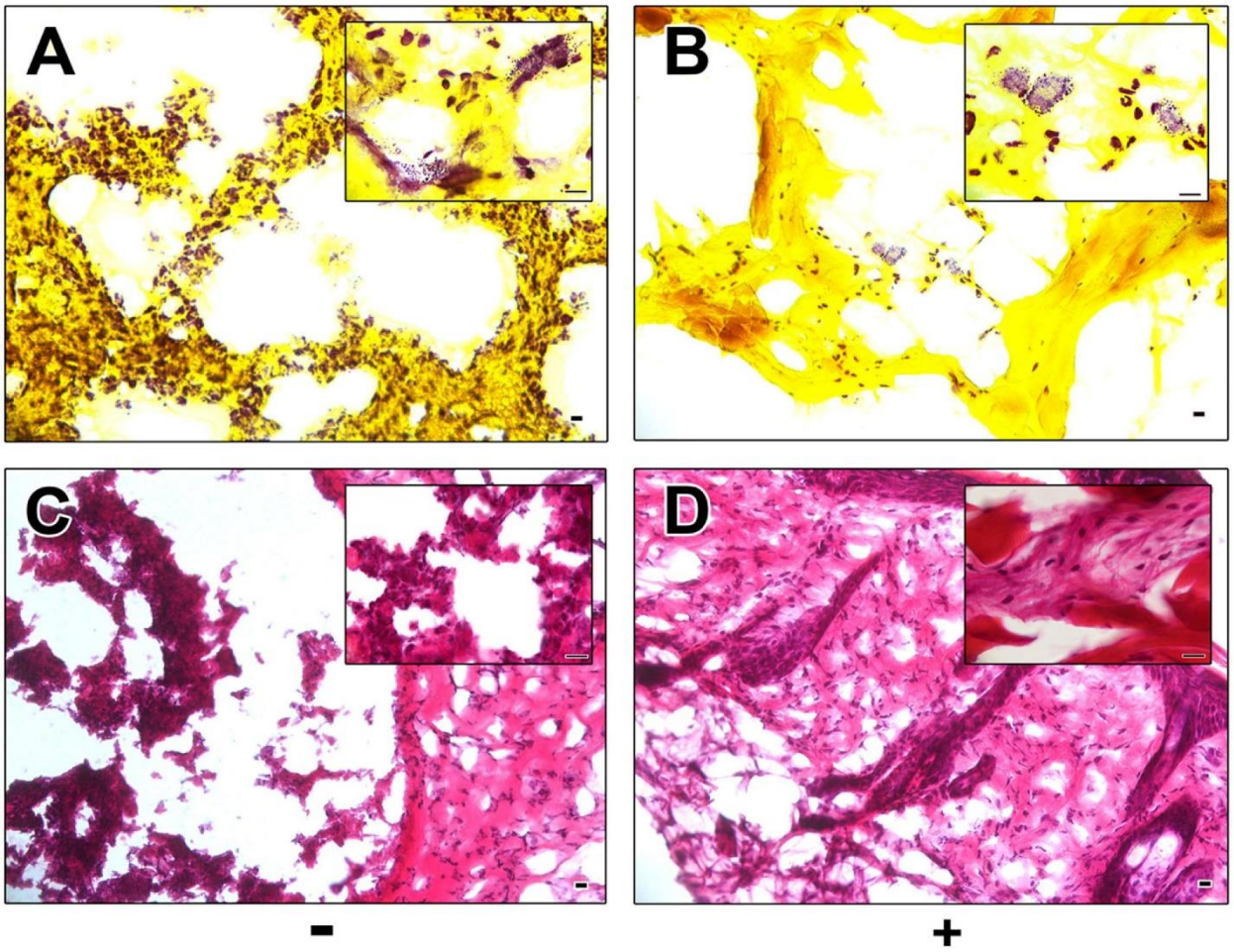

Figure 2. Histological analysis of control and AP183 treated wounds. Histological analysis of control and AP183 treated wounds. Gram **(A,B)** and H&E **(C,D)** stained sections of excised wounds infected with *S. aureus* Xen29 in the absence **(A,C)** and presence **(B,D)** of AP183 co-administration. Clusters of Gram-positive cocci were observed in higher frequency in untreated wounds than those treated with *B. velezensis* AP183. Scale indicates 100 μM. **(This data and figure were generated by Shamima Nasrin and Andrew D. Brannen)**

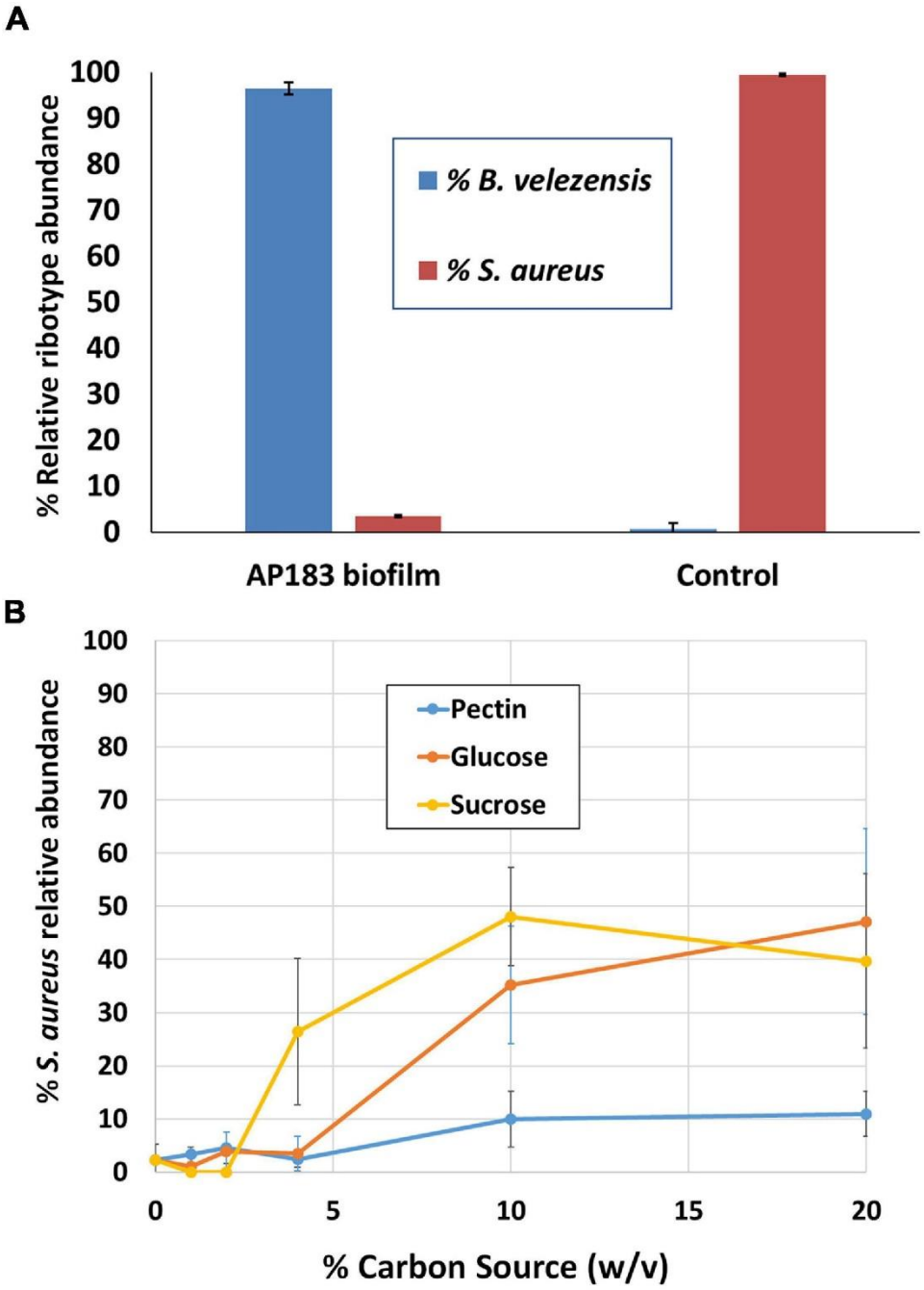

Figure 3. Competition between B. velezensis AP183 and S. aureus NE1260F. Competition between *B. velezensis* AP183 and *S. aureus* NE1260F when **(A)** the *B. velezensis* AP183 culture forms a biofilm on a tracheostomy tube inner cannula section prior to inoculation with *S. aureus* NE1260F, or **(B)** when *B. velezensis* AP183 and *S. aureus* NE1260F are co-inoculated into M9

minimal medium containing either glucose (can be used by both cultures), sucrose (can be used by both cultures), or pectin (can only be used by *B. velezensis* AP183).

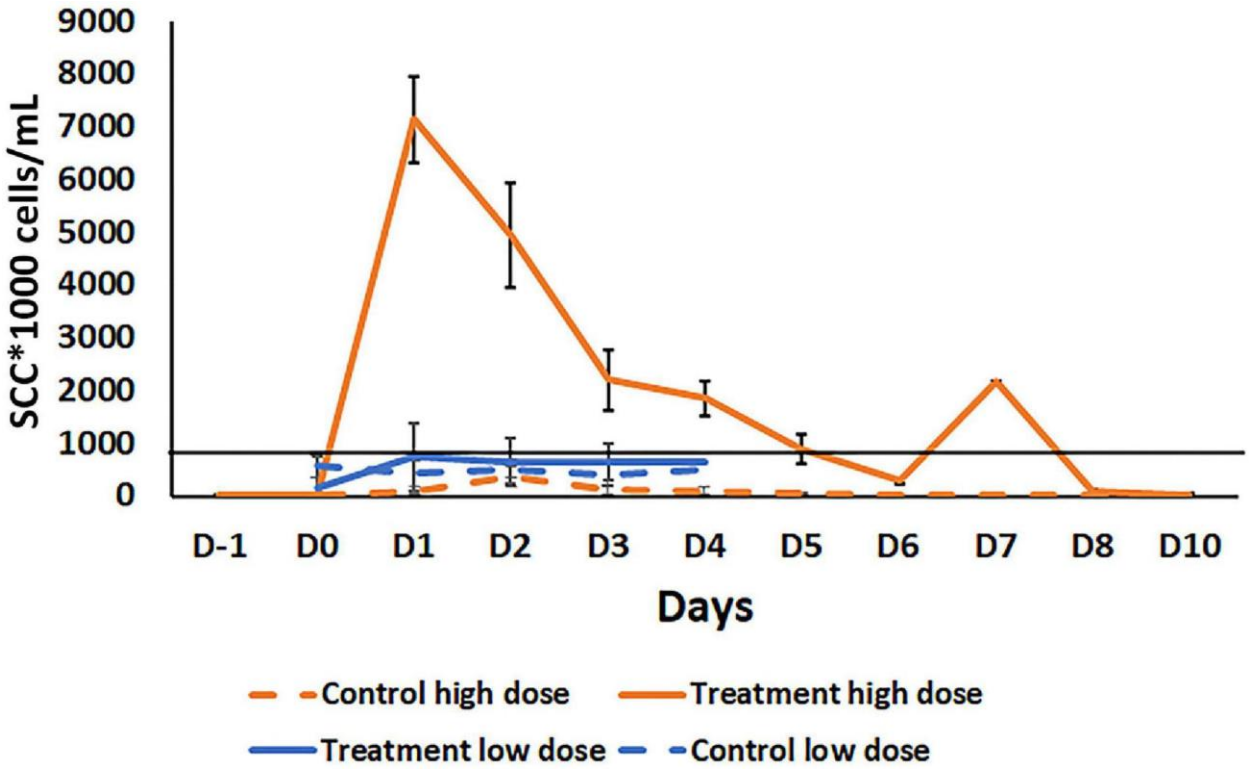

Figure 4. Comparison of somatic cell count (SCC) in milk samples after intramammary inoculation of bovine with either a low (10^3 CFU) or high (10^9 CFU) dose of *B. velezensis* AP183. Comparison of somatic cell count (SCC) in milk samples after intramammary inoculation of bovine with either a low (10^3 CFU) or high (10^9 CFU) dose of *B. velezensis* AP183. The black line across the graph indicates the bulk-tank SCC threshold that is 750,000 according to USDA. Treatment high dose: Treatment with a high dose (10^9 CFU) *B. velezensis* AP183; Control high dose: No treatment in high dose group; Treatment low dose: Treatment with low dose (10^3 CFU) of *B. velezensis* AP183; Control low dose: No treatment in a low dose group. Milk samples in high dose study were collected from pre inoculation day (D-i) to post inoculation days D_o to D_{10}. In the low dose study, milk samples were collected from D_o to D_4. Though the low dose and high dose

inoculation were two different experiments with different animals at different times, all other procedures were the same. Significant differences were found between the low dose and high dose treatment groups in SCC count in milk samples collected from D_o to D_4 (Di, $P = 6.8 \times 10^{-6}$; D_2, $P = 0.0005$; D_3, $P = 0.04$; D_4, $P = 0.03$).

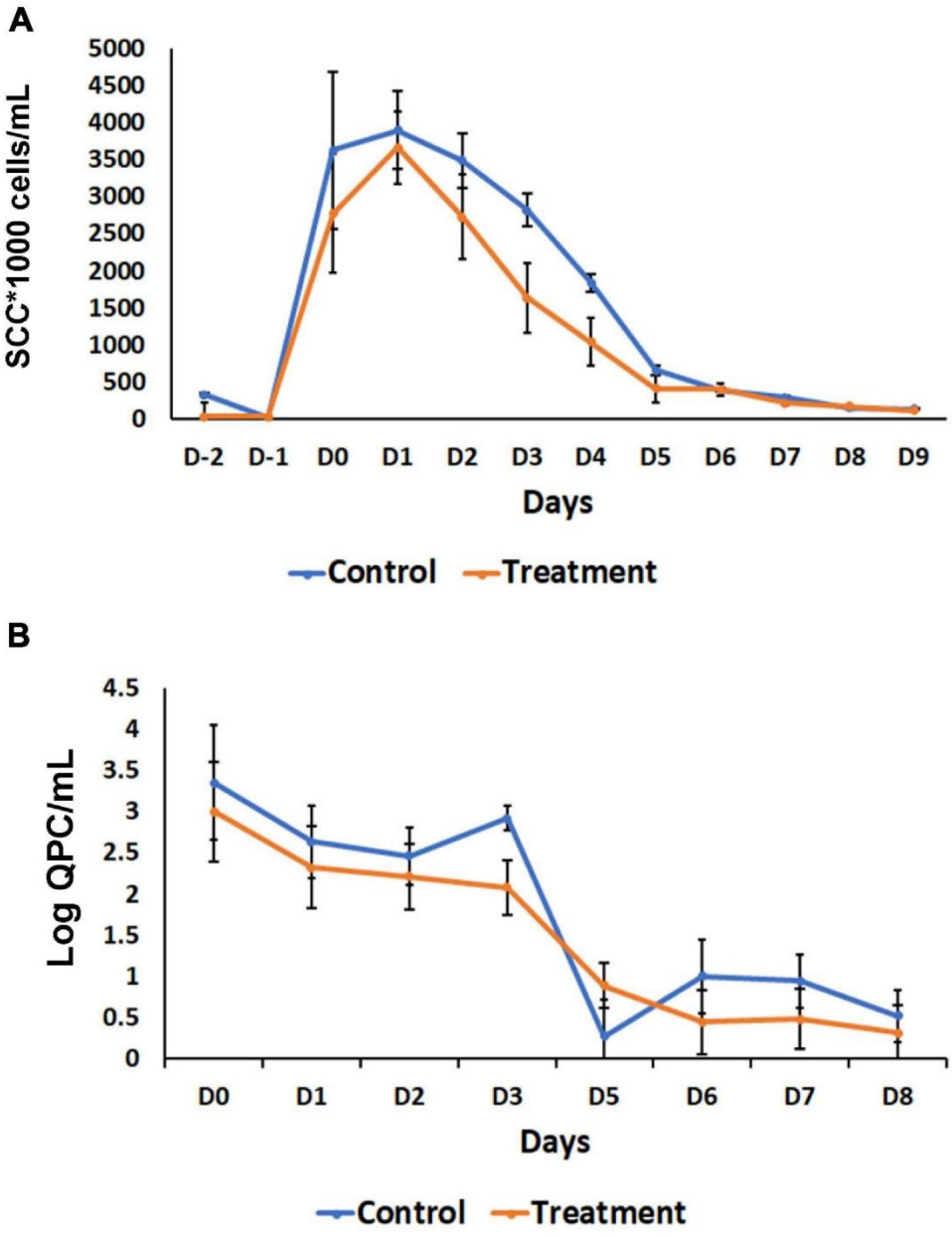

Figure 5. Effect of probiotic treatment on somatic cell counts (SCC) and *Staphylococcus aureus* abundance. Effect of probiotic treatment on somatic cell counts (SCC) and *Staphylococcus aureus* abundance. **(A)** SCC counts decrease after day one in *B. velezensis* AP183 treated group, when compared with its corresponding control, then both groups return to baseline levels after day five. **(B)** The relative count of *S. aureus* decreased steadily in treatment groups, when compared with

the control. The data were expressed as mean ± standard error of log of quantitative plate counts (QPC).

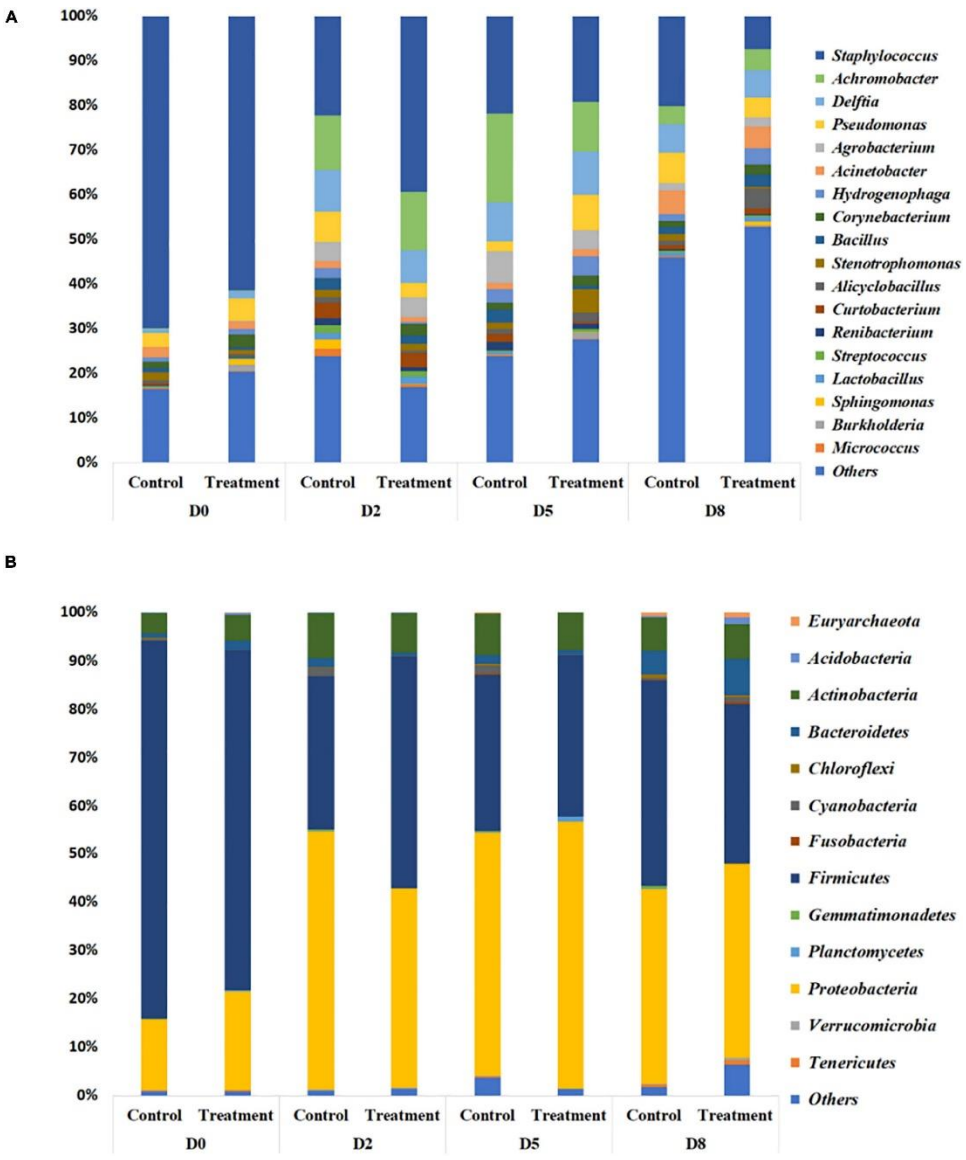

Figure 6. Relative abundance of bacteria in milk from cows with clinical mastitis at the genus level. Relative abundance of bacteria in milk from cows with clinical mastitis at the genus level **(A)** and the phylum level **(B)**, with or without *B. velezensis* AP183 probiotic treatment, based on

16S rRNA gene amplicon sequencing. The treatment group corresponds to bovine quarters treated with both *S. aureus* RF122 and *B. velezensis* AP183, whereas the control group corresponds to treatment with *S. aureus* RF122 alone. D_o, Day before the treatment; D_2, 2 days post treatment; D_5, 5 days post treatment; D_5, 8 days post treatment. The most abundant taxa were sorted by descending order of relative abundance; remaining phylum and genera were grouped as 'Others.' Each bar plot of the corresponding category in both phylum and genus level represents the relative abundance of bacteria in each sample.

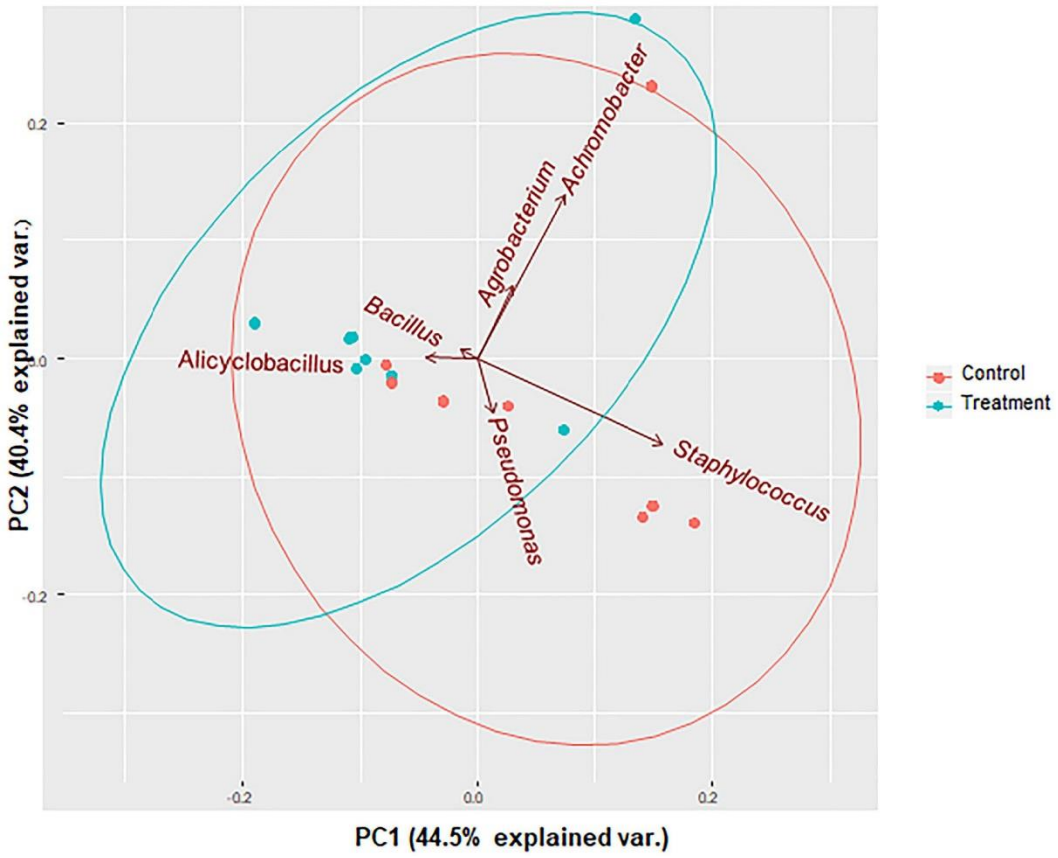

Figure 7. PCA bi-plots of 16S rRNA-based relative abundance according to treatment group. PCA bi-plots of 16S rRNA-based relative abundance according to treatment group. Microbial community profile using beta diversity represented in biplot using weighted Unifrac distances for PCoA analysis. Colors correspond to the control group (red) in which animals were inoculated with only *S. aureus* RF122 and the treatment group (blue) in which animals were inoculated with both *S. aureus* RF122 and *B. velezensis* AP183. PCA indicates distinct differences between the two groups.

Table 1. Bacterial isolates used in this study.

Organism	Strain	Source or reference
B. velezensis	AP143	This study[1]
B. velezensis	AP191	This study[1]
B. velezensis	AP183	This study[1]
B. velezensis	AP218	This study[1]
B. velezensis	AB01	This study[1]
S. aureus	Xen29	PerkinElmer (Akron, OH, United States)
S. aureus	NE1260F	Bose et al., 2013
S. aureus	4366-13	This study[2]
S. aureus	1171-09	This study[2]
S. aureus	1052-1	This study[2]
S. aureus	2226-11	This study[2]
S. aureus	1779-09	This study[2]
S. aureus	3851-07	This study[2]
S. aureus	3651-09	This study[2]
S. aureus	RF 122	This study[2]
S. uberis	22272-08	This study[2]
K. pneumoniae	1583-07	This study[2]
B. cereus	NRS 1595	This study[2]

[1]*Department of Entomology and Plant Pathology, Auburn University Microbial Culture Collection.*
[2]*College of Veterinary Medicine, Auburn University.*

Table 2. Comparison of α diversity between treatment and control groups at different time points.

Day	Group	Species Richness	chao1	Shannon	Simpson
D-0	Control	13.3 (±8.9*)	35.4 (±27.7)	3.8 (±0.1)	0.97 (±0.0)
	Treatment	14.9 (±9.3)	41.2 (±35.3)	3.8 (±0.1)	0.97 (±0.0)
D-2	Control	17.8 (±5.6)	34.7 (±15.4)	3.9 (±0.0)	0.97 (±0.0)
	Treatment	15.8 (±5.9)	32.7 (±13.7)	3.9 (±0.0)	0.97 (±0.0)
D-5	Control	16.3 (±7.5)	33.3 (±15.4)	3.9 (±0.0)	0.97 (±0.0)
	Treatment	17.3 (±5.8)	30.0 (±10.0)	3.9 (±0.0)	0.97 (±0.0)
D-8	Control	22.0 (±8.0)	37.3 (±12.4)	3.9 (±0.0)	0.97 (±0.0)
	Treatment	24.9 (±9.7)	44.3 (±16.5)	3.9 (±0.0)	0.97 (±0.0)

**Results are expressed as mean values ± standard deviation.*

Supplementary figures and tables

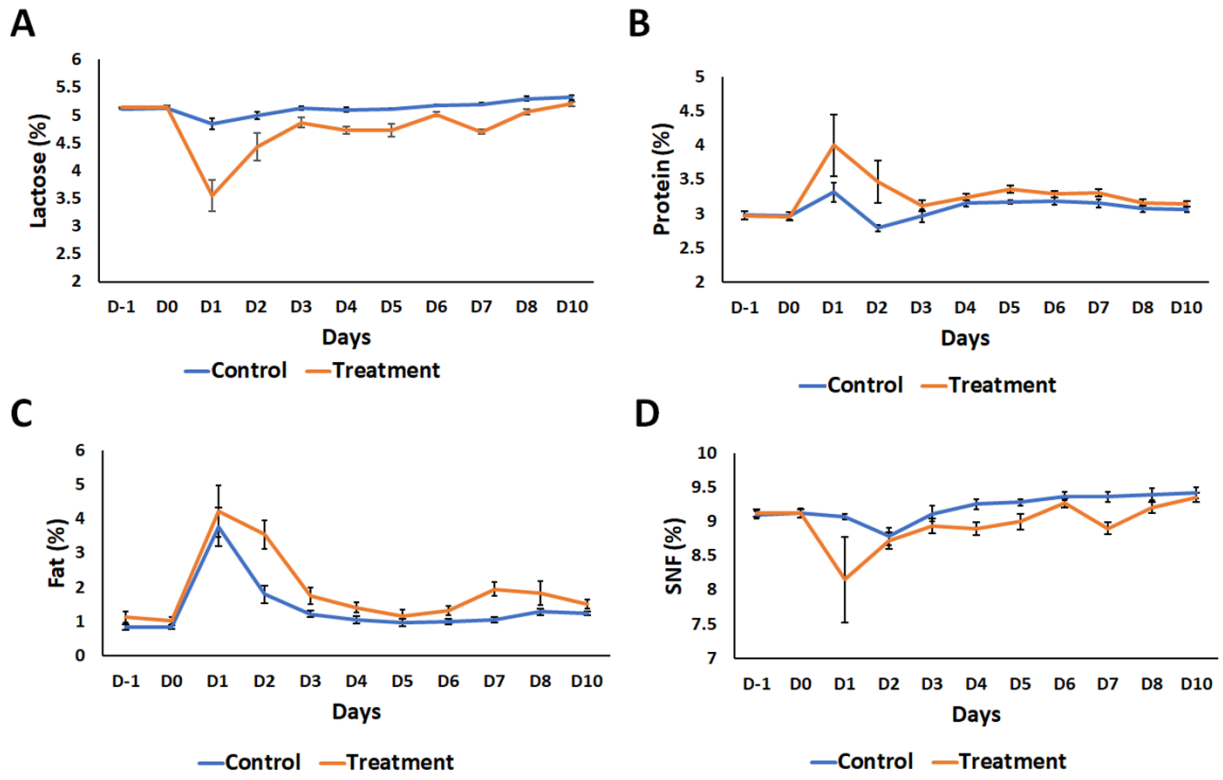

Figure S1. Effect of high dose probiotic *B. velezensis* AP183 treatment on (A) Lactose, (B) Protein, (C) Fat and (D) SNF in response to treatment (*B. velezensis* AP183 only) and control (healthy mammary glands).

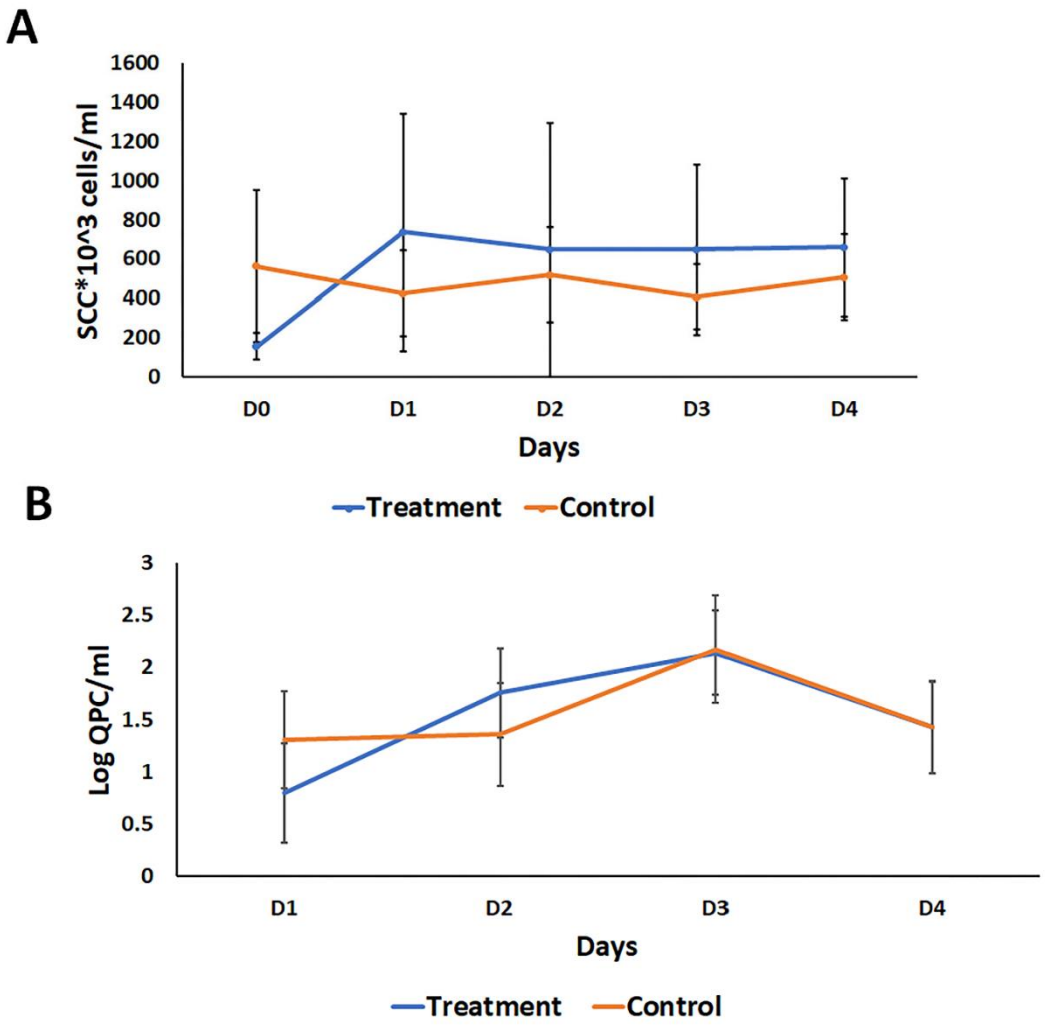

Figure S2. Effect of low dose probiotic *B. velezensis* AP183 on (A) somatic cell counts and (B) *Bacillus* count in response to treatment and control to treatment (*B velezensis* AP183 only) and control (healthy mammary glands)

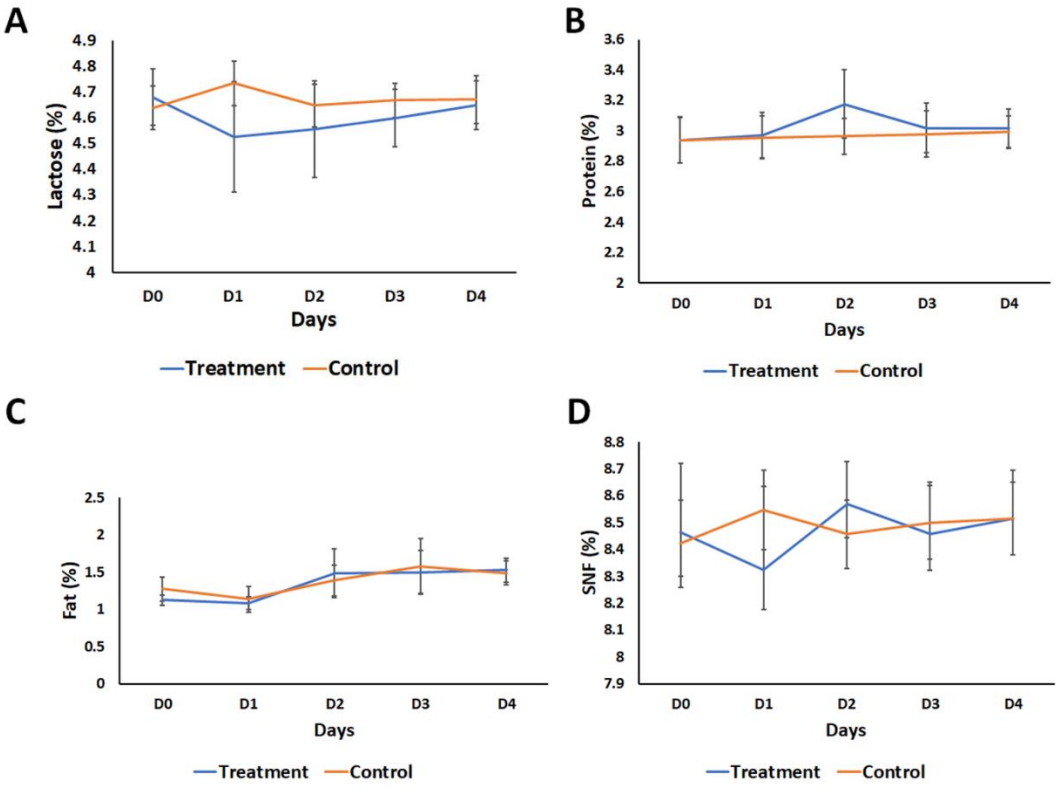

Figure S3. Effect of low dose probiotic *B. velezensis* AP183 treatment on (A) Lactose, (B) Protein, (C) Fat and (D) SNF in response to treatment (*B. velezensis* AP183) and control (healthy mammary glands)

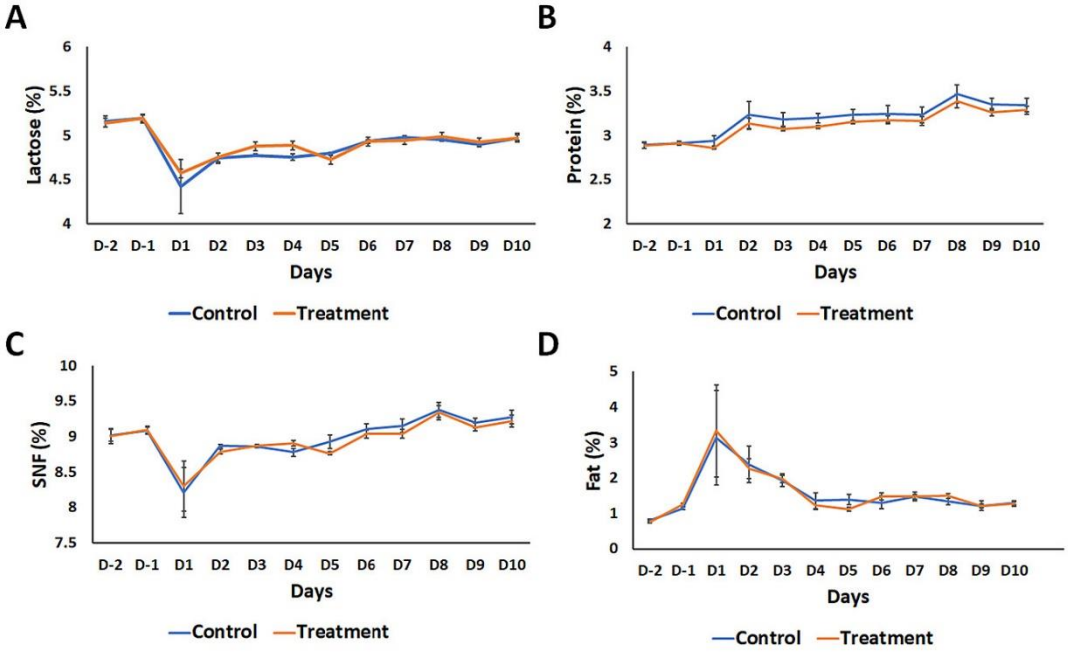

Figure S4. Effect of low dose probiotic *B. velezensis* AP183 treatment on (A) Lactose, (B) Protein, (C) Fat and (D) SNF in response to treatment (*B. velezensis* AP183 plus *S. aureus*) and control (*S. aureus* only).

Table S1. Antimicrobial activity of *B. velezensis* AP183 against mastitis-causing bacteria.

Organism	Strain	Test result[1]
S. aureus	1171-09	S
S. aureus	4366-13	S
S. aureus	1052-1	S
S. aureus	2226-11	S
S. aureus	1779-09	S
S. aureus	3851-07	S
S. aureus	3651-09	S
S. aureus	RF122	S
S. uberis	22272-08	S
K. pneumoniae	1583-07	R
B. cereus	NRS 1595	SS

[1]S = Susceptible to *B. velezensis* AP183 metabolites, based on a zone of growth inhibition >5 mm; SS = Slightly susceptible to *B. velezensis* AP183 metabolites, based on a zone of growth inhibition <5 mm; R = Resistant to *B. velezensis* AP183 metabolites, no growth inhibition was observed.

Chapter IV

Identification of novel bacteriocins from a soil metagenome

1. Abstract

According to CDC, every year 48 million foodborne illness occur in United States, which cause 128,000 hospitalizations and an estimated economic loss of $77.7 billion. Recent years have seen the alarming emergence of antibiotic resistant foodborne pathogens and a need for new natural antimicrobials. Bacteriocins are small ribosomally synthesized natural products that can have inhibitory activity against foodborne pathogens. The goal of our project is to identify and express novel bacteriocins from soil samples using a metagenomic approach.

In our study we used a metagenomic approach to discover novel bacteriocins encoded by soil microorganisms sampled from a long-term agricultural rotation (Cullars Rotation) at Auburn University. A previous study from our lab generated a soil metagenomic library containing 19,200 clones in a bacterial artificial chromosome vector with an average insert size of 110kb. An Illumina HiSeq next generation sequencer was used to sequence those clones in a pooled format (plate, row and column) and the trimmed sequences were assembled to generate contigs. The contig sequences were compared to the BAGEL3 and antiSMASH3.0 bioinformatics pipelines for bacteriocin mining. In silico screening of soil metagenome identified a total of 136 different types of bacteriocins. Among these putative identified bacteriocins, 98 bacteriocins were class I, 2 were class II and 36 were class III. BAGEL-3 screening showed that the class I bacterion contained 44 lasso peptide, 22 sactipeptide, 15 lanthipeptide, 8 bottromycin, 4 LAP, 2 head to tail cyclized peptide, 1 thio peptide, 1 micromycin and 1 cyanobactin. Further, in case of class II, it has one unmodified class IIa and one class IIc. In addition, out of 36 class III, 8 linocin M18, 13 zoocin, 2 pyocin and 1 collicin and the rest of the class III are peptidase M23. Few class I and class III

bacteriocins were selected for further study. For the expression of class III bacteriocin, the selected clones were identified from the cryopreserved metagenomic library, amplified by PCR and subcloned and expressed using the Expresso Rhamnose SUMO Cloning and Expression system. For class I bacteriocin expression, the structural gene were amplified first and subcloned in the Expresso Rhamnose SUMO Cloning and Expression system. Later the plasmid construct was transformed into corresponding metagenomic clones containing the whole BGC and expressed. The expressed protein was purified by affinity chromatography using Ni-NTA resin. The predicted bacteriocin gene sequences were highly divergent from known bacteriocins in the GenBank nr/nt database. The PCR results confirmed successful cloning of most of the respective bacteriocin gene into the expression vector and the expression of bacteriocin was confirmed by SDS-PAGE analysis. The protein expression for class III bacteriocin was partly soluble as we observed significant amount of protein in the pellet on SDS PAGE gel, it could be due to the aggregation of the expressed protein. We did not observe any antimicrobial activity against the indicator food borne organisms used in this study. For protein expression of class I bacteriocin, we observed successful expression of the bacteriocin but did not observe the expected modification which accompanied an expected loss of molecular weight due to dehydration step in post translational modification. Similarly, we did not observe any antimicrobial activity of those expressed putative bacteriocin against the selected food borne pathogens, confirming that no post translational modification happened. As we know that bacteriocins have potential application in the control of foodborne pathogens, the novel bacteriocins identified from this study could be of potential use as safe and effective antimicrobial agents in the food industry. We suggested that some strategies can be used in future to successfully express the bioactive putative bacteriocins that we identified from this study. The strategies for class I bacteriocin could be to place the structural gene and post

translational modification gene assemble into a single plasmid under strong promoter to get the active modified lanthipeptides. For the expression of class III bacteriocin, optimization of the expression condition at correct temperature, culture media to increase the solubility and yield of purified putative bacteriocin.

2 Introduction

The spread of multidrug resistance among pathogenic bacteria is increasing worldwide, which poses a great threat to public health (Tang, Wu, Wang, Gu, & Li, 2018). Overuse and misuse of antibiotics are one of the major causes for this increasing bacterial antibiotic resistance problem. There is also scarcity of new antibiotics developed in the last few decades (Brown & Wright, 2016). Customers also demand natural antimicrobials compounds and food preservatives in food products. Therefore, the discovery of new natural antimicrobials such as bacteriocin can be an alternative to the conventional antibiotic (Cotter et al., 2013; Mills, Ross, & Hill, 2017). Bacteriocins are produced by most bacteria as their defense system. Bacteriocins are ribosomally-synthesized peptides or proteins, which are natural, nontoxic, poses bacteriostatic or bactericidal activity against closely related bacterial species (Field, Cotter, Hill, & Ross, 2007). Bacteriocin can be divided into three classes; class I bacteriocins subjected to post-translational modification, class II is unmodified, and class III bacteriocins are unmodified heat-sensitive large peptides (Arnison et al., 2013; Cotter et al., 2013). The majority of the bacteriocin, especially class I bacteriocin, were initially synthesized as inactive precursor molecules, later subjected to many enzymatic post-translational modifications (Arnison et al., 2013).

Class I lanthipeptides have special structural, physiological, and functional characteristics, which make them a good candidate for antimicrobials. Lanthipeptides contain thioether amino acids, including lanthionine and/or methyllanthionine, that give them a polycyclic structure

(Dischinger, Chipalu, & Bierbaum, 2014). The post-translational modifications (PTMs) of lanthipeptides are carried out by Lanthipeptide synthetases enzymes. Two different kinds of proteins found in class I lanthipeptide, Lan B and Lan C, which have hydratase and cyclase activity, respectively. For class II lanthipeptide, the dehydration and cyclization are carried out by a single protein LanM that has both dehydratase and cyclase activity. LanA is the structural protein of class I lanthipeptide composed of a leader peptide and a core peptide (Arnison et al., 2013; Knerr & van der Donk, 2012). After the post translational modification of the core-peptide, the bioactive mature lanthipeptide forms which is later transported to outside of the cell by LanT, a ATP-binding cassette (ABC) transporter (Knerr & van der Donk, 2012).

Lan B hydrolyzes the serine and threonine residue of the core peptide, and the cyclase domain of Lan C performed the Michael-type addition-cyclization that Dhb/Dha and thiol groups of cysteines in the core structure to generate methyllanthionine/lanthionine (Dabard et al., 2001).The same reactions are done in the lanthipeptide II by Lan M proteins which have both dehydratase and cyclase domains. Linocin M18 is a non-lanthionine-containing class III bacteriocin that was isolated and characterized from red smear cheese bacteria *B. linens* (Valdes-Stauber & Scherer, 1996)(Ref). The molecular mass of this single protein subunit is 31 kDa, determined by SDS-PAGE though it forms a high molecular mass in native form by aggregation (Valdes-Stauber & Scherer, 1996). Linocin M18 are encapsulins that forms nano compartments containing ferritin-like proteins or peroxidases involved in oxidative stress response (Valdes-Stauber & Scherer, 1996). This bacteriocin has broad-spectrum activity against *Listeria monocytogenes* and some other Gram-positive bacteria such as *Bacillus*, *Arthrobacter*, *Corynebacterium*, *Micrococcus* (Valdes-Stauber & Scherer, 1996).

Few strategies can be used to identify novel bacteriocin, for example, culture-based approach and Insilco based approach (Marsh, O'Sullivan, Ross, Cotter, & Hill, 2010). Microbial diversity in the soil is huge, and culture-based techniques cannot capture most of them. The Insilco based technique has the advantage over the culture-based one as huge sequencing data can be generated by the metagenomic approach, which revealed many novel bacteriocins such as lantibiotic, microcin, sactibiotic (Begley, Cotter, Hill, & Ross, 2009; Lawton, Cotter, Hill, & Ross, 2007; Marsh et al., 2010; McClerren et al., 2006; Singh & Sareen, 2014). Bacteriocin gene cluster has special features, for example, the presence of post-translational modification genes, immunity gene, and ABC transporter gene. These features helped to discover and characterize bacteriocins using different bioinformatic tools. In recent years the bacteriocin mining software BAGEL3/BAGEL4 (Heel et al., 2013; van Heel et al., 2018) has become a valuable tool for in silico bacteriocin discovery. BAGEL discovered new bacteriocins based on the structural gene and the associated genes responsible for post-translational modification or other activity. antiSMASH is another tool that is used to detect bacteriocin. Specifically, it is helpful to detect the core peptide in class I bacteriocin (Blin et al., 2019). Soil is rich in different kinds of the diverse microbial community. Only 0.1 to 1 % of viable bacteria in soil grow in cultural methods (Torsvik, Sørheim, & Goksøyr, 1996). It is possible to identify largely unrevealed soil microbial communities using a metagenomic approach consisting of direct extraction of DNA and preparing clone libraries. A previous study from our lab constructed a soil metagenomic library from a soil plot that had not received fertilizer for at least 100 years (Pereira, 2021). The DNA from soil was subjected to high throughput sequencing on Illumina, and finally, clone location was identified using Geneious R10. This study used those cleaned contigs sequences to identify novel bacteriocins using the bioinformatic tool.

For heterologous expression, *E. coli* is the ideal host due to some specific advantages. They have a short generation time, easy to grow, have high cell density and product yield, and can be easily manipulated to investigate a target compound (Ortega & Van Der Donk, 2016). As a class I bacteriocin needs post-translational modification, it is required to supply the PTM enzyme from external sources. The structural genes and PTMs genes contained in a single fragment can be inserted into a single vector, or they can be inserted in a single vector under the control of the different promoters. It is not feasible to insert a big fragment containing the structural gene, PTM genes, and all other accessory genes; as for active expression, accessory genes are not required for the host (Ongey et al., 2018).

The specific objectives of the study were to (1) identify different types of bacteriocins from a soil metagenome, (2) examine any homology between structural gene and associated genes with previously characterized bacteriocin gene clusters (3) Finding the location of those bacteriocins from the metagenomic library using Geneious software, (4) Heterologous expression of few selected putative bacteriocins and check antibacterial activity against foodborne pathogens.

In this study, to express class I bacteriocin, the genes encoding the structural class I bacteriocin peptide were amplified from soil metagenomic library and then ligated to the Rhamnose vector and transformed into *E. coli* host. Later, the vector containing the structural bacteriocin gene was transformed into the corresponding metagenomic clone of *E. coli* that contains the whole BGC. Furthermore, for the expression of class III bacteriocin, the structural genes were amplified and ligated into the *E. coli* host of lucigenin rhamnose cloning system and expressed.

3 Methods and materials

3.1 Bacterial strains and growth conditions

E. cloni 10G Chemically Competent Cells were used for cloning and protein expression. After transformation, *E. coli* was grown in Luriae Bertani (LB) medium at 37°C. The indicator strains used in the bacteriocin assays were *L. monocytogenes, S. aureus, B. cereus, E. coli, Salmonella, V. parahaemolyticus and Campylobacter jejuni. L. monocytogenes, E. coli and Salmonella* were grown on Typtic Soya Agar plate, *S. aureus* and *Bacillus cereus* were grown on Brain Heart infusion agar. *V. parahaemolyticus* was grown on marine agar and *Campylobacter jejuni* was grown on Mullar Hinton broth medium. All the organisms were grown at 37°C except *Listeria monocytogenes, Vibrio parahaemolyticus* and *Campylobacter jejuni* which required 32°C, 32°C and 42°C respectively.

3.2 *Insilico* screening to identify novel bacteriocin genes

A previous study constructed a soil metagenomic library from a soil plot at the Cullars Rotation at Auburn University that had not received fertilizer for at least 100 years. The DNA from soil was subjected to high throughput sequencing on Illumina and was used to predict the bacteriocin genes. The library contained 50 plates of 384 wells, a total of 19200 clones. The pooled sample were prepared from each of the 50 plates, 24 columns and 16 rows separately. Then, each pool was bar-coded and sequenced using an Illumina HiSeq next generation sequencer. Next, the FASTA formatted contig sequences were obtained from each of the pooled samples and uploaded to the web version of BAGEL3/ BAGEL4 and antiSMASH software for screening of putative bacteriocin gene clusters.

The class III bacteriocin Linocin M18 were further selected for this study and linocin M18 sequences were aligned using the Multiple Sequence Alignment (MSA) tool MUSCL, included a sequence of commercially linocin M18. The predicted bacteriocins lanthipeptides were further investigated using antiSMASH tool. AntiSMASH tool can predict core and leader peptide of lanthipeptids. The fasta sequence of the contigs containing the genes of putative lanthipeptides were extracted from BAGEL3 and using the software RAST, the sequences were manually annotated. Then the predicted coding regions were analyzed using the BLASTp web server on NCBI and the nr database. For the lanthipeptide identification, modified enzyme or lanthipeptide associated genes were screened to identify different types of clusters of lanthipeptides. Putative Linocin M18 and lanthipeptides were aligned with Multiple sequence alignment tool MUSCLE.

3.3 Identification the location of clone in metagenomic library

Contig sequences were obtained from soil metagenome library from plates, rows and columns. To find the location of the structural genes of the putative bacteriocins, the structural gene as well as all the sequences of plates, rows and columns were imported to the Geneious R10. Then a blast search was performed for putative bacteriocin gene against the databases of plates, rows, and columns. Clone location was confirmed if blast matched 100 % with plate, row, and column. If only plate and any other one (row or column) matched 100%, then we used all the wells for other one (row or column) which did not match. After getting the position, the DNA was extracted from those clones. Primers were designed using those open reading frame of those bacteriocin genes in a way so that entire open reading frame was amplified (Table 1, Table 2).

3.4 Isolation of DNA from soil metagenome

DNA extraction was carried out with the help of Omega BAC/PAC DNA isolation Kit following the manufacturer protocol. PCR was performed using those template DNA with different

bacteriocin gene-specific primers which were designed in ours study. The focus of our study was to express open reading frames of those bacteriocin heterologously in *E. coli*.

3.5 PCR Condition

The DNA from soil metagenome was used for PCR amplification using a total of 50µl volume containing 25 µl of Green master mix, 20 µl of PCR water, 2 µl of 20 µM of primer pair and 3 µl of DNA. The program was done as follows: initial denaturation for 5 min at 95 °C, followed by 30 cycles of 15 sec at 95°C, 15 sec at 55 °C, and 1min at 72 °C then 1 cycle of 72 °C for 10 min. Then, 10µl of the PCR product was loaded onto 1.5 % agarose gel in 1X SB buffer and ran for 1 hour at 100 volts. The PCR products were analyzed in gel electrophoresis to identify bands after the gel was stained using ethidium bromide. Alpha Imager software was used to analyze images (AlphaView® software, San Leandro, CA).

3.6 Bacteriocin Quantification

Bacteriocin was quantified using UV absorption at 280 nm and BCA method (BCA1-1KT; Sigma-Aldrich, USA).

3.7 Induction conditions for class III bacteriocin genes expression in *E. coli*

To get the expressed protein, 10 ml of LB medium containing 30 µg/mL kanamycin was inoculated with a single colony of *E. cloni* 10G cells which contained a pRham expression construct and incubated at 37 °C with shaking. After the OD600 reached approximately 0.6, cultures were induced with 2% rhamnose and allowed to grow for overnight at 37 °C with shaking. Cells were collected by centrifugation at 4,000 × *g* for 15 min at 4 °C), after centrifugation 1 ml of lysis buffer the containing 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, was added to the pellet Then, lysozyme (1mg/ml) was added to assist cell lysis and incubated on ice for 30 min. Next, sonication was done to lyse the cell further and the samples were kept on the ice. In this procedure,

6-10 pulses of 10 sec were used and between the pulses the samples were allowed 1 minute to cool. A small amount of whole cell lysate was collected to perform gel analysis. The rest of the samples were centrifuged at 12000 × *g* for 10 min at 4 ℃ and supernatant was collected to get soluble protein. The pellet was also resuspended with equal volume of lysis buffer.

3.8 Transformation of class I bacteriocin constructs to corresponding BAC clones, growth and protein expression

To achieve expression of the designated genes, plasmid DNA containing the structural genes were transformed to corresponding BAC (Bacterial Artificial Chromosome) clones. The empty vector of rhamnose cloning system also transformed to empty vector of pSMART BAC clones. So, there are four different kind of expression vectors created for each clone; one is BAC clone (B), BAC clone containing the plasmid includes structural gene (B+S), structural gene alone (S) and rhamnose empty vector plus pSMART BAC empty vector clones (EVB+EVS). These four different expression strains were induced at four different kinds of inductions: rhamnose, arabinose, rhamnose+ arabinose and no induction.

For the expression purpose, all these four different kinds of strains grew overnight in LB broth with appropriate antibiotic. Next, 150 µl of culture from each strain were transfer to 15 ml of LB supplying with appropriate concentrations of required antibiotics. When the OD600 was around 4, appropriate kinds of inducers were added and grew another 24 h at 30°C to express the genes. After 24 h, 1 ml of culture was taken in Eppendorf tube and centrifuge at 12000g for 1 min. The supernatant was discarded and 100 µl of 2X SDS loading buffer was added and resuspended the cell. The sample was stored at -20 for later SDS PAGE analysis. 10 ml of culture of each type was taken in a falcon tube and centrifuge at 4000 × *g* for 15 min and then media was discarded, and the cell pellet kept at -20 for further preparations and analysis.

3.9 Extraction and purification

Cell pellets were stored in -20 °C, thawed and resuspended in lysis buffer (50 mM NaH2PO4_H2O, pH 8, 300 mM, 10 mM Imidazole). The cells were disrupted using sonication and proteins were released. To collect the soluble lysate, cell debris was separated by centrifugation for 15 min at 16,000 × *g* at 4 °C. The clarified lysates were applied to IMAC mini column for purification screening. In order to purify the 6XHis tagged proteins, the supernatant containing the soluble fractions were loaded onto Ni- NTA spin columns (Proteus IMAC Mini Column). Before loading the sample, the column was equilibrated with 0.65 ml binding buffer pH 7.4 (10Mm imidazolate) by centrifuging 1,800 × *g* for 1min. To purify the loaded protein, the manufacturer instructions were followed. Finally, the His-tagged proteins were eluted with 300 Mm imidazole by centrifuging at 1800 x *g* for 1 min. Purified His6-protein was dialyzed (SLIDE A-LYZER mini dialysis devices, thermo scientific fisher) using dialysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol) for 24 h at 4 °C to remove imidazole. The dialyzed purified bacteriocin was analyzed by SDS-PAGE analysis and protein concentrations were measured using BCA protein assay method. The dialyzed protein samples were concentrated using Amicon Ultra centrifugation tubes with 3.5 kDa molecular weight cut-off.

3.10 Spot lawn assay

A single colony of indicator organisms were inoculated in TSB and grew at 37 °C except *Listeria monocytogenes*, *Vibrio. parahaemolyticus* and *Campylobacter jejuni* which grew at 32 °C, 32 °C and 42 °C respectively 24 h. Later, 50 µl of culture was inoculated into 3 ml of fresh TSB and grown for another 5 h. With a sterile cotton swab, indicator cultures were spread onto appropriate microbiological plates and allowed for few min to dry. Later, to test antibacterial

activity, 20 µl of each tested whole lysate and purified putative bacteriocins were added to the plate in triplicate and incubated for 24 h at respective temperatures.

3.11 LC-MS

3.11.1 Sample Preparation

Samples were precipitated with a fourfold dilution of ice-cold acetone, centrifuged, and the solvent was removed. The precipitant was allowed to dry at room temperature and redissolved with 1 µL formic acid followed by 9 µL of 0.1% trifluoroacetic acid in 95% water with 5% acetonitrile.

3.11.2 LC-MS analysis

Analysis was performed on an ultra-performance LC system (ACQUITY, Waters Corp., USA) coupled with a quadrupole time-of-flight mass spectrometer (Q-Tof Premier, Waters) with electrospray ionization (ESI) in positive mode using Masslynx software (V4.1). Injection of 1 uL of the sample was made on a C4 column (Aeris™ 3.6 µm WIDEPORE C4 200 Å, LC Column 50 x 2.1 mm, Phenomenex) with a 300 µL/min flow rate of mobile phase solution A (0.1% formic acid in 95% water and 5% acetonitrile) and solution B (0.1% formic acid in 95% acetonitrile and 5% H2O) beginning at 10% B ramping to 35% B in 2 min, followed by a linear ramp to 62% B in 18 min, then to 100% B at 22 min, held 3 min, and back to 10% B with 7 min of re-equilibration. The MS spectral range was 150-3000 m/z, analysis occurred in continuum mode, with a scan time of 1.0 s and 0.02 interscan delay. The capillary voltage was set at 3.1 kV, the sample cone voltage was 30 V, and the extraction cone was 3.0 V. The source and desolvation temperature were maintained at 105 and 300 °C, respectively, with the desolvation gas flow at 300 L/h. The spectra were deconvoluted with the MaxEnt 3 algorithm within Masslynx 4.1.

4 Results

4.1 *In silico* screen of putative bacteriocin gene clusters

In silico screening of soil metagenome identified a total of 136 different types of bacteriocins. Among these putative identified bacteriocins, 98 were class I, 2 were class II and 36 were class III. BAGEL-3 screening showed that the class I bacterion contained 44 lasso peptide, 22 sactipeptide, 15 lanthipeptide, 8 bottromycin, 4 LAP, 2 head to tail cyclized peptide, 1 thio peptide, 1 micromycin and 1 cyanobactin. Further, in case of class II, there was one unmodified class IIa and one class IIc. In addition, out of the 36 class III, 8 linocin M18, 13 zoocin, 2 pyocin and 1 collicin were identified and the rest of the class III were peptidase M23 (Figure 1). For further analysis, 13 class I bacteriocin were selected and screened in *silico* for Lan C, Lan B, Lan M or other class I modifying enzymes homologues. Among them 10 were putative/homolog lanthipeptides, 1 was LAP, 1 was carocin D, and the last one was cerecidine. Based on the availability of clone location, we selected 8 of those putative class I bacteriocins for further investigation. Further analysis of the ORF of these clones using BlastP was performed for specific PTMs (Figure 2, Table 3). Out of these 8 class I bacteriocins, 3 of the lanthipeptides had both Lan C and Lan B, one cluster contained only Lan C, and for one cluster no lantibiotic-modifying genes were identified in close association (Table 3). One cluster of LAPs contained the required modifying gene such as cyclodehydration and LapbotD. For most of the lanthipeptides, structural peptides Lan A were not showed in insilico screening. Only 2 cluster showed contained putative *lan A, lan B* and *lan C* genes (Figure 2, Table 3).

For this study, 13 Linocin M18 were selected. Out of those, only 7 were further selected based on the availability of the clone position. The additional information of the ORF of Linocin

M18 was showed in Table 4. The putative Linocin M18 sequences and lanthipeptide sequences were aligned using multiple sequence alignment tool MUSCLE (Figure 3 and 4).

4.2 PCR amplification and construction of vectors for *E. coli* expression of bacteriocin

The location of the clone was successfully identified for some selected clones and the structural genes were amplified successfully from the DNA of soil metagenomic library. Out of 13 selected class I bacteriocins, 9 amplified were from soil metagenome and out of seven selected class III bacteriocin Linocin M18, six were amplified from soil metagenome (Figure 1 lanthipeptide+ Linocin M18 PCR). After transformation of amplified PCR products of bacteriocin genes into *E cloni* 10G using Expresso Rhamnose SUMO cloning and expression system, colony PCR was performed to confirm the transformation. Positive clone gave expected band size after gel electrophoresis of the PCR products and confirmed the subcloned by sequencing.

4.3 Expression and purification of bacteriocins

After successful transformation of putative lanthipeptide amplified PCR products into *E. coli* 10G, the constructs were extracted from *E. coli* 10 G and later the constructs were successfully transformed to corresponding BAC clones. The samples were analyzed after induction on SDS PAGE and found that out of nine samples, 7 expressed strongly, one expressed weakly and one did not express. The lysate and pellet were collected after sonication, applied to SDS PAGE gel and soluble protein was observed (Figure 5). Interestingly, among six linocin M18, three showed expressions with molecular size ~30 KDa, which w corresponds to the size of commercially available Linocin M18 (Figure 6).

4.4 Purification and concentration of putative bacteriocin

After successful expression, putative bacteriocin were purified and ran on SDS gel to confirm the purification (Figure 7).

4.5 LC MS analysis

The concentration of purified putative lanthipeptides were determined using BCA protein kit and the samples were sent to Auburn University mass spectrometry facility. The results here suggested that there was no successful post translational modification happened for lanthipeptides as there were no expected mass loss of molecules observed (Table 5).

4.6 Antibacterial activity of expressed putative bacteriocins

The whole lysate as well as purified concentrated putative bacteriocin orthologs were tested against several strains of *S aureus, B cereus, B subtilis* and *L monocytogenes* using a spot lawn assay. However, antibacterial activity was not observed.

5 Discussion

In the present study, we identified different types of bacteriocin producing genes from the soil metagenome through *in silico* screening. Many lactic acid bacteria produce antimicrobial peptides known as bacteriocins, which are natural, safe and there are no known drug resistance so far and have other benefits (Cotter et al., 2005). Some of those bacteriocins have narrow antimicrobial activity and lower production capability. Among the different bacteriocin discovered so far, nisin is the only commercially available bacteriocin used in the food industry. Therefore, it is very necessary to discover novel bacteriocins with desired characteristics as an alternative way to overcome the scarcity of new bacteriocin. Metagenomic based approach can be used to identify novel small molecule antibiotics and small antimicrobial proteins (Banik & Brady, 2010). To our knowledge, only one study by Gargi el al., 2013, identified bacteriocin genes from soil metagenome directly by amplifying bacteriocin-like genes, using different bacteriocin specific

primers and heterologously expressed those (Pal & Srivastava, 2014). In this study, we first screened the soil metagenome library to identify bacteriocin like genes, then cloned the genes and finally express some of those genes in *E. coli*. We were able to identify different class of bacteriocin and further selected some potential class I and class III bacteriocin based on the availability of location of the clone in the metagenomic library. We identified only 2 class II bacteriocin from the library which is relatively low frequency compared to class I and class III. A study by Calum el al., 2015, also identified relatively low frequency of class II bacteriocin compared to other classes from gastrointestinal tract (Walsh et al., 2015) This can be due to the lack of appropriate approaches that can identify class II bacteriocin (Walsh et al., 2015).

For the lanthipeptide clone and expression, we hypothesized that using a separate plasmid for structural gene and other plasmids for whole BGC could do the post translational modification. To test our hypothesis, we transformed the plasmid contains the structural genes into the corresponding metagenomic *E. coli,* clones containing whole BGC for bacteriocin that could help post translationally modification to the bacteriocin core peptide. The specific hypothesis was that after posting translational modification, the dehydratase domain of the PTMs will catalyze the dehydration of the threonine and serine residues from the core peptide of lanthipeptides, which is associated with loss of few molecules of water from post translationally modified peptide. In this, we did not observe the expected loss of molecular weight in LC-MS analysis after expression of clone, which contains two plasmids separately having structural gene and whole BGC. Therefore, our conclusion is that this strategy did not generate expected modification. One of the reasons for improper expression of the PTMs genes in the BAC clone, could be because the clone has 110 kb long inserts with different types of genes and the genes are not under strong promoter. Another reason could be that the structural gene and modification genes should be on the same plasmid for

post translational modification to occur. A study done by Elvis et al., 2018, also found that when they used two plasmids separately to express lanthipeptide RumA, it did not generate the expected modification. Similarly, we did not observe any antimicrobial activity of those expressed putative bacteriocin against the selected food borne pathogens, confirming that no post translational modification happened as active lanthipeptides must go for post translational modification to get the cyclic structure required for antimicrobial activity. There was also earlier study that attempted to express the lantibiotics but only expressed the unmodified precursor peptide (Karakas-Sen & Narbad, 2012).

Further study was required to determine the optimum condition for the expression of lanthipeptide. We were in need of the structural gene and post translational modification gene to assemble into a single plasmid under strong promoter to get the active modified lanthipeptides. Previously, a few studies assembled the required genes for lanthipeptides into a single plasmid under strong promoter and successfully expressed the bioactive lanthipeptide (Kuthning, Mösker, & Süssmuth, 2015; Ongey et al., 2018)

Later, we selected few putative class III bacteriocin Linocin M18 from soil metagenome. As reported previously, linocin M18 is a bacteriocin with antimicrobial activity that inhibits many important food borne pathogens (Valdes-Stauber & Scherer, 1996; Valdés-Stauber & Scherer, 1994). This class of bacteriocin does not require any post translational modification, because of this nature linocin M18 is considered as a good choice for the selection. The putative linocin M18 construct was made successfully with the His-tag fusion, and then it was over expressed after induction as a full-length protein of above 40 kDa size. The protein expressed was partly soluble as we observed significant amount of protein in the pellet on SDS PAGE gel, that could be due to the aggregation of the expressed protein. We did not observe any antimicrobial activity against the

selected foodborne organisms used in this study. It could be due to the low concentration of purified linocin M18, not correctly folded protein or not proper optimization condition of the expressed protein or this putative linocin M18 orthologs were not active against the selected food borne pathogens.

Further study will be required for active putative linocin M18 expression to determine the optimum condition that can enable us to obtain soluble protein of the correctly folded peptide. This can be done by optimizing temperature, inducer, media composition, choosing a correct heterologous host. Kraken which is a new sequence classification tool can be used to assign a taxonomic label to an unknown metagenome sequence (Wood & Salzberg, 2014). This can predict the origin of the sequence that will help the selection of indicator organisms as bacteriocins mostly work against closely related organisms (Hatakka & Saxelin, 2008).

In conclusion, this study identified many putative novel bacteriocins through *in silico* analysis of a soil metagenome. Though it is not hard to clone and express those putative bacteriocin but the functional expression of putative bacteriocin in a host could be a complicated specially for class I bacteriocin lanthipeptide. In addition, we mentioned some of the future strategies to successfully express the bioactive putative bacteriocins that we identified from this study.

Figures and tables

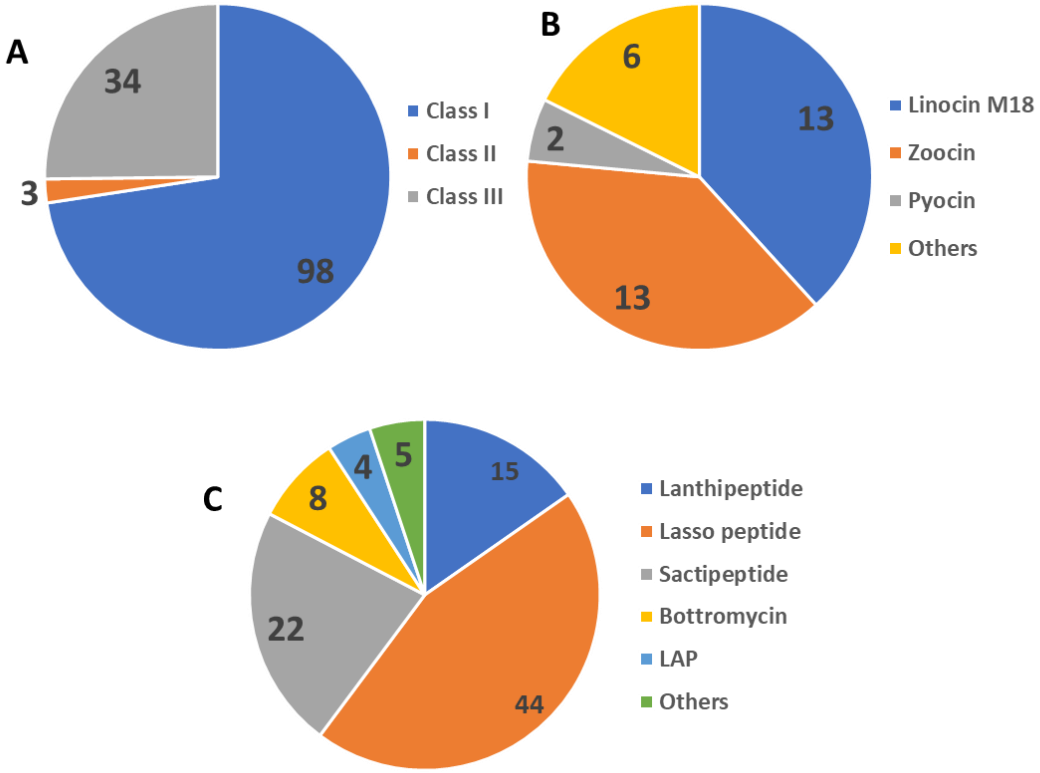

Figure 1. Frequency of bacteriocin class (A), class III (B) and class I bacteriocin (C) from soil metagenome.

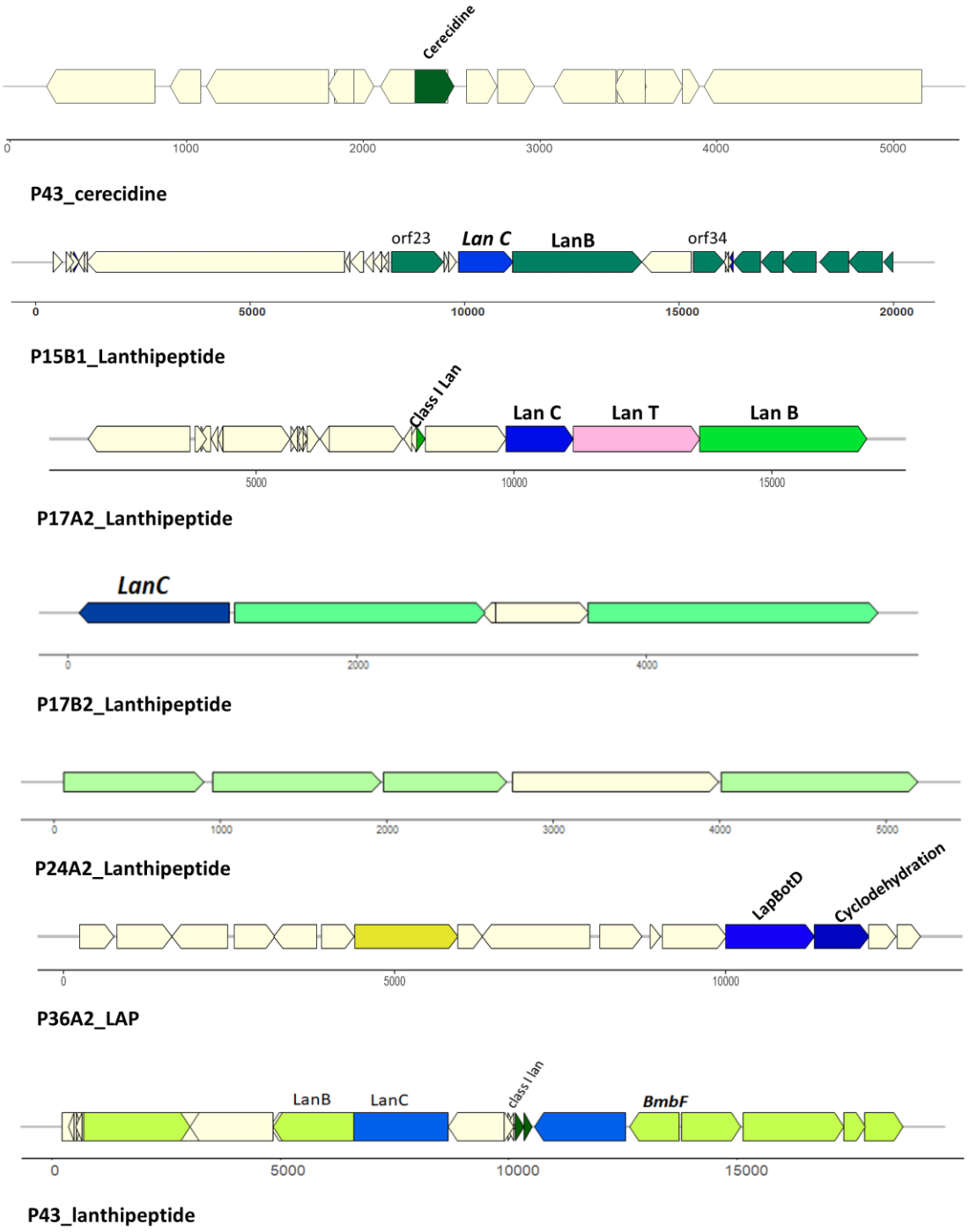

Figure 2. Class I bacteriocin cluster types predicted by BAGEL3/4.

Figure 3. Multiple sequence alignment of predicted putative Linocin M18 orthologs.

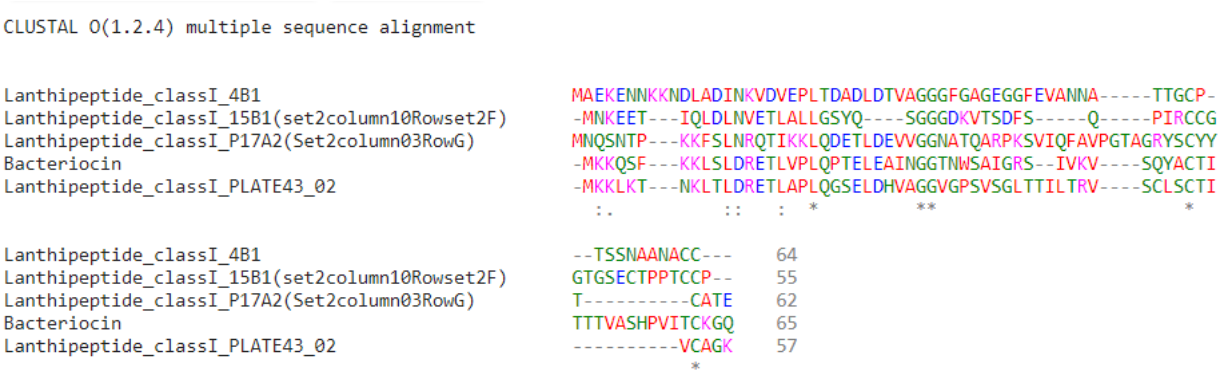

Figure 4. Multiple sequence alignment of predicted putative lanthipeptide orthologs.

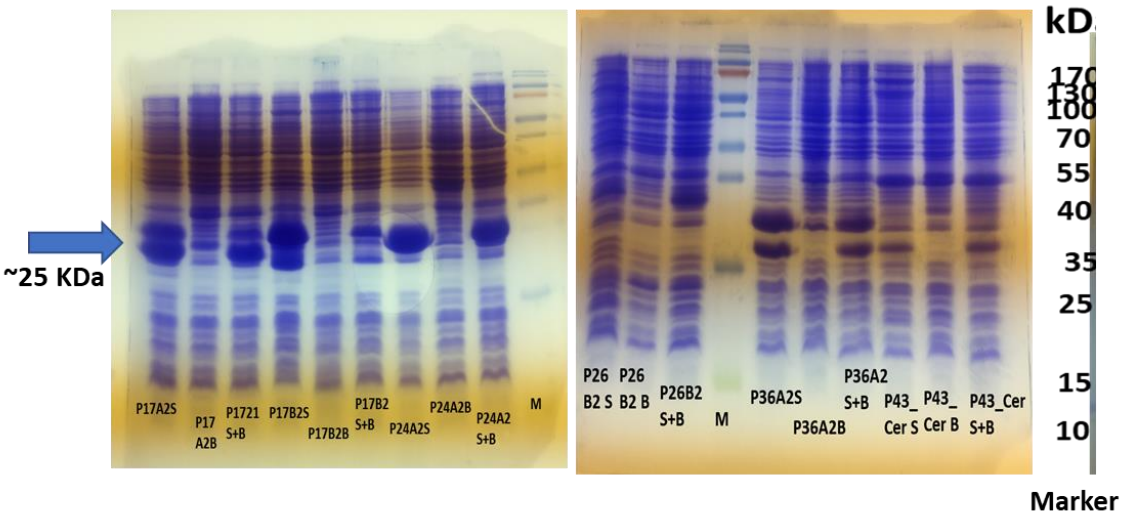

Figure 5. Expression of His_6Tag fusion putative lanthipeptides in *E. coli*.

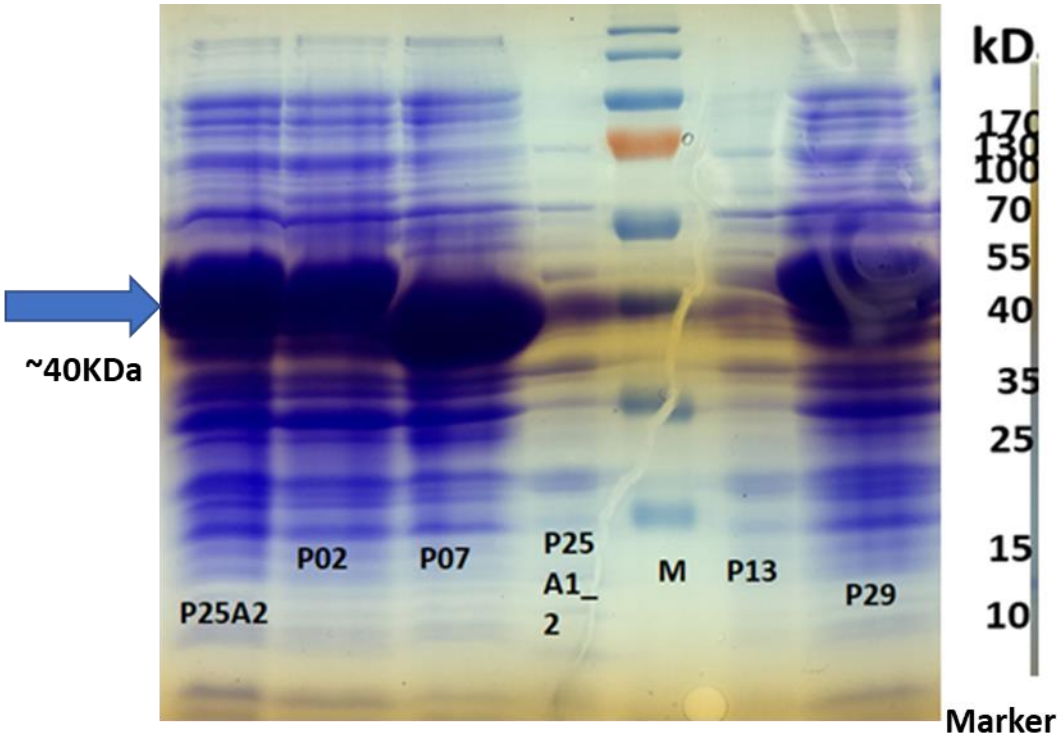

Figure 6. Expression of His_6 Tag fusion putative Linocin M18 in *E. coli.*

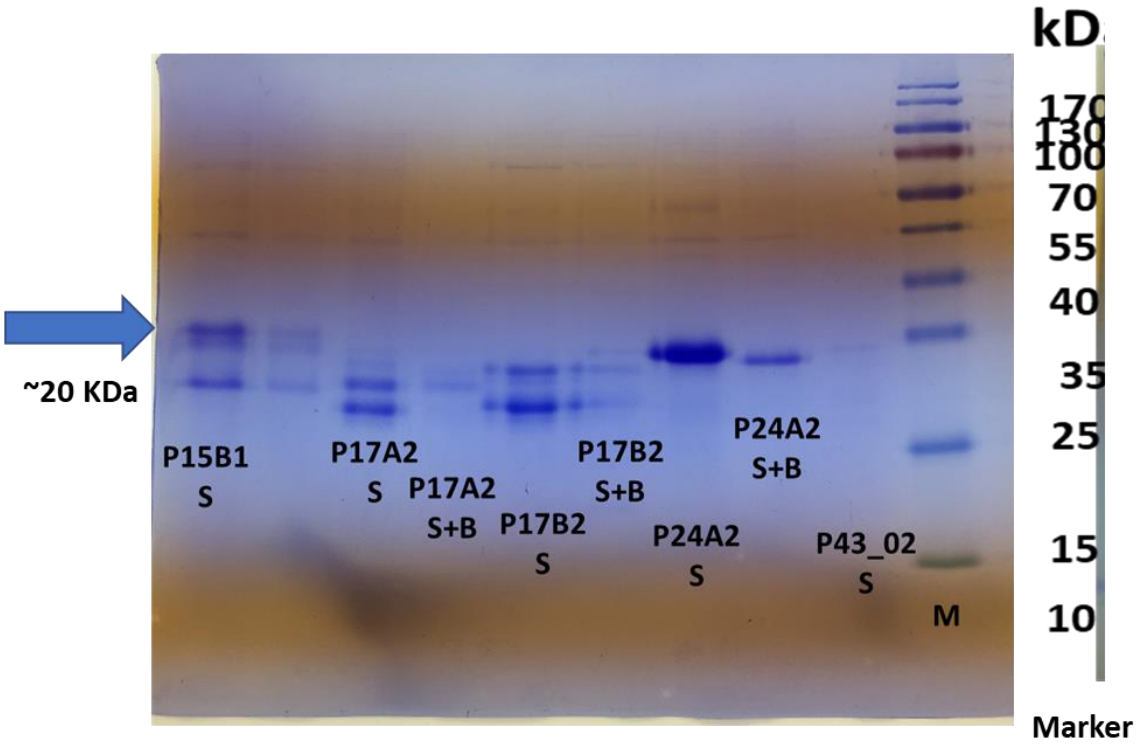

Figure 7. SDS analysis of purified putative fusion lanthipeptides in *E. coli.*

Table 1. Class I bacteriocin specific primers used in this study.

Serial	Primer name	Sequence	Tm	GC%
1	Lan_P43_02 F	CGCGAACAGATTGGAGGTAAGAAGCTCAAGACCAAC	71.9	61.7
	Lan_P43_02_R	GTGGCGGCCGCTCTATTACTTCCCCGCGCAGACGAT	80.6	67.3
2	Lant_15B1_F	CGCGAACAGATTGGAGGTAACAAGGAAGAAACCATCCAG	74.6	62.6
	Lan_15B1_R	GTGGCGGCCGCTCTATTACGGACAACAGGTGGGGGGAG	81.5	69.1
3	Lan_26B2_F	CGCGAACAGATTGGAGGTGGGAATAACGATCATCTT	73	60.5
	Lan_26B2_R	GTGGCGGCCGCTCTATTAATTTCTGCAATGATATCC	70.7	59.4
4	Lan_4B1_F	CGCGAACAGATTGGAGGTGCTGAGAAAGAGAACAAC	72.8	61.7
	Lan_4B1_R	GTGGCGGCCGCTCTATTAGCAGCAGGCGTTTGCCGCGTTAG	83.5	69.4
5	Lan_P17B2_F	CGCGAACAGATTGGAGGTAACAAGAAGACGGACG	72.6	61.8
	Lan_P17B2_R	GTGGCGGCCGCTCTATTAGCAAGGCCAGCAACTGTTG	78.7	66
6	Lan_P17A2_F	CGCGAACAGATTGGAGGTAACCAATCGAACACGCCCAAG	78.8	64.7
	Lan_P17A2_R	GTGGCGGCCGCTCTATTACTCGGTCGCGCAGGTGTAG	79.9	68.3
7	Lan_24B2_F	CGCGAACAGATTGGAGGTCAGACCCCCGAAATTCTGAAC	78.1	64.7
	Lan_24B2_R	GTGGCGGCCGCTCTATTAGTTCTGGGTGCACTTGTT	75.4	63.9
8	Lan_P38B1_F	CGCGAACAGATTGGAGGTGCTAAGAAAGGTAATCGCGTTCAG	77.1	64.3
	Lan_P38B1_R	GTGGCGGCCGCTCTATTACTTGATTTCTTTGTGAGTCG	74.1	62.6
9	Lan _S2C12_F	CGCGAACAGATTGGAGGTTGCTGGAAAGCCGCCGTTTTG	81.7	65.7
	Lan_S2C12_R	GTGGCGGCCGCTCTATTACGGCGTGTGCACGACGAC	82.1	68.5
10	LAP_ S4RF_36A2_F	CGCGAACAGATTGGAGGTTTCACCGAGCTCAAGGAG	76.3	63.9
	LAP_ S4RF_36A2_R	GTGGCGGCCGCTCTATTAGCTCCAAGAAAGCTTGCAG	76.6	64.9
11	Lan_43_old_F	CGCGAACAGATTGGAGGTAAGAAGCAGTCGTTCAAGAAG	73.8	62.6
	Lant_old_R	GTGGCGGCCGCTCTATTACTGACCCTTGCAGGTGAT	76.7	65.1
12	Carocin D_24A1_F	CGCGAACAGATTGGAGGTGCTAATGACGATACGATTTAC	72.7	61.5
	Carocin D_24A1_R	GTGGCGGCCGCTCTATTACTCTCCATATACTTGTTTAATTTCTG	72.2	62.3
13	Cerecidin__43_ 02_F	CGCGAACAGATTGGAGGTATTGCACCGACGACGAT	76.4	62.9
	Cerecidin__43_ 02_R	GTGGCGGCCGCTCTATTAGCGCTTGACTCTGCAGGT	78	66.2

Table 2. Class III bacteriocin specific primers used in this study.

Serial	Name of the primers	Sequences	Tm	%GC
1	Lin29A2C12RE_F	CGCGAACAGATTGGAGGTAGCCATCTGTTTCGC	73.4	54.5
2	Lin29A2C12RE_R	GTGGCGGCCGCTCTATTAGCGCTTGGGAGACTT	75.3	60.6
3	Lin29A2C12RE_F2	CGCGAACAGATTGGAGGTAGCCATCTGTTTCGCGAC	76.6	55.6
4	Lin29A2C12RE_R2	GTGGCGGCCGCTCTATTAGCGCTTGGGAGACTTTTC	76.6	58.3
5	Lin25A1C2RE_F	CGCGAACAGATTGGAGGTGTGGTCGGCGTGCAG	79.6	63.6
6	Lin25A1C2RE_R	GTGGCGGCCGCTCTATTAGCTCTTGGCTTTCTCGCC	77.8	61.1
7	Lin25A1C2RE_F2	CGCGAACAGATTGGAGGTGTGGTCGGCGTGCAGGGTTC	83	63.2
8	Lin25A1C2RE_2F	CGCGAACAGATTGGAGGTAATAATCTTCACCGCGAGCTT	75.2	48.7
9	Lin25A1C2RE_2F2	CGCGAACAGATTGGAGGTAATAATCTTCACCGCGAGCTTGC	77.7	51.2
10	Lin25A1C2RE_2F3	CGCGAACAGATTGGAGGTAATAATCTTCACCGCGA	73	48.6
11	Lin25A1C2RE_2R	GTGGCGGCCGCTCTATTAGCTCTTGGCTTTCTCG	74.9	58.8
12	LinP25A2C11RB_F	CGCGAACAGATTGGAGGTGTGGACGAGATCGCCAACCA	81.3	57.9
13	LinP25A2C11RB_R	GTGGCGGCCGCTCTATTACTTGATCACGCAGATGGCTTC	78.5	56.4
14	LinP25A2C11RB_F2	CGCGAACAGATTGGAGGTGAATGGTTAAGACGCAGC	75.3	52.8
15	LinP25A2C11RB_R2	GTGGCGGCCGCTCTATTACTTGATCACGCAGATGGC	77.5	58.3
16	Lin13B2C5RE_F	CGCGAACAGATTGGAGGTAACCACCTGCTGCG	75	59.4
17	Lin13B2C5RE_R	GTGGCGGCCGCTCTATTAGCGATCGTTCTTGCTCGC	79	61.1
18	Lin13B2C5RE_F2	CGCGAACAGATTGGAGGTAACCACCTGCTGCGCGAGCTGGC	84.2	63.4
19	LinP02A2C1RA_F	CGCGAACAGATTGGAGGTGTGATAGACATGGATCTGCTCA	76.6	50
20	LinP02A2C1RA_R	GTGGCGGCCGCTCTATTAGGACGCCACGAGCGG	80.3	69.7
21	LinP02A2C1RA_2F	CGCGAACAGATTGGAGGTGATCTGCTCAAACGAGAGCTCGCAC	81.5	55.8
22	LinP02A2C1RA_2F2	CGCGAACAGATTGGAGGTGATCTGCTCAAACGAGAGCTCG	79.1	55
23	LinP02A2C1RA_2R	GTGGCGGCCGCTCTATTAGGACGCCACGAGCGGCA	82.8	68.6
24	LinP07B2C12RF_F	CGCGAACAGATTGGAGGTAACCACCTGCTACGCGAGTTCG	79.3	57.5
25	LinP07B2C12RF_R	GTGGCGGCCGCTCTATTAGCTGTAGCGCAGC	74.3	64.5
26	LinP07B2C12RF_F2	CGCGAACAGATTGGAGGTAACCACCTGCTACG	71.5	56.3
27	LinP07B2C12RF_R2	GTGGCGGCCGCTCTATTAGCTGTAGCGCAGCG	76.5	65.6
28	LinP03B2C5RA_F	CGCGAACAGATTGGAGGTAGCCATCTGCTGCGCGA	79.6	60
29	LinP03B2C5RA_R	GTGGCGGCCGCTCTATTAGGGGGTGAGCGCGAC	79.7	69.7

Table 3. Additional information based on the presence of PTMs associated genes in the putative class I bacteriocins.

ORF	Top BLAST hit function	Top BLAST hit neighbor	%Identity	E val	Accession number
15B1_ orf23	MULTISPECIES:	Myxococcus	45.6%	4.00E-	WP_141252
15B1_ LanC	lanthionine synthetase C	Myxococcus	41.7%	6.00E-	WP_141640
15B1_ orf31/Lan	lantibiotic	Corallococcus	38.1%	2.00E-	WP_164933
P17A2_orf0026	thiopeptide-type bacteriocin	Pyxidicoccus sp.	46.0%	0	WP_206720
P17A2_LanB	lantibiotic dehydratase	Corallococcus	43.4%	0	WP_120609
P17A2_LanT	peptidase domain-	Deltaproteobacte	54.2%	0	MBI4511350
P17A2_LanC	lanthionine synthetase C	Phycisphaerae	38.3%	1.00E-	MBN256264
P17A2_class I lanthipeptide	class I lanthipeptide	Archangium	65.5%	0.003	WP_204494
Plate 43_ class I lanthipeptide	class I lanthipeptide	Deltaproteobacte	50.0%	4.00E-	MBX315834
Plate 43_ LanC	lanthionine synthetase C	Deltaproteobacte	68.3%	6.00E-	MBA254515
Plate 43_ ORF13/lanthib	lantibiotic dehydratase	Deltaproteobacte	71.1%	0	MBA254127
P17B2_LanC	lanthionine synthetase C	Acidobacteria	75.9%	0	MBW88768
P17B2_orf6	chorismate synthase	Acidobacteria	94.6%	0	MBW88768

ORF	Top BLAST hit function	Top BLAST hit neighbor	%Identity	E val	Accession number
36A2_ Lank	HAMP domain-containing	Actinobacteria	71.5%	0	TML70489.1
36A2_Cyclodehydration	SagB/ThcOx family	Sorangium	49.3%	3.00E-	WP_012239
36A2_LapBotD	YcaO-like family protein	Actinobacteria	53.9%	2.00E-	MBA341208

Table 4. Additional information based on the BLASTp analysis of putative Linocin M18.

Linocin ORF	Top BLAST hit function	Top BLAST hit neighbor	%Identity	E value	Accession number
13B2	bacteriocin	Myxococcales bacterium	58.1%	1.00E-102	MAC26574.1
25A1	bacteriocin family	Rhodoplanes sp.	84.4%	5.00E-137	WP_0680216
P03B2	bacteriocin	Actinobacteria bacterium	79.0%	9.00E-149	TMK25053.1
P07B2	bacteriocin family	Actinomarinicola tropica	61.8%	9.00E-119	WP_1537586
P02A2	bacteriocin family	Polyangium sp. SDU3-1	72.0%	8.00E-130	WP_1369255

Table 5. Molecular masses of putative lanthipeptides by LC-MS analysis.

SL#	His-tagged protein	Mass
1	P15B1C19R(S)	5396,14113, 18206
2	P15B1C19R(B+S)	14113, 18208
3	P17A2C6RM(S)	15648, 16964, 17108, 17112
4	P17A2C6RM(B+S)	16964, 7108, 17112
5	P17B2C18RB(S)	12541, 19088, 19312, 14136, 15643
6	P17B2C18RB(B+S)	4370, 15648
7	P24C12RB(S)	12427
8	P24C12RB(B+S)	19532
9	P43-02(S)	12427
10	P43-02(B+S)	12427
11	P36C12RK(S)	12547, 14917, 15081, 13986
12	P36C12RK(B+S)	14920,15081, 15152
13	P43-OLD(S)	13047
14	P43-OLS(B+S)	26971, 13046

Chapter V

Conclusion

Soil is both ubiquitous and rich in bacterial diversity that are great resource of secondary metabolites. Only less than 1% of bacterial species are identified from soil and 99 % of bacterial species are not able to be cultivated in the laboratory since many of them are not readily cultured under laboratory conditions. Therefore, despite of abundance of microbial diversity in soil, many of the secondary metabolites are not discovered from soil. In this study both culture-dependent and culture-independent approaches were used to discover antibiotics or antimicrobials against *Staphylococcus aureus* infection and other food borne pathogens.

Chapter 2

In this study, a type I modular PKS biosynthetic gene cluster was identified through genome mining that was predicted to synthesize bacillusin A in *B. velezensis* AP183. To verify that this BGC was responsible for biosynthesis of bacillusin A, a 44 kb region was deleted from the predicted BGC and found by LC-MS analysis that mutant lost to produce bacillusin A and anti-MRSA activity. In addition to generate a knockout mutant that lacked bacillusin A biosynthesis in *B. velezensis* AP183, the entire large (~70 kb) *basA* BGC was cloned and expressed in *B. subtilis* 168 and was found to produce bacillusin A that had anti-MRSA activity. Surprisingly, LC-MS analysis identified that *B. velezensis* SQR9 which has a similar comparable type I modular BGC like *B. velezensis* AP183, not only produce bacillunoic acid but also produces bacillusin A. However, the bacillusin A production are at a lower level than *B. velezensis* AP183, and this has not been previously reported. In conclusion, a type I modular PKS BGC was discovered that was responsible for bacillusin A production in *B. velezensis* AP183.

Chapter 3

B. velezensis AP183 was evaluated in inhibiting *Staphylococcus aureus* infection in mouse wound model and bovine mastitis model. Mice were subcutaneously injected with bioluminescent *S. aureus* strain Xen29 with and without AP183 spores and metabolites. It was observed that *B. velezensis* AP183 significantly reduced the relative abundance of *S. aureus* as well as the number of *S. aureus* compared to the control group. Another experiment was conducted to evaluate if *B. velezensis* AP183 biofilm formation in a tracheostomy tube inner cannula can reduce the subsequent *S. aureus* colonization on tracheostomy tube. It was observed that *B. velezensis* AP183 biofilm significantly inhibited the colonization of *S. aureus.* Next, we evaluated the impact of *B. velezensis* AP183 against *S. aureus* induced bovine mastitis model. First, the impact of *B. velezensis* AP183 was evaluated for the immune response on mammary gland with high dose and low dose of *B. velezensis* AP183 intramammary inoculation. It was observed that high dose increased the short-term somatic cell count and cause clinical mastitis. On the other hand, no clinical mastitis formed with low dose inoculum and lower increase of somatic cell count observed that was below the threshold of bulk tank somatic cell count in milk in US. In addition, the inhibition capability of *B. velezensis* AP183 in *S. aureus* induced bovine mastitis was investigated and found that AP183 significantly reduce the relative abundance of *S. aureus* in treatment group after 8 days post inoculation.

Chapter 4

In this study, a metagenomics approach was used to discover novel bacteriocin from soil metagenome. In silico screening of soil metagenome identified a total of 136 different types of bacteriocins orthologs including 98 class I, 3 class II and 36 class III, most of the identified bacteriocins were novel. We cloned and expressed few putative lanthipeptides and linocin M18

orthologs using rhamnose sumo cloning expression system. We did not observe post translational modification of lanthipeptides by LC-MS analysis. No antimicrobial activity was observed for lanthipeptides and linocin M18 against selected foodborne pathogens. Further study will be required for active putative bacteriocin expression to improve the expression.

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