

# Bacteriocin producing *Bacillus* and their potential applications in fish farming

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Received: 04 October 2023 / Accepted: 24 February 2024 / Published online: 03 March 2024  
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**Abstract** Studies on the bacteriocins of *Bacillus* species are increasing due to enhanced research interests and benefits for diverse applications ranging from bio-preservatives to probiotics, providing natural antimicrobial functions in fish farming and related aquaculture production needs. These benefits are due to their broad spectrum of antimicrobial activity and mode of action, their ability to secrete these important peptides in large amounts (g/L level) in a culture medium, and the ubiquitous nature of producing strains. It is well known that fish farming industries are challenged with pathogenic infection, indiscriminate antibiotic use, and now linked to antibiotic resistance of pathogens due to poor sewage disposals and persistence in nature. To overcome these challenges and the quest to stop the unregulated use of conventional antibiotics in fish farming industries has encouraged studies toward more eco-friendly options such as probiotics and associated bacteriocins with antimicrobial potential. Therefore, this review summarized and discussed recent studies on the effectiveness of *Bacillus* strains probiotic application in fish farming, emphasizing the importance of their proper characterization, extended field trials and the need for specifications in the selection criteria of the potential *Bacillus* probiotic strains of interest before further investigations, approvals and registrations.

**Keywords** Aquaculture . Antimicrobials . Probiotics

## Introduction

Fish farming and related aquaculture production systems are robust global agricultural sectors contributing to food security, better nutrition and health with benefits of added economic value from a range of food products. One of the major challenges to sustainable advances in this sector is that it is affected by infectious pathogenic microbes (*A. hydrophila*, *S. agalactiae*, *A. salmonicida*, *P. fluorescens*, *Vibrio* spp. and others.), which can result in fish production underperformance and losses from death in severe cases affecting farmers and associated production system of aquaculture (Banerjee et al. 2017; Haenen et al. 2023; Sanches-Fernandes et al. 2022). Of the techniques adopted by farmers (improved sanitation, light and water control and others) against these pathogens, antibiotics usage was a significant breakthrough in controlling

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fish pathogens as early as the 1990s (Hernández Serrano 2005). However, the recent overuse of antibiotics and the resulting antibiotic resistance of the surrounding microbes are environmental and public health concerns. This is largely due to poor technical know-how, overuse, and abuse of the application as a fish growth promoter, and its unregulated release into the surrounding environment via sewage disposals, resulting in the rise of antimicrobial-resistant microbes (AMR) (Cabello 2006). This has prompted the banning of some antibiotics in fish farming in some nations across the globe (Chowdhury et al. 2022; Lulijwa et al. 2020). The enacting of strict laws on restricted use by some regulatory bodies to ensure this compliance by farmers has occurred; Thailand is a case study (Sommanustweechai et al. 2018). To overcome the prohibited use of antibiotics, more eco-friendly alternatives, such as probiotics, were adopted as alternatives to conventional antibiotics in fish farming (Merrifield et al. 2010).

Probiotics usage in fish farming is proven to improve the immune status of the farmed fish, increase their resistance to diseases and reduce their stress response (Cruz et al. 2012; Merrifield et al. 2010). Over the years, the probiotics of the lactic acid bacteria (LAB) group have mostly been studied due to their long history of discovery and generally regarded as safe (GRAS) status (FAO/WHO 2006). Subsequently, the probiotics of *Bacillus* species have also gained recognition due to the additional advantages they offer, such as a broad antimicrobial spectrum, wider stress resistance and the formation of resilient spores for commercial formulation and use (Abriouel et al. 2011). To ensure safety regulations on potential microbial probiotic strains, FAO/WHO set out five (5) main *in vitro* studies regulatory guidelines; resistance to gastric acidity, bile acid resistance, adherence to mucus and human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria and ability to reduce pathogen adhesion to surfaces (FAO/WHO 2006). Their antimicrobial activity study against an indicator is considered the first line of study among these guidelines. Furthermore, the potential of the strains bacteriocin-producing ability is evaluated (Corr et al. 2007).

Therefore, in this review, we have discussed the bacteriocins of *Bacillus* species and the need for their characterization to help advance an effective path for their classification and to regulate proper probiotic strain selection in different fields of application, especially in fish farming.

#### Fish farming and some of its pathogenic challenges

Fish farming, or pisciculture, refers to the controlled commercial rearing of fish aimed at providing accessible animal protein for human consumption and producing other valuable products for human and animal welfare. The advantages of fish farming are multifaceted. It seamlessly integrates into existing farming systems, offering farmers an additional income stream. Furthermore, fish farming can enhance water quality for other agricultural activities, as fish excrete ammonia, which serves as a nitrogen source readily utilized by crops. Additionally, fish farming promotes efficient land use, particularly beneficial in waterlogged soils where traditional agricultural drainage methods can be financially burdensome (Benbrook 2002).

Unfortunately, despite the enormous positive impact that fish farming provides on both economic and nutritional levels, it also faces a major challenge; particularly diseases caused by pathogenic organisms such as *A. hydrophila*, *P. fluorescens*, *E. tarda*, *A. salmonicida*, *P. putida*, *B. mycoides*, *V. vulnificus*, *V. ordalii*, *V. carchariae*, *M. viscosa*, *P. skyensis*, *F. psychrophilum*, *F. branchiophila* and *E. ictaluri*. These pathogens are associated with fish diseases such as cottonmouth, dropsy, fin/tail rot, swim bladder, winter ulcer, bacterial gill disease, piscirickettsiosis, vibriosis, pasteurellosis and columnaris disease (Sudheesh et al. 2012).

This challenge has resulted in great economic losses to the farmers and tremendous stress to the fish as these diseases affect the growth performance of the fish and may lead to their death, hence, the high mortality rate. Therefore, to solve this challenge, most farmers adopted the use of antiseptics, disinfectants, improved sanitation techniques, light and water control, vaccines, and antibiotics such as oxytetracycline, sulphadiazine, florfenicol, chloramphenicol, sulphadimethoxine, erythromycin, amoxicillin, and enrofloxacin, amongst others (Lulijwa et al. 2020). The use of antibiotics was a significant breakthrough in controlling fish pathogens as early as the 1990s (Hernández Serrano 2005). Unfortunately, beyond the use of antibiotics to inhibit and/or kill fish pathogens, farmers also used them as feed additives to promote fish growth, thus leading to the indiscriminate use of conventional antibiotics (Cabello et al. 2023).

This unregulated usage of antibiotics in fish farming, or pisciculture has given rise to environmental,



food safety, and public health challenges, as most pathogens are now resistant to conventional antibiotics. A study by the American Society of Microbiology (ASM) 1994 showed that antibiotics used in an aquatic system could be unintentionally released into the environment, entering open waterways such as the sewage system. This will then expose these antibiotics to other environmental microbial pathogenic contaminants leading to acquired or crossed resistance (Cabello et al. 2023). Nevertheless, antibiotic resistance is not a new occurrence since the discovery of antibiotics. But nowadays, its overuse is causing great concern due to the increase in the frequency of antibiotic resistance occurrence. The resulting detrimental effects are increasingly severe, rendering nearly all prior strategies aimed at eliminating these pathogenic microbes ineffective (Cabello et al. 2023).

According to Cesur and Demiröz (2013), antibiotic resistance tends to occur when a target pathogenic microbe can withstand the antagonizing effect of an administered antibiotic. These resistant pathogens can easily come into contact with humans via fish handling and consumption, further endangering human health. The fish pathogens antibiotics resistance challenge has prompted stakeholders such as farmers, researchers, health, and some government agencies to seek effective alternatives for overcoming this challenge by first introducing regulatory governance that prohibits the use of antibiotics as a feed additive in pisciculture and overall aquaculture as in Europe, for instance (Authority 2009). In Thailand, two laws were enacted: the Drug Act of 1967 and the Animal Feed Quality Control (AFQC) Act of 2015 to regulate antibiotics and medicated feed use in animal farms (Sommanustweechai et al. 2018). Under the AFQC Act, the Thailand Ministry of Health has banned the use of some drugs and their salts in animal feed, including chloramphenicol, nitrofurazone, nitrofurantoin, furazolidone, furaltadone and malachite green (Sumpradit et al. 2021). Currently, few antibiotics are approved in aquaculture, such as tetracycline, oxytetracycline, amoxicillin, sulfadimethoxine/normetoprim, trimethoprim, and enrofloxacin (Baoprasertkul et al. 2012).

In addition, the Thai Department of Fisheries (DOF) and the Thai Frozen Foods Association were also established to regulate the development, management and export of fish and shrimp. This is to ensure that farmers comply with export quality rules regarding the administration of antibiotics (Thongsamer et al. 2021). Also, in 2017, Thailand adopted the National Action Plan against Antimicrobial Resistance (NSP-AMR) under the One Health approach, encompassing human, animal, and environmental health. This plan outlined five targets, including a 50% reduction in morbidity from AMR, a 20% decrease in antibiotic use in humans, a 30% reduction in antibiotic use in animals, a 20% increase in public awareness regarding appropriate antibiotic usage, and enhancing national capacity for AMR management to level 4 (Sumpradit et al. 2021).

#### Probiotics as an alternative to conventional antibiotics usage in fish farming

The understanding of probiotics has evolved since the initial discovery of *Lactobacillus delbrueckii* subsp. *bulgaricus* by Grigorov, a Bulgarian medical student who first examined the microflora of Bulgarian yoghurt in 1905. This beneficial microbe was later suggested to be essential for healthy living and longevity by Elie Metchnikoff in 1907 (Brown and Valiere 2004). In 1953, a German professor, Werner Georg Kollath, introduced the word probiotics. He defined probiotics as active substances that can counter harmful microbial metabolites and are essential for a healthy life (Park 2009; Rusch 2002).

However, the history of microbes with antimicrobial properties dates back to 1925 when Belgian scientist Andre Gratia first discovered colicin of *Escherichia coli* (Wainwright 2000). In addition, to the announcement of probiotics in 1953, a study by Jacob et al. in the same year introduced the concept of bacteriocin (Khelissa et al. 2021). This study proposed the definition of bacteriocin as a specific antibacterial protein produced by certain bacterial strains, which exhibit activity against other strains of the same species (Khelissa et al. 2021). However, this definition has been modified as bacteriocin studies have advanced.

Conversely, probiotics have had diverse definitions. However, in 2002, FAO and WHO experts defined probiotics as live microorganisms that, when administered in adequate amounts, confer health benefits on the host (FAO/WHO 2006). Furthermore, a study by Merrifield et al. (2010) defined probiotics in aquaculture as microbial cells that can be administered as a feed or water supplement to strengthen the immune system of host fish by increasing their resistance to diseases, reducing stress response, improving gastrointestinal tract morphology, and also benefit the farmer and the final consumer by improving fish appetite, health, growth performance, feed utilization, flesh quality and reduced malformations (Merrifield et al. 2010).



The promising benefits of probiotics are innumerable and have led researchers to explore areas such as the probiotic mode of transmission, which based on these studies (Korkea-aho et al. 2012; Lazado et al. 2011; Luis et al. 2011; Mahdhi et al. 2012; Sorroza et al. 2012; Sugimura et al. 2011), can be achieved through immunomodulation and also through the ability of most probiotic organisms to attach to the mucosal epithelium of the gastrointestinal tract to assist the autochthonous microbiota in inhibiting invading pathogens. A study by Das et al. (2022) highlights the properties of good probiotics as a strain that is capable of exerting a beneficial effect on the host animal. For instance, increased growth and disease resistance; being non-pathogenic and non-toxic; having living cells preferably in large numbers; capable of surviving in low pH and organic acids; ability to maintain viable stability of the desired characteristics under storage and field conditions.

#### Beneficial characteristic features of *Bacillus* strain

Bacteria of the genus *Bacillus* belong to the Bacillota phylum, as they mostly possess Gram-positive cell wall structures (Oren and Garrity 2021). However, their Gram-staining character can be heterogeneous as they can be Gram-positive at the early stage of growth and Gram-negative at the later stage of their development. This has been attributed to the difference in their carbohydrate metabolism as well as differences in the molecular composition of individual strains (Becerra et al. 2016). Studies have proved that they are either obligate aerobes or facultative anaerobes, endospore-formers and rod-like in shape (Beladjal et al. 2018). The endospore formation ability of *Bacillus* is indicated through their survival strategy in response to a lack of nutrients in their immediate environment (Buehler et al. 2018). The composition of *Bacillus* spores consists of a core which is surrounded by a coat and/or endosporium. This core consists of DNA, enzymes, and dipicolinic acid. Dipicolinic acid maintains the spore dormancy by resisting DNA-damaging substances usually bound to divalent cations, such as  $\text{Ca}^{2+}$ , at a 1:1 ratio in the core (Setlow 2014). Thus, the formed endospores are not true reproductive spores (Buehler et al. 2018).

This sporulation ability, however, contributes to their ability to withstand heat, radiation, disinfectants, desiccation, antibiotics and other toxic chemicals (Christie and Setlow 2020). Hence this genus is useful in a wide range of industrial processes, such as in the production of enzymes, antibiotics, and fine biochemicals, including flavour enhancers, food supplements and insecticides (Bahaddad et al. 2023).

Although the safety of the utilization of some *Bacillus* spp. as probiotics is of great concern regarding their public health and food safety challenges such as food poisoning in the case of *B. cereus* and anthrax by *B. anthracis* (Christie and Setlow 2020), most species in this genus, especially those of *B. subtilis*, have been pronounced GRAS (generally regarded as safe) by the Food and Drug Administration (Abdel-Mohsein et al. 2010). This status conferment is attributed to their lack of toxic effects including cytotoxicity, hemolysis and enterotoxins. Also, their high antimicrobial susceptibility with a slight resistance to streptomycin and tetracycline which is revealed intrinsic, thus non-transferable but of an advantage to the restoration of their hosts gut microbiota after exposure to antibiotics (Adimpong et al. 2012; Gueimonde et al. 2013; Kim et al. 2022; Parveen Rani et al. 2016). Therefore all potential novel *Bacillus* strains must align with these characteristic safety features before acceptance, commercialization and utilization (FAO/WHO 2006).

Additionally, *Bacillus* species of bacteria are ubiquitous as they have been isolated from a range of diverse sources such as soil, gastrointestinal tracts of animals, fermented Miang tea leaves, fermented seafood and dairy samples and aquatic environments, among others (Joseph et al. 2013; Parveen Rani et al. 2016; Ruiz-García et al. 2005; Sumpavapol et al. 2010; Unban et al. 2020).

Another attribute of *Bacillus* species, which has amplified their utility in both industry and research, besides their aforementioned safety features, is their inherent capacity to excrete significant quantities of beneficial substances/proteins into their growth medium. These include alpha-amylase, protease, pectinase, xylanase, amylase, cellulose, and  $\beta$ -mannanase, as well as lipopeptides such as surfactins and mycosubtilins. Additionally, they produce bacteriocins and/or bacteriocin-like substances (BLIS), for the yet-to-be-characterized bacteriocins (Khatthongngam et al. 2019; Nigris et al. 2018; Unban et al. 2020).

#### Proposed classifications of *Bacillus* spp. bacteriocins

The classification of *Bacillus* spp. bacteriocins are experiencing some drawbacks as detailed information

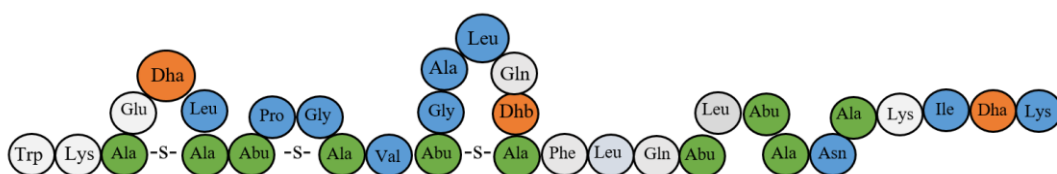


about the molecular, physical, and biochemical properties of some already identified bacteriocins is lacking. However, some studies have proposed a direction for this classification based on the biosynthetic pathway of individual bacteriocin. Bacteriocins are ribosomally synthesized peptides derived from short amino acid precursors and, through posttranslational modification, are processed into mature peptides (Mathur et al. 2021; Willey and van der Donk 2007).

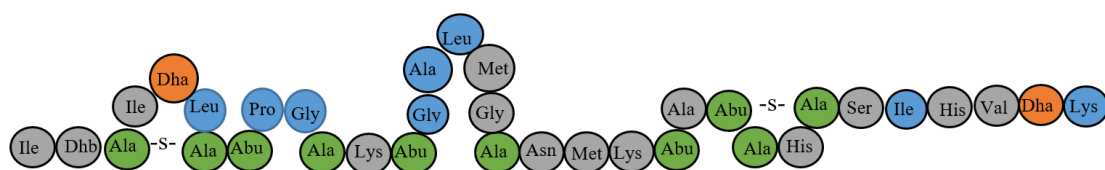
Although some other enzymes are ribosomally synthesized (Caulier et al. 2019), however, bacteriocins are distinguished by their distinct mode of action, which include: bacteriostatic, bactericidal, sporostatic, sporicidal, bacteriolytic, bacteriocin-induced cell damage, physical adsorption and quantal killing (Egan et al. 2016). Overall, the antimicrobial interaction of bacteriocins against an indicator cell occurs by physically adsorbing the bacteriocin molecules into the indicator-exposed cell-envelope receptors. This action can be reversible based on the interaction time frame, the amount and concentration of the applied bacteriocin, biochemical features of the interaction, the site of interaction and the environmental factors (Zimina et al. 2020). This initial contact can result in bacteriostatic and sporostatic modes of inhibition, where the bacteriocin inhibits the growth of the indicator cell or its spore without any physiological damage. The next method of interaction results in an irreversible pathological change via biochemical lesion as in the case of bactericidal, sporicidal and bacteriolytic activities. Therefore, a transition between these two phases of interaction may exist as some bacteriocins may require a longer time to penetrate their indicator cell envelope (Darbandi et al. 2022).

The biosynthetic pathway of bacteriocins includes various posttranslational modifications and the proteolytic cleavage of the leader peptide at the N-terminal end. Some studies have proposed that the already identified bacteriocins can be classified on this basis (Caulier et al. 2019). Abriouel et al. (2011) proposed that bacteriocins of *Bacillus* spp. be classified into three classes. Class 1 has been presented as posttranslationally modified, such as the lantibiotics, which are of smaller linear peptides ranging from 19.0 – 38.0 amino acid sequences and below 5.0 kDa in molecular size. This class is subdivided into four subclasses. In addition, the similarities of subclass 1.1 - 1.3 are in their lantibiotics structures which possess inter-residual thioester bonds made of modified amino acid residues, which are lanthionine and methyllanthionine bridges (Willey and van der Donk 2007). Fig. 1 depicts the similarities and differences in the amino acids in the peptide of subtilin and Nisin A. The green colour represents their similarities, while the shades of ash show their differences. Therefore, in orange, Dha (2, 3-didehydroalanin) and Dhb (2, 3-didehydrobutyrine) represent the dehydrated serine and threonine. Intra-molecular addition of Dha or Dhb on a cysteine residue leads to the formation of lanthionine and methyllanthionine bridges which is the posttranslational modification (Willey and van der Donk 2007). Subclass 1.4 are those with a unique cyclic peptide. An example is subtilisin A (Fig. 2), which contains a head-to-tail cyclic peptide and sulfide bridges formed between

#### A) Subtilin



#### B) Nisin A



**Fig. 1** Similarities and differences between subtilin (A) and nisin A (B), modified from Willey and van der Donk (2007)



cysteine groups and dehydrated amino acid residues (Kawulka et al. 2004). Other proposed classes include class 2 with three subclasses to contain the non-modified pediocin-like, thuricin and other linear peptides within the molecular mass of <10 kDa, high heat and pH stability (Abriouel et al. 2011), and class 3 is for bacteriocins of larger molecular weight with phospholipase activities. These classifications are summarized in Table 1.

However, these enlisted proposed classification systems of the bacteriocins of *Bacillus* spp. are recently experiencing some level of drawbacks due to the inability of some studies to elucidate the molecular properties of their potential bacteriocins/bacteriocin-like peptides. Therefore, in this review, we suggest the integration of whole genome sequencing of the producing *Bacillus* strain and molecular characterization of their purified bacteriocin peptides, to foster ease with these classification challenges.

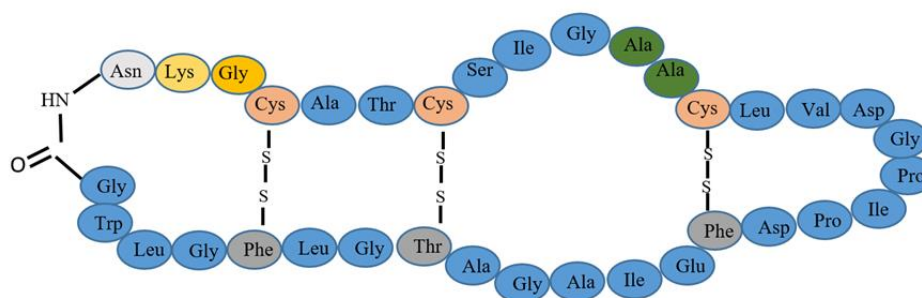
#### Bacteriocin production ability: The criteria for probiotics strain selection

The bacteriocin production ability is the fundamental quality feature based on which probiotic strains are selected (Corr et al. 2007). This has been demonstrated in some studies where bacteriocins have been proven to be non-pathogenic and non-toxic, having the ability to colonize the gastrointestinal tract of a host organism, thereby exerting its beneficial effects on the organism (Dobson et al. 2011; Gillor and Ghazaryan 2007). Therefore, the proposed strategy for probiotic strain selection is based on its bacteriocin production ability (Desriac et al. 2010). The first step is always to identify a source of isolation. In aquacultural application, studies have emphasized the need to consistently isolate strains indigenous to the target host organism or its environment and within the GRAS family (Chauhan and Singh 2019; Soltani et al. 2019). This is to satisfy efficiency over temperature and salinity variations of the aquatic environment. Nevertheless, strains from other sources can be applied to marine life, provided the necessary safety steps and evaluations are undertaken.

Isolation of the microbial strain is the next step, followed by evaluating the antimicrobial ability against an indicator strain. It is important to note that the power of a potential strain to inhibit an indicator strain *in vitro* is not enough to confirm as a probiotic characteristic hence the need for further characterization of its molecular, biochemical, physical features, and safety properties according to FAO/WHO (Desriac et al. 2010; FAO/WHO 2006). These steps are summarized in Fig. 3 below.

#### Some *Bacillus* spp. bacteriocin-producing strains and their properties

Bacteriocins are ribosomally synthesized polypeptides of the Gram-positive or heterogeneous group of the GRAS bacteria, which shows antimicrobial effects on either its closely related and/or broader organisms but not on the producing strain (Perumal et al. 2019; Wei et al. 2021). These ribosomally synthesized molecules have also been attributed as one of *Bacillus* spp. survival strategies and are always directed against competitive microorganisms, generating a selective advantage for the producer species (Riley and Wertz 2002). Studies have revealed that these synthesized molecules show high target specificity against related bacteria while maintaining self-immune protective pathways against their bacteriocin (Sumi et al. 2015). Recently, *Bacillus* spp. bacteriocins have gained much attention as they can secrete large amounts (in g/L level) of



**Fig. 2** The sulfide bridges formed in a cyclic peptide of subtilisin A, modified from Kawulka et al. (2004)



**Table 1** The proposed classification of *Bacillus* species bacteriocins based on their biosynthetic pathways

Class/subclass	Description	Examples
Class 1	Post-translationally modified peptides	
Subclass 1.1	Single-peptide, elongated lantibiotics	Subtilin, ericin S, ericin A
Subclass 1.2	Other single-peptide lantibiotics	Sublancin 168, mersacidin, paenitbacillin
Subclass 1.3	Two-peptide lantibiotics	Haloduracin, lichenicidin
Subclass 1.4	Other post-translationally modified peptides	SubtilosinA
Class 2	Non-modified peptides	
Subclass 2.1	Pediocin-like peptides	Coaguln, SRCAM 37, SRCAM 602, SRCAM 1580
Subclass 2.2	Thuricin-like peptides	Thuricin H, thuricin S, thuricin 17, bacthuricin F4, cerein MRX1
Subclass 2.3	Other linear peptides	Cerein 7A, cerein 7B, lichenin, thuricin 439
Class 3	Large proteins	Megacin A-216, megacin A-19213

Adapted from Abriouel et al. (2011)



these peptides into a culture medium and possess a broad spectrum of antimicrobial activity. Furthermore, their spore-forming ability has proven them to be highly stable through extraction, purification and storage processes (Beladjal et al. 2018). Some of the already studied bacteriocins of the *Bacillus* spp. are as follows.

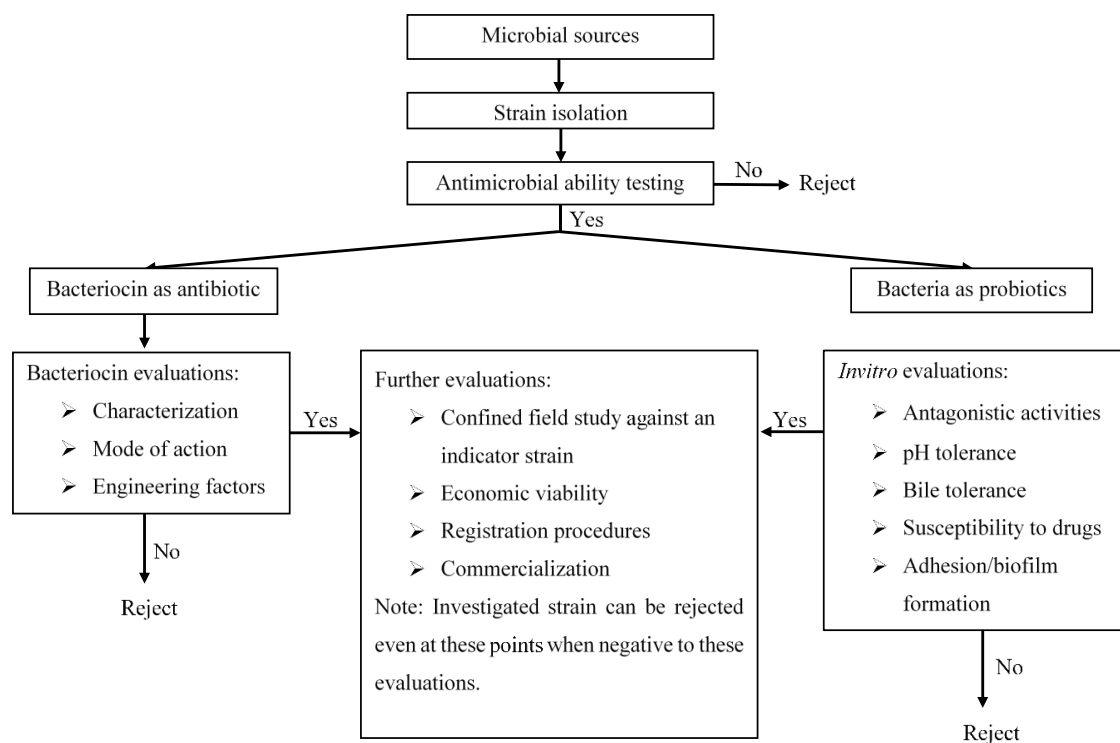
### Subtilin

This ribosomally synthesized peptide is grouped under the family of lanthionine antibiotics. It consists of 32.0 amino acid residues that form a cationic pentacyclic structured antimicrobial peptide and are commonly synthesized by *B. subtilis* strains with a molecular mass of 3317.0 Da (Guder et al. 2000; Lee and Kim 2011). Subtilin antimicrobial activity is via permeabilization of the cytoplasmic membrane of sensitive bacteria, and it is always against a broad spectrum of Gram-positive bacteria (Parisot et al. 2008; Stein 2005). Due to the unstable nature of its compound, previous reports have shown that the antibacterial activity can be lost during isolation and storage (DePaz et al. 2002).

Subtilin amino acid sequence shares 57.0% and 61.0% similarities with the nisin Z leader sequence and pro-regions, respectively, hence their structural similarities (Barbosa et al. 2015). The biosynthesis of subtilin is regulated through positive feedback by the binding of the regulatory system SpaK (sensor histidine kinase), which is controlled by sporulation transcription sigma H factor and SpaR (protein regulator) in an extracellular subtilin to a DNA motif (*spa-box*), thereby, promoting the expression of genes for both the subtilin biosynthesis (*spaS* and *spaBTC*) and immunity (*spaIFEG*) (Kleerebezem 2004; Stein et al. 2004; Stein et al. 2003). Another subtilin production pathway can be regulated via cell culture density in the quorum sensing mechanism, where it displays pheromone ability in response to growth (Bongers et al. 2005; Burkard et al. 2007).

### Subtilosin A

This is one of the bacteriocins associated with *B. subtilis*, commonly isolated from fermented foods (Eparti et al. 2022). A study on the 3-dimensional structure of subtilosin reveals that it consists of 35 amino acids (X-Gly-Leu-Gly-Leu-Trp-Gly-Asn-Lys-Gly-Cys-Ala-Thr-Cys-Ser-Ile-Gly-Ala-Ala-Cys-Leu-Val-



**Fig. 3** An essential study pathway to ensure safe and reproducible probiotics based on bacteriocin production ability of microbial strain.





Asp-Gly-Pro-Ile-Pro-Asp-Glx-ne-Ala-Gly-Ala) sequence which forms a circular frame with 3-cross-links between the sulphurs of cysteine and the  $\alpha$ -carbon of the two phenylalanine and the threonine (Kawulka et al. 2004). This structural feature distinguishes subtilisin A from other bacteriocins. Barbosa et al. (2015) further explained that subtilisin A secretion by *Bacillus* spp. starts at the end of their vegetative growth and finishes before it sporulates. Mature subtilisin peptide has been found to result from the loss of an unusually short seven amino acid leader peptide which causes the cyclization of N and C terminals and modifications of Cys, Thr and Phe residues (Zheng et al. 1999).

This formed mature subtilisin is resistant to proteolytic enzymes and stable at moderate temperatures and acid treatments. Its posttranslational modification has been suggested to occur via the oxidative linkage of cysteine sulfur to the  $\alpha$ -carbon of another amino acid residue (Kawulka et al. 2004). Subtilisin A has broad antimicrobial activity against aerobic, anaerobic, Gram-positive and negative microbes, but its antimicrobial activity is substantially reduced in capsulated strains (Shelburne et al. 2007). Although there is still consensus on the antimicrobial pathway of subtilisin A. However, some studies have proposed it to occur as a function of interaction with membrane-associated receptors and also by binding to the outer membrane which results in membrane permeabilization that leads to its bactericidal effect on the susceptible microbes (Thennarasu et al. 2005; Wiedemann et al. 2001).

### Ericins

A study by Stein et al. (2004) on *B. subtilis* A1/3 revealed a lantibiotic gene cluster which shows a very close similarity with that of the subtilin gene cluster and is also located on the same genetic locus as the *spa* genes in *B. subtilis*. However, this lantibiotic gene cluster possesses two subtilin-like genes separated by a sequence similar to a portion of the *lanC* gene. These two subtilin-like genes are denoted ericin S and A, having molecular weights of 3442.0 Da and 2986.0 Da, respectively. The amino acid sequence of ericin S differs from that of subtilin by four interchanged amino acid residues, and it still maintains a subtilin-like, lanthionine bridging pattern hence its antimicrobial similarity to subtilin.

In contrast, ericin A has thirteen amino acids interchange with the absence of three amino acid residues at its C terminal, and this was said to be linked to its weak antimicrobial activity. Ericins biosynthesis is dependent on LanB functionality. Despite their amino acid sequence difference, ericin A and S maintain the same physical characteristics.

### Thurincin 17

This is a bacteriocin of *B. thuringiensis*. This Gram-positive bacteria is commonly known for producing delta-endotoxins, an insecticidal protein. However, *B. thuringiensis* can also secrete bacteriocins which are used in food industries as a food preservative. Studies by Kamoun et al. (2005) revealed that the amino acid sequence of bacthuricin F4 was 22.0, though incomplete. However, the N-terminal amino acid sequence unique to this bacteriocin is of 8.0 residues (DWTXWSXL) where X is unknown, and the molecular mass is 3160.0 Da. This bacteriocin is very stable at 70.0°C as over 70.0% of its activity was maintained after 30.0 mins of incubation and on a low pH of 3.0. This physical strength has made it essential for use in agro-industries. Its antimicrobial activity, which is highly expressed against *B. cereus*, is also targeted against its close relatives and other Gram-positive bacteria.

### Sublancin

This is a bacteriocin of *B. subtilis* 168 strain and it is made up of 37.0 amino acid sequences (Hsieh et al. 2012). Studies by Paik et al. (1998) reveal that the mature amino acid sequence of sublancin consists of one serine, one threonine, and five cysteine residues. The mature region of the sublancin peptide is cationic. Its predicted molecular mass is approximately 3900.0 Da; this molecular mass depends on the posttranslational modification pathway. The N-terminal region of the mature peptide was shown to have these sequences (Gly-Leu-Gly-Lys-Ala-Gln-Xaa-Ala-Ala-Leu-Trp-Leu-Gln-Xaa-Ala-Xaa-Xaa), where (Xaa) represents the unidentifiable amino acid residues.

Sublancin genes sunA and sunT are the genes responsible for the regulation of biosynthesis of sub-



lancin together with PepT and Pep5 transporter proteins. Although sublancin antimicrobial activity is only against Gram-positive bacteria, it can also strongly inhibit a bacterial spore outgrowth but has little effect in inhibiting the vegetative growth of bacteria. Its strong storage stability was proven by its ability to retain biological activity after 2 years of storage in an aqueous solution without degrading. The pH stability falls within the range of 1.5 – 9.5, and the temperature range is up to 121°C for 3 mins (Paik et al. 1998).

#### Megacin

This is a bacteriocin synthesized by different strains of *B. megaterium*, which are also targeted against its strains and a few other bacterial strains (Kiss et al. 2008). Due to differences in the molecular and physical properties of individual strain megacin, they are divided into three classes: A, B and C. This was based on their inducibility pathway, mode of action and spectrum of activity (Kiss et al. 2008). Megacins of class A are known to be inducible by UV irradiation (Kiss et al. 2008), and this is one of the characteristic features of bacteriocins as they are not affected by large doses of ultraviolet irradiation, thus, differentiating bacteriocins from phages (Reeves 2012). Megacin B and C are not inducible. Although megacin B have not been well characterized, it is known to exhibit the intermediate activity of spectrum, which was only demonstrated on solid media (Ginting et al. 2023). In addition, megacin A displays phospholipase activity which can be detected at the conversion of phospholipids to corresponding lysophospholipids (Kiss et al. 2008). All megacins have low antimicrobial spectra; the C megacins are highly homogenous in their activity, while the others exhibit some differences (Abriouel et al. 2011).

#### Cerein

This is a bacteriocin of *B. cereus*, an aerobic spore-forming bacteria commonly isolated from the soil and groundwater and often found on plants and animals at the point of harvest or slaughter (Oscáriz et al. 2006). Cerein are usually named after the synthesizing *B. cereus* strains by researchers, and this has been attributed to their possible chemical differences (Oscáriz et al. 2006).

Oscáriz and Pisabarro (2000) revealed that cerein7A, which is produced by *B. cereus* Bc7 (CECT 5148), is a peptidic antibiotic and highly hydrophobic, with an N-terminal end as GWGDVL and a molecular weight of about 3940.0 Da. Cerein7A hydrophobicity contributes to its ability to aggregate in an aqueous solution, thereby preventing diffusion and loss of its antibiotic nature and maintaining its concentration at high levels in the surroundings of the bacterial population. In addition, cerein7A can inhibit the proteolytic activity of aminopeptidase, suggesting that the N-terminal end of its peptide is modified. Its antimicrobial activity targets the cell wall or the cytoplasmic membrane of their targeted microbes, implying that they are bactericidal and highly effective against Gram-positive bacteria with little effect on Gram-negative bacteria. They are heat-stable up to 100.0°C and stable under a wide pH range.

Cerein8A is not yet well characterized; however, its antibacterial effects were investigated by Bizani et al. (2005); their study revealed that cerein8A has a bactericidal and bacteriolytic ability against a broad spectrum of both Gram-positive and Gram-negative bacteria. Bizani and Brandelli (2002) showed that the maximum stable temperature was 80.0°C, pH ranged from 5.0 to 8.0, and its bacteriocin activity was retained after cooling or freezing. Other cereins include cerein7B described by Oscáriz et al. (2006). These are produced by the same *B. cereus* Bc7 of cerein7A. The N-terminal amino acid of 7B was GW-WNSWGK. The molecular weight of 7B was calculated to be 4893.0 Da, slightly cationic (net charge of 0.77 at pH 7.0), hydrophobic (42.8% of apolar residues), and with a pI of 8.38. Other bacteriocins of *Bacillus* spp. are highlighted in Table 2.

#### Diverse applications of *Bacillus* spp. bacteriocins in fish farming

The utilization of bacteriocins from *Bacillus* spp. in fish farming is varied and encompasses both feed and water applications, serving as a biocontrol agent (Pereira et al. 2022). While the direct use of pure bacteriocin may not be economically viable, targeting probiotics as live cultures proves to be a practical alternative (Desriac et al. 2010). The effectiveness of probiotics is more pronounced as a preventive measure rather than a treatment, particularly when the host is already contending with a pathogen (Tegege and Kebede 2022). Table 3 outlines several isolated bacteriocins and their potential applications in diverse aspects of



**Table 2** A variety of reported *Bacillus* bacteriocins: their synthetic pathways, mode of action and producing gene

Bacteriocin	Producing strain	Indicator strain	Mode of action	Producing gene	Synthetic pathway	Possible class	Reference
Mersacidin	<i>Bacillus</i> spp. HIL-y85/54728	<i>M. luteus</i> ATCC 4698	Inhibition of the bacterial peptidoglycan biosynthesis at the cell wall level.	<i>mrsA</i>	Posttranslational modification	Lantibiotics type B	Altena et al. (2000)
Lichenin	<i>B. licheniformis</i>	<i>S. bovis</i>	Oxygen labile	NS	NS	UC	Patnaik et al. (2001)
Coagulins A	<i>B. coagulans</i>	<i>L. innocua</i> , <i>L. monocytogenes</i>	NS	<i>coaA</i>	NS	UC	Le Marrec et al. (2000)
Cytolisin	<i>B. halodurans</i>	<i>L. lactis</i>	NS	<i>blh0453</i>	Posttranslational modification	Lantibiotic	McClerren et al. (2006)
Subpeptin JM4B	<i>B. subtilis</i>	<i>C. glutamicum</i> , <i>S. flexneri</i>	NS	NS	Posttranslational modification	UC	Wu et al. (2005)
Haloduracin	<i>B. halodurans</i> C-125	<i>L. lactis</i>	NS	<i>halAI</i>	Posttranslational modification	Lantibiotic	McClerren et al. (2006)
Thiocillin	<i>B. cereus</i> (strain ATCC 14579 / DSM 31)	<i>B. subtilis</i> PC1219, <i>B. anthracis</i> , <i>S. aureus</i> FDA209P	NS	<i>tcI</i>	Posttranslational modification	UC	Wieland Brown et al. (2009)
Thurincin H	<i>B. thuringiensis</i> SF361	<i>L. ivanovi</i> ATCC 19119, <i>C. piscicola</i> CU216	NS	<i>tucAI</i>	Posttranslational modification	Lantibiotic	Lee et al. (2009)
Bacilloecin 1580	<i>B. citriculans</i>	<i>C. jejuni</i>	NS	NS	NS	Pediocin like	Svetoch et al. (2005)
Thurandacins A&B	<i>B. thuringiensis</i> serovar <i>andalousiensis</i> BGSC 4AW1	<i>B. thuringiensis</i>	NS	<i>thuA</i>	Posttranslational modification	UC	Wang et al. (2014)
Thusin alpha	<i>B. thuringiensis</i> strain BGSC 4BT1	<i>B. amyloliquefacien</i> , <i>B. cereus</i>	Bacteriocidal via Synergistic effect	<i>lanM</i>	Posttranslational modification	Lantibiotics II	Xin et al. (2016)
Thusin beta							
Thusin beta 2							
Plantazolicin	<i>B. amyloliquefaciens</i> FZB42	<i>B. megaterium</i> and <i>B. subtilis</i> HB0042	NS	<i>micD</i>	Posttranslational modification	Lantibiotics	Scholz et al. (2011)
Entianin	<i>B. subtilis</i> subsp. <i>spizizenii</i> DSM15029 <sup>r</sup>	<i>S. aureus</i> ATCC 43300 (MRSA), <i>E. faecalis</i> ATCC 51299 (VRE)	Bacteriocidal	<i>lanBC</i>	Posttranslational modification	Lantibiotics	Fuchs et al. (2011)
Lichenicidin VK21	<i>B. licheniformis</i> VK21	<i>M. luteus</i> B1314, <i>B. megaterium</i> VKM41	Bacteriocidal via synergistic effect	<i>lanM</i>	Posttranslational modification	Lantibiotics	Shenkarev et al. (2010)
Cerecidins A7	<i>B. cereus</i> As1.1846	<i>S. aureus</i> (MDRSA), <i>E. faecalis</i> (VRE)	Bacteriocidal	<i>lanM</i>	Posttranslational modification	Lantibiotics II	Wang et al. (2014)

Note: NS indicates not stated; UC indicates unclassified



**Table 3** *In vivo* and *in vitro* applications of *Bacillus* spp. bacteriocins in fish farming

Bacteriocin producing strains	Source of isolation	Indicator strains	Study host/mode	Bacteriocin properties	Bacteriocin applications	References
<i>B. stratosphericus</i> CM1FG7, <i>B. aerophilus</i> CM1HG5 <i>B. licheniformis</i> CM3FG19 <i>B. subtilis</i> LR1	Mrigal ( <i>Cirrhinus mrigala</i> ) gastrointestinal tract of <i>Labeo rohita</i>	<i>A. hydrophila</i> , <i>B. mycooides</i> , <i>P. fluorescens</i> <i>B. mycooides</i> , <i>A. salmonicida</i> , <i>P. fluorescens</i> and <i>A. hydrophila</i>	<i>Cirrhinus mrigala</i> Fingerlings of <i>C. catla</i> and <i>L. rohita</i> .	Mode of action: Bacteriocidal pH range: 4 – 9 Heat stability: up to 90°C Mode of action: Bacteriocidal pH range: 7 Heat stability: 40°C	Animal feed additive as a probiotic. Feed additive	Mukherjee et al. (2016) Banerjee et al. (2017)
<i>B. siamensis</i> JFL15	the gastrointestinal tract of the hairtail	<i>E. coli</i> , <i>E. tarda</i> , <i>P. aeruginosa</i> , and <i>A. hydrophila</i>	<i>In vitro</i> study	Mode of action: Bacteriocidal and/or synergistic effect pH range: Heat stability:	Biocontrol agent	Xu et al. (2018)
<i>Bacillus</i> sp. SW1-1	Stock	<i>E. tarda</i> , <i>S. iniata</i> , <i>S. parauberis</i> , <i>V. anguillarum</i> , and <i>V. harveyi</i> .	<i>In vitro</i> study	Heat stability: Mode of action: Bacteriolytic pH range: 2 - 11 Heat stability: 100°C	Biocontrol agent	Kim et al. (2014)
<i>B. amyloliquefaciens</i> JFP2	Jeotgal traditional fermented seafood.	<i>A. hydrophila</i> , <i>E. tarda</i> , <i>P. damsela</i> sup-sps, <i>P. phosphoreum</i> , <i>S. parauberis</i> , <i>S. iniata</i> and <i>V. anguillarum</i>	<i>Juvenile flounder</i>	Mode of action: Bacteriolytic pH range: 2-12 Heat stability: 4 - 45°C	Antibacterial agent	Heo et al. (2021)
<i>B. subtilis</i> NCIMB 3610	Stock	<i>E. faecalis</i> , <i>V. anguillarum</i> 408 O1 and <i>P. damsela</i> subsp. <i>piscicida</i>	<i>In vitro</i> study	Mode of action: Bacteriocidal pH range: 6 – 8.5 Heat stability: 70°C	Prophylactic treatment of fish against bacterial infections	Touraki et al. (2012)
<i>B. subtilis</i>	Gastrointestinal of mrigal ( <i>Cirrhinus mrigala</i> )	<i>A. hydrophila</i> , <i>E. tarda</i> , <i>P. aeruginosa</i> , <i>P. fluorescens</i> , <i>F. branchiophilum</i>	<i>In vitro</i> study	Mode of action: Bacteriocidal pH range: 7 Heat stability: 30°C	Antimicrobial	Nayak and Mukherjee (2011)
<i>B. licheniformis</i> P40	GIT of teleost fish ( <i>Leporinus</i> sp).	<i>L. monocytogenes</i> , <i>B. cereus</i>	<i>In vitro</i> study	Mode of action: Bacteriocidal and bacteriolytic pH range: 3 - 11 Heat stability: 100°C	Antimicrobial and bio-preservative	Cladera-Olivera et al. (2004)
<i>Bacillus</i> spp. NM 12	Dragonets ( <i>Callionymus</i> sp).	<i>V. vulnificus</i> RIMD 2219009	<i>In vitro</i> study	Mode of action: Bacteriocidal pH range: 6.5 – 8.5 Heat stability: 100°C	Biocontrol agent	Sugrta et al. (1998)
<i>Bacillus</i> spp.	Nile tilapia ( <i>Oreochromis niloticus</i> ) intestine	<i>S. typhi</i> CFP/IAL1472, <i>A. hydrophila</i>	<i>In vitro</i> study	Mode of action: Bacteriostatic pH range: 5 – 8 Heat stability: 25°C	Biocontrol agent	Pacheco et al. (2018)



fish farming. Moreover, certain *Bacillus* strains (such as *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, etc.) are now being incorporated with other microbial strains (*Enterococcus* spp., *Lactobacillus* spp., *Pediococcus* spp., etc.) to create multi-strain commercial probiotics like AquaStar® and MicroPan® (El-Kady et al. 2022), these combinations are ideally employed as water supplements or feed additives.

However, their single strain *in vivo* studies majorly as a feed additive, as shown in Table 4, are also proven to confer health benefits such as strengthening the immune system of the host fish, increasing their resistance to diseases, improving their feed utilization, improve the water quality, their gastrointestinal microbiota and morphology as well as reducing their stress response, amongst others (Han et al. 2015; Jang et al. 2023; Zhao et al. 2023).

#### Immune system strengthening and increased disease resistance

The immune system of fish is revealed to comprise specific and non-specific immune response defence mechanisms. Both response systems are known to operate synchronously, with the non-specific immune response encompassing physical barriers, and cellular, and humoral components serving as the initial line of defence when pathogens invade the fish (Sarder et al. 2001). Their physical defence barriers include the skin, scales, epithelial layers in the gills and gastrointestinal tract, and mucus coverings tagged mucosa-associated lymphoid tissue (MALT) (Shija et al. 2023). These mucus coverings contain antimicrobial substances such as lectins, lysozymes, complement proteins, and antimicrobial peptides (AMPs) that can effectively neutralize and eliminate invading pathogens. These components are also associated with the humoral and cellular components, including phagocytic cells such as macrophages, circulatory monocytes, and neutrophils (Sarder et al. 2001; Wang et al. 2023).

Therefore, the application of probiotic *Bacillus* strains in fish is known to interact with these immune cells to increase their sensitivity to an invading pathogen via pathogen pattern recognition receptors (PRRs) and recognition of the pathogens microbe-associated molecular patterns (MAMPs) (Chu and Mazmanian 2013; Iwashita et al. 2015). Thus, it increases the transcription of immune cell signalling molecules, pro-inflammatory cytokines and chemokines, which recruit these innate immune cells (Standen et al. 2016). Furthermore, probiotic *Bacillus* strains can increase white blood cells in fishes, thus increasing their blood leukocyte counts, improving lysozyme activity, and modifying the respiratory burst process in fish by generating antioxidant molecules which enable reduction of reactive oxygen species (ROS) production by cells, hence minimizing the resulting oxidative damage which in turn enhances the host immune system (Kuebutornye et al. 2020; Liu et al. 2017; Opiyo et al. 2019; Wu et al. 2021).

#### Enhancement of the gastrointestinal tract morphology, feed utilization and growth performance

The gastrointestinal tract (GIT), also referred to as the digestive tract or alimentary canal, is a vital functional component in fish. It comprises various anatomical structures, including the mouth, teeth, gill rakers, oesophagus, stomach, pylorus, pyloric caeca, pancreatic tissue (both exocrine and endocrine), liver, gall bladder, intestine, and anus. It is important to note that the presence of these components can vary among fish species and is dependent on their feeding habits. However, in most commonly farmed fish, their GIT is characterized by a microscopic and saclike stomach (Tesfahun and Temesgen 2018), separated from the intestine by a pyloric sphincter. Their stomach and oesophagus are commonly composed of four undifferentiated layers: serous layers, double muscle layer/muscular containing striated fibres, submucosal layer, and the mucosal layer (Ekele et al. 2014; Moawad et al. 2017; Palladino et al. 2023).

The mucosal layer is particularly important as it allows the fish to accommodate variations in feed availability by permitting distension, thereby enhancing feed digestion efficiency. This layer is shielded from the acidic stomach environment by mucus cells in the tubular glands and the neck cells of the gastric glands (Palladino et al. 2023; Tesfahun and Temesgen 2018). The double muscle layer/muscularis, situated beneath the bottoms of the gastric glands, exerts contractile activity, preventing the blockage of gastric mucosal cells during food ingestion. The submucosal layer houses immune-competent cells such as lymphocytes, macrophages, granulocytes, and mast cells, which play a role in protecting the fish from invading pathogens (Ekele et al. 2014; Moawad et al. 2017; Palladino et al. 2023). The serosa layer is linked to the external muscular layer through a dense fibrous structure, consisting of two sublayers: the lamina subserosa



**Table 4** Health benefits associated with *Bacillus* spp. applying as probiotics in fish farming

<i>Bacillus</i> species	Host fish samples	Probiotic health benefits	Probiotic application mode	Field trial time (days)	References
<i>B. velezensis</i>	Channel catfish ( <i>Ictalurus punctatus</i> )	Increase in growth, improved water quality	Feed amendment	70	Thurlow et al. (2019)
<i>B. megaterium</i> PTB 1.4	Catfish ( <i>Clarias</i> sp.).	Increased feed utilization and growth	Feed additive	30	Afilasari and Meryandimi (2016)
<i>B. megaterium</i>	Songpu mirror carp ( <i>Cyprinus spectularis</i> Songpu)	Improved growth, feed utilization and intestinal tract microbiota	Feed amendment	56	Luo et al. (2020)
<i>B. subtilis</i> SB3615	Nile tilapia, ( <i>Oreochromis niloticus</i> )	Improved immunity	Feed amendment	21	Addo et al. (2017)
<i>B. pumilus</i> AQAHBS01	Nile tilapia, ( <i>Oreochromis niloticus</i> )	Improved resistance to pathogens	Feed additive	30	Srisapoomee and Areechon (2017)
<i>B. licheniformis</i>	Juveniles Nile tilapia, ( <i>Oreochromis niloticus</i> )	Increased growth, immune response, disease resistance and anterior intestinal development and integrity	Feed additive	70	Han et al. (2015)
<i>B. licheniformis</i> Dabb1	Mozambique tilapia ( <i>Oreochromis mossambicus</i> )	Increased growth, feed utilization, and disease resistance.	Feed additive	28	Gobi et al. (2018)
<i>Bacillus</i> sp. PM8313	Red sea bream ( <i>Pagrus major</i> )	Improved growth performance, feed utilization and intestinal tract microbiota	Feed additive	56	Jang et al. (2023)
<i>B. velezensis</i> MVCR2	Rainbow trout ( <i>Oncorhynchus mykiss</i> )	Improved growth, immunity, serum enzyme activity, gastrointestinal tract morphology, intestinal microbiota, and disease resistance	Feed additive	30	Zhao et al. (2023)
<i>B. cereus</i>	Pengze crucian carp ( <i>Carassius auratus</i> var. Pengze)	Improved flesh quality, growth performance, immunity, feed utilization	Feed additive	70	Yang et al. (2019)



(comprising loose connective tissue with numerous blood vessels) and the lamina epithelialis serosa (containing mesothelial cells). This layer provides mechanical support to the GIT (Awaad et al. 2014; Palladino et al. 2023).

Moreover, the gastrointestinal tract of fishes is abundant in beneficial microbes: Actinobacteriota, Chloroflexi, Actinobacteriota, Chloroflexi, Cyanobacteria, Firmicutes, etc., which play an essential role in supporting the host GIT fundamental physiological functions for their effective adaptations (Bereded et al. 2022). However, these microbial biodiversity and abundance are significantly influenced by the fish diet and their composition (Wang et al. 2023). Studies have proven the application of *Bacillus* probiotics in fishes to enhance the functionality of these GIT structures via improvement of the intestinal histomorphology which includes an increase in the number of goblet cells in the anterior, middle and posterior intestine and reduction of the microvilli density (Ghalwash et al. 2022; Kuebutornye et al. 2020). Single-strain *Bacillus* probiotics as feed additives also contribute to improved growth performance, as demonstrated by Jang et al. (2023), who observed significant enhancements in weight gain, specific growth rate, and feed conversion rate in their fish models. This improvement is linked to the ability of the probiotic strain to colonize the host GIT microbiota, thereby enhancing metabolism, nutrient digestion, and absorption.

### Water quality improvement

Water pollution is always an unavoidable challenge associated with aquaculture, as fishes excrete into their aquariums. These excreta are revealed to contain a high concentration of nitrogen which creates a favorable condition for the growth of algae, thus leading to microbial bio-flocculation and the building of biofilms in the waterbodies (Ling et al. 2010). However, the application of *Bacillus* probiotics as feed additives has been found to play a crucial role in the biodegradation of ammonia, addressing an organic waste concern in aquaculture (Hlordzi et al. 2020; Mohammadi et al. 2020). This biodegradation of ammonia leads to a reduction in the growth of pathogenic microbes, thereby enhancing aquacultural water quality and promoting healthier fish.

Nonetheless, to ensure unbiased public health and environmental safety measures in the context of commercialization, it is essential to extend the field trial time of the future potential novel aquacultural probiotic *Bacillus* strains.

### Biosafety concerns of probiotics application in fish farming

Research on emerging *Bacillus* probiotic strains is progressing, and investigators must adhere to the FAO/WHO (2006), guidelines for *in vitro* probiotic safety, which includes examining their mechanism of action (Oscáriz and Pisabarro 2001). This adherence is essential to establish proper safety regulations for these strains. To maintain uniformity, contemporary molecular techniques can be employed to thoroughly understand the genetic profile of the specific probiotic strain, including the identification of their antimicrobial or virulence genes. This precaution aims to prevent the potential transfer of such genes, which may be linked to the probiotic strains, to pathogenic bacteria, posing a biosafety concern in fish farming. Modern molecular techniques encompass various methods such as ribotyping, random amplified polymorphic DNA (RAPD), pulse field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), PCR-denaturing gradient gel electrophoresis (DGGE), fluorescent activated cell sorting (FACS), nucleic acid-based enumeration methods, and fluorescent *in situ* hybridization (FISH) (Stefanis et al. 2016; Yadav and Shukla 2017) can be adopted. However, it is important to note that these advanced molecular study techniques can be costly, presenting a challenge in allocating resources for the investigation of potential probiotic strains.

While the positive effects of incorporating probiotics in fish farming are undoubtedly apparent, there are also certain drawbacks associated with their application. One notable drawback is their slow mode of action, as the strains must adhere to and colonize the target host gut microbiota before providing health benefits (Hosain and Liangyi 2020). This necessitates their consistent long-term application. Primarily, the probiotic target site is the gastrointestinal tract of the host fish. A potential theoretical disadvantage of probiotic use is the risk of the strain migrating to untargeted sites, leading to systemic infection and the possible death of host fish (Adel and Dawood 2021).



## Conclusions

Bacteriocins of *Bacillus* spp. have recently gained much attention and are the target of research interest. This increasing focus has been attributed to their broad spectrum of inhibition and ability to retain viability through all purification and storage processes. Therefore, in this review, we have highlighted the need for proper identification and characterization of these peptides as it will reduce the challenges associated with their classifications in the future. Furthermore, for applying these bacteriocins in fish farming, investigators and other stakeholders should avoid using strains that are not granted GRAS to align eco-friendly application and human safety. Again, for any strain to be adopted as an antimicrobial, feed additive or biocontrol agent, a small-scale field trial should be observed for the long term to ensure unbiased risk assessment practices. Since the bacteriocin purification process is not economically viable, we also suggest that the bacteriocin production ability of a strain of interest be the basis of its selection as probiotics and therefore use as a live inoculant.

**Competing interests** The authors declare no competing interests.

**Authors' contributions** Chioma Stella Anyairo and Kridsada Unban: Conceptualization, methodology, software, investigation, resources, data curation, writing—original draft preparation. Chartchai Khanongnuch and Kalidas Shetty: Writing—review and editing, visualization. Chartchai Khanongnuch: Supervision, project administration, validation, funding acquisition. All authors have read and agreed to the published version of the manuscript.

**Acknowledgements** The authors express profound gratitude to the Chiang Mai University Presidential Scholarship Program, the Multidisciplinary and interdisciplinary school of Chiang Mai University and the Faculty of Agro-industry, Chiang Mai University for all support and research facilities.

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