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Prevalence of virulence genes and antibiotic susceptibility of *Bacillus* used in commercial aquaculture probiotics in China

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ABSTRACT

Disease is a significant constraint faced by aquaculture, and its prevention and control bring together a hub of recent research. Several resources and resorts have been applied to prevent diseases in aquaculture. Probiotics are known to be beneficial natural derivatives that have several benefits in aquaculture. Currently, several commercial probiotics are used in the aquaculture industry that contains one or more live microorganisms. In the aquaculture industry, *Bacillus* species is one of the most widely used probiotic organisms. They are considered distinctive and are found to be natural members of the gut microbiota of some fish species. The safety of beneficial microorganisms is essential since some of these organisms are reported to harbor traits that might be transferable to their hosts. In this study, the safety of some *Bacillus*-based commercial probiotics used in aquaculture in terms of virulence and drug resistance were assessed. Commercial *Bacillus* species after isolation were screened for the presence of virulence genes (*nheA, nheB, nheC, hblA, hblC, hblD, cytK, and entFM*) and one emetic gene (*ces*), as well as their resistance to some antibiotics. Most isolates did not possess any of the virulence genes assessed. Nonetheless, three isolates harbored the *nheABC* and *entFM* enterotoxin genes, while two had the *hblA, hblC, hblD, cytK* genes. None of the isolates possessed the *ces* emetic gene. Antibiotic resistance assessment revealed most of the isolates to be resistant to β-lactam antibiotics, including penicillin, ampicillin, oxacillin, cefuroxime, and ceftriaxone, and also to minocycline.

1. Introduction

Aquaculture refers to the farming or propagation of aquatic organisms including fish, mollusks, crustaceans, and aquatic plants, for diverse purposes such as; protein source, medicine, aesthetic value, research, etc. (FAO, 2016; Troell et al., 2017), and is noted to have a great history existing since 2000−1000 B.C (Swann, 1992). Recently, aquaculture is responsible for a much-increasing share of global aquatic food production and accounted for 65 % of the increase in fish production from 2005 to 2014 (FAO, 2016). Over the years, aquaculture has succored in the deficit in capture fisheries, contributing to global fish production reaching 46.0 % in 2018, up from 25.7 % in 2000 (FAO, 2020).

Aquaculture reportedly to contributes more than 50 % to the world's fish and seafood production meant for human consumption and needs (Thilsted et al., 2014) and is estimated to reach 62 percent by 2030 (Msangi & Batka, 2015), showing the significant role aquaculture continues to play in the modern-day. Nevertheless, the occurrence of disease has been among the tremendous constraints to aquaculture development. Even though economic losses resulting from diseases in aquaculture globally have not been assembled, it is sure to be devastating since research has shown the severity of disease outbreak in aquaculture environments, which in some cases can lead to total mortality (Faruk et al., 2004; Kalaimani et al., 2013; Rodger, 2016; Tavares-Dias & Martins,

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2017).

Bacterial diseases are prime causes of high mortality in wild and cultured fish (Pérez-Sánchez et al., 2018). Various measures such as antibiotic usage have been employed to control and eradicate diseases and their related impacts confronting aquaculture production (Scarfe et al., 2008; Subasinghe, 2009a). Antibiotics have been applied in managing diseases in aquaculture for several decades, but recent research has pointed out its adverse effects and has classified antibiotic use as a global health problem (Landers et al., 2012; Marshall & Levy, 2011; Pérez-Sánchez et al., 2014). The utilization of probiotics that can control pathogenic organisms through various means has been considered a sure alternative in place of antibiotics in aquaculture (Hoseinifar et al., 2018; P et al., 2012; Ringø et al., 2020).

Probiotics are live microorganisms that present several health benefits to the host when administered in an adequate dosage (Alayande et al., 2020). The benefits of probiotics in aquaculture go beyond the enhancement of the health status of aquatic organisms (George Kerry et al., 2018; Yirga, 2015), disease resistance (Kuebutornye et al., 2020a, b), and growth improvement (Patil et al., 2015; Ringø et al., 2020). Some probiotics are known to assist in improving water quality (Hasan & Banerjee, 2020; Hlordzi et al., 2020) and aid in maintaining an environmental balance (Jahangiri & Esteban, 2018; Mohamed, 2013).

Based on concerns raised on the use of antibiotics as feed additives, including acquired resistance against commonly used antibiotics (Aly & Albutti, 2014; Sun et al., 2020), and the European Union's ban placed on the application of antibiotics as feed additives beginning form the year 2006 (European Union, 2006), probiotics have been considered as a replacement for these antibiotic additives (Patil et al., 2015; Yirga, 2015). Even though probiotics have been widely accepted not only in the aquaculture industry but in many other animal rearing sectors and also in human food and medicine industries (George Kerry et al., 2018), its safety must be thoroughly studied and analyzed to make sure this helpmeet does not turn to cause any adverse effects on the host organisms.

Research and studies have centered on and have proven the importance and effectiveness of different probiotics, and these studies have helped in scientifically establishing the several benefits of probiotics (Kuebutornye et al., 2020c; P et al., 2012; Patil et al., 2015). Meanwhile, there are not many studies to elaborate and prove its safety to their host organism (Alayande et al., 2020; Doron & Snydman, 2015)

In 2017 the global market for probiotic supplement ingredients in food reached USD 47.1 Billion in 2018 and was projected to grow at a Compound Annual Growth Rate (CAGR) of 6.8 % from 2019 to 2026 (GlobalNewswire, 2020). Probiotics are administered as food supplements or directly added to the culture medium, including tanks or ponds in the case of aquaculture (Verschuere et al., 2000).

In expanding probiotic use in the human market, much concern on some safety issues such as mislabelling of the probiotics, microbial contamination, and pathogenic probiotics have been raised (Jackson et al., 2019; Sorokulova, 2013). There have been reports concerning the contamination of final probiotic products due to violation of good manufacturing practices, which releases life-threatening pathogens in the public domain (Cohen, 2018). For example, reports such as fatal infections in an immune-compromised patient due to *Bacillus* strain (Oggioni et al., 1998) and death of a premature 8-day old infant battling gastrointestinal mucormycosis after fungal contamination of probiotics supplement has been witnessed (Vallabhaneni et al., 2015). While few studies have assessed the safety of human and animal use of probiotics (Salvetti et al., 2016; Wisener et al., 2014), there is still a lack of systemic surveillance in detecting post-marketing hazards microorganisms used in probiotics might cause (Kolaček et al., 2017). Several commercial probiotics are used in the aquaculture industry, including *Lactobacillus, Bifidobacteria*, yeast, *Bacillus*, and many others since they are considered and scientifically proven beneficial (Fijan, 2014).

Bacillus species are gram-positive anaerobic bacteria known to exhibit a wide range of physiological characteristics (Jahangiri & Esteban, 2018; Standards Unit & Public Health England, 2018). Even though some *Bacillus* species are known to possess some disadvantageous traits (Doron & Snydman, 2015) and are also producers of toxins in their human and animal hosts (Elshaghabee et al., 2017), most of them are not pathogenic and are used as a dietary supplement for the improvement of human and animal health (George Kerry et al., 2018; Lee et al., 2019; Mongkolthanaruk, 2012). *Bacillus* genus belongs to the phylum Firmicutes with 293 species and subspecies, constituting a phylogenetically incoherent group (Patel & Gupta, 2020).

In aquaculture, the *Bacillus* species is one of the most widely used probiotic organisms. They have also been found to be natural members of the gut microbiota of some fish species (Kuebutornye et al., 2020b; Lavrador et al., 2018). The genus *Bacillus* is pervasive in almost all environments, including terrestrial, aquatic, and the atmosphere (Hong et al., 2005). *Bacillus* species are considered distinctive and are presently used as probiotic organisms (Soltani et al., 2019). Dietary supplementation of *Bacillus* species has enhanced immune responses, improved disease resistance, growth performance, and resistance against pathogenic bacteria infections (Guo et al., 2016; Selim & Reda, 2015). Besides playing a beneficial role in eliminating unwanted products from aquaculture environments, *Bacillus* species are also known for sustaining an optimum and ideal water quality which in turn reduces stress that can continuously cause an increase in survival and also aid in achieving an immune and physiological balance (Elsabagh et al., 2018; Hlordzi et al., 2020). They also perform some inhibitory activity against the growth of plant pathogens (Fira et al., 2018). Nevertheless, much study is required on the efficiency of some species in specific aquatic organisms and their water quality management characteristics.

The safety of beneficial microorganisms is essential because of the ability of some of these organisms to harbor virulence and drug resistance traits that might be transferable to their hosts (Jackson et al., 2019). Several studies have elaborated on the poor microbiological quality of many commercial probiotic formulations regarding identification, viability, or the number of microorganisms (Celandroni et al., 2019). For instance, a considerable number of probiotics produced in Asia were found to be poorly defined on the species level (Hong et al., 2005). With this, it can be argued that these formulations may not possess all the expected health benefits, and they may also pose health risks to consumers. In addition, virulence genes in beneficial bacteria are a major safety concern associated with their use (Cohen, 2018).

Virulence and antibiotic resistance factors are associated with both pathogenic organisms and some named beneficial microorganisms (Berthold-Pluta et al., 2019; Owusu-Kwarteng et al., 2017). Several studies conducted on probiotics used in both animal and human diets indicated the prevalence of virulence genes and also observed adverse effects which attributed to these virulence genes detected (Taras et al., 2006; Vallabhaneni et al., 2015; Wisener et al., 2014). The *Bacillus cereus* group belonging to the genus *Bacillus* has been identified by several researchers to possess some virulent traits and labeled as pathogenic (Ehling-Schulz et al., 2019; Gao et al., 2018; Park et al., 2009). They are known to be a principal cause of food poisoning. Also, antibiotics resistances, together with their Antibiotic Resistance Genes (ARGs), have in recent times been identified in aquatic environments (Shen et al., 2020) and has been attributed to several factors, including the indiscriminate use of antibiotics in aquaculture, among others (Zhang et al., 2009).

Nevertheless, the failure to ascertain and intuit the possibility of beneficial microorganisms used in aquatic environments as a source may also cause the prevalence of ARGs. As far as the 1970′s some *Bacillus* species were identified to possess certain antibiotic resistance traits (Bernhard et al., 1978); yet still, some of these species are used in both human and animal field without thorough investigation on their composition and safety (Lefevre et al., 2017; Sun et al., 2020). This study seeks to evaluate the safety of some *Bacillus* used in commercial probiotics formulation in aquaculture in terms of the presence and prevalence of some virulence encoding genes and their antimicrobial

susceptibility

2. Materials and method

2.1. Acquisition of commercial probiotic formulations

Thirty-two *Bacillus*-based commercial probiotic formulations used in aquaculture were purchased for this study. All formulations were selected randomly and purchased from the market and investigated before the expiration date.

2.2. Isolation and bacteria

All samples were in powdery form. For isolation, the samples were dissolved in sterile phosphate-buffered saline (PBS) in 150 mL tubes and homogenized using 15 mL Borosilicate glass tissue homogenizer (Shanghai Lenggu Instrument Company, Shanghai, China) for 2 min. The homogenized solution was serially diluted (10-fold) in sterile PBS, and 0.1 mL of each aliquot was inoculated onto Luria broth (LB) agar plates. Each inoculation was done in triplicates. Plates and content were incubated at 37 °C for 24 h and observed for growth. Distinct bacterial colonies were randomly selected and re-incubated in LB liquid media for 12 h. Re-streaking was performed to ensure the isolation of pure strain from a single species as described in (Owusu-Kwarteng et al., 2017).

2.3. Identification of bacteria

The bacteria isolated in the current study were characterized based on their morphological, biochemical tests and identified by molecular 16S rRNA gene sequence analysis using universal bacterial primers 27 F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Chen et al., 2017) through Polymerase Chain Reaction (PCR) to identify the isolates further. The PCR reaction system contained 1 μL of each primer, 1 μL template of each isolate, 12.5 μL of 10 × rTaq buffer, and 9.5 μL of double-distilled water. The PCR amplification was initiated with denaturation at 96 °C for 5 min followed by 33 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min 30 s; the amplification was completed by holding the reaction mixture at 72 °C for 10 min. The selected bacteria strains biochemical characterization was performed using commercial kits procured from Guangdong Huankai Microbial Sci. and Tech. Co., Ltd. (Guangdong, China) following the company's instructions. With the help of a *Bacillus cereus* identification bar (HBIG07-1) purchased from the Qingdao Hope Bio-Tech. Co., Ltd. (Qingdao, China), the biochemical tests conducted were confirmed. The PCR products were analyzed by one percent agarose gel (1% w/v) electrophoresis, and sequencing was performed by Sangon Biotech Co., Ltd. (Guangzhou, China). The sequence obtained was homologically compared with 16S rRNA gene sequences available in the National Centre for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) program to identify the bacteria strain isolated.

2.4. Genomic DNA extraction

The genomic DNA of various *Bacillus* species isolated from the commercial probiotics samples was extracted using the TaKaRa MiniBEST Bacteria Genomic DNA Extraction Kit Ver.3.0 following the manufacturer's instruction.

2.5. Resistance to antibiotics

Antimicrobial Susceptibility Testing of all identified *Bacillus* species was conducted using commercial antibiotics discs purchased from Hangzhou Microbial Reagent Co., Ltd., Hangzhou, China. Antibiotic discs were carefully placed on Mueller-Hinton agar plates previously spread with the probiotic bacteria and incubated for 24 h at 37 °C. Susceptibility inhibition zones were measured and interpreted, referring to the zone diameter interpretive described by Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute (CLSI, 2013) and also following manufacturer's users' guidelines. Twelve antibiotics were tested for including; Erythromycin (E, 15 μg), Minocycline (MI, 30 μg), Tetracycline (TE, 30 μg), Kanamycin (K, 30 μg), Penicillin (P, 10 μg), Ampicillin (AMP, 10 μg), Oxacillin (OX, 1μ), Deoxycycline (DX, 30 μg), Cefuroxime (CXM, 30 μg), Gentamicin (CN, 10 μg), Neomycin (N, 30 μg), Ceftriaxone (30, μg)

2.6. Detection of virulence factor genes

PCR screening was conducted to detect enterotoxigenic genes *hblA, hblC, hblD, nheA, nheB, nheC, cytK, entFM*, and one cereulide synthetase gene (ces). The PCR reaction system contained 1 μL of each primer, 1 μL template of Genomic DNA extracted, 10 μL of rTaq buffer (Takara, China), and 7 μL of double-distilled water. Also, double distilled water was used as the template for negative control in all reactions. Sequences of all primers used are provided in Table 1. The PCR amplification was initiated with denaturation at 95 °C for 5 min followed by 33 cycles of denaturation at 95 °C for 30 s, annealing at the specific temperature of each gene for 40 s, and extension at 72 °C for 40 s; the amplification was completed by holding the reaction mixture at 72 °C for 5 min. The PCR products were analyzed by agarose gel (1% w/v) electrophoresis. Following electrophoresis, gels were photographed under UV illumination.

2.7. Statistical analysis

All the experiments were performed in triplicate. Microsoft Excel (2010) was used to compute mean, percentages, and constructing tables.

3. Results

3.1. Isolation and identification of bacteria isolates

Results showed that some commercial probiotics did not contain or

Table 1
Sequences of PCR primers targeting various virulent factor genes in this study.

Target gene	Primer 5′ to 3′	Amplicon bp
nheA	TACGCTAAGGAGGGGCA GTTTTTATTGCTTCATCGGCT	480 (Owusu-Kwarteng et al., 2017)
nheB	CTATCAGCACTTATGGCAG ACTCCTAGCGGTGTTCC	754 (Owusu-Kwarteng et al., 2017)
nheC	CGGTAGTGATTGCTGGG CAGCATTCGTACTTGCCAA	564 (Owusu-Kwarteng et al., 2017)
hblA	GTGCAGATGTTGATGCCGAT ATGCCACTGCGTGGACATAT	301 (Owusu-Kwarteng et al., 2017)
hblC	AATGGTCATCGGAACTCTAT CTCGCTGTTCTGCTGTTAAT	731 (Owusu-Kwarteng et al., 2017)
hblD	AATCAAGAGCTGTCACGAAT CACCAATTGACCATGCTAAT	411 (Owusu-Kwarteng et al., 2017)
entFM	ATGAAAAAAGTAATTTGCAGG TTAGTATGCTTTTGTGTAACC	1,269 (Owusu-Kwarteng et al., 2017)
Ces	GGTGACACATTATCATATAAGGTG GTAAGCGAACCTGTCTGTAACAACA	1,271 (Owusu-Kwarteng et al., 2017)
cytK	GTAACTTTCATTGATGATCC GAATACATAAATAATTGGTTTCC	505 (Stenfors & Granum, 2001)

have fewer microorganisms than declared on their label. In some others, no bacteria growth was observed when spread on agar plates. Some samples also did not have any known *Bacillus species,* but rather some contained bacteria considered as pathogenic. In total, 49 isolates were obtained from the 32 commercial probiotics used, of which 44 were *Bacillus species* required for this study. (*see* Table 2 *for distribution of isolates*)

3.2. Resistance to antibiotics

The results of the antibiotic susceptibility assessment of the isolated *Bacillus* species are shown in Table 3. Almost all of the isolates were resistant to Penicillin (90.9 %), Ampicillin (47.7 %), Oxacillin (25 %), Erythromycin (18.2 %), Cefuroxime(11.4), Tetracycline (4.5 %), Gentamycin (4.5 %), Ceftriaxone (4.5 %), Minocycline (2.3 %), Neomycin (2.3 %) except for Kanamycin and Deoxycycline which recorded no resistance. Nevertheless, a good percentage of isolates were susceptible to Ceftriaxone (91 %), Gentamycin (91 %), Deoxycycline (86.4 %), Kanamycin (84.1 %), Cefuroxime (84.1 %), Minocycline (79.5 %), Oxacillin (72.7 %), Erythromycin (68.2 %), Tetracycline (61.4 %), with a few fractions being susceptible to Ampicillin (36.4 %) and Penicillin (2.3 %)

3.3. Detection of virulence factor genes among isolates

Enterotoxin genes *nheA, nheB, nheC, hblA, hblC, hblD*, and *entFM* together with cytotoxin K *(cytK)* and cereulide *(ces)* were amplified with PCR reactions. All designated primers used produced amplicons of the expected size from their respective target virulence genes. Each PCR was repeated three times to confirm the presence of the gene. Results from the PCR reaction are presented in Table 4. Three isolates CMPF 4 (*Bacillus cereus*), CMPF 6 (*Bacillus mojavensis*), CMPF 41(*Bacillus paranthracis*), were found to harbor the *nheA*, *nheB, nheC*, and *entFM* enterotoxin genes. CMPF 4 (*Bacillus cereus*), CMPF 41(*Bacillus paranthracis*) expressed the *hblA, hblC*, and *hblD* hemolytic genes. Only CMPF 41(*Bacillus paranthracis*) expressed an amplicon for the cytotoxin K (*cytK*) gene. None of the *Bacillus* species isolates expressed an amplicon for the cereulide (*ces*) gene

Table 2
Microorganisms Detected and as Declared on Label.

Sample No.	*Microorganism Detected*	*Microorganism Declared on Label*
1	*B. subtilis*	*Not stated*
2	*B. subtilis*	*B. subtilis, B. licheniformis*
3	*B. subtilis, B. cereus*	*B. subtilis, B. licheniformis, B. gelatins*
4	*B. subtilis*	*Not stated*
5	*B. mojavensis*	*Not stated*
6	*Enterobacter cloacae*	*Bacillus*
7	*B. subtilis*	*Bacillus*
8	*B. subtilis, B. velezensis*	*Bacillus*
9	*B. subtilis, B. mojavensis*	*Bacillus*
10	*none*	*B. subtilis*
11	*B. subtilis*	*Bacillus*
12	*B. subtilis*	*Bacillus*
13	*B. velezensis, B. subtilis*	*B. subtilis*
14	*B. subtilis, B. velezensis, B. tenquilensis*	*B. subtilis*
15	*B. velezensis, B. tenquilensis*	*Bacillus*
16	*B. velezensis*	*B. subtilis*
17	*B. tenquilensis, B. subtilis*	*B. subtilis, B. licheniformis*
18	*B. tenquilensis, B. subtilis*	*B. subtilis*
19	*Klebsiella pneumonia, Enterobacter cloacae*	*B. subtilis, B. licheniformis*
20	*B. subtilis*	*B. subtilis, B. licheniformis, B. cereus*
21	*B. velezensis, B. subtilis*	*B. subtilis*
22	*B. tequilensis, B. subtilis*	*B. subtilis, B. licheniformis*
23	*B. velezensis, B. tenquilensis*	*B. subtilis, B. licheniformis*
24	*B.tenquilensis*	*B. subtilis*
25	*B. amyloliquefaciens, B. velezensis*	*B. subtilis*
26	*Cronobacter sakazakii, Klebsiella pneumoniae*	*B. subtilis, B. licheniformis*
27	*B. paralicheniformis*	*B. subtilis*
28	*B. paralicheniformis*	*compound Bacillus*
29	*B. mojavensis*	*B. subtilis, B. cereus*
30	*B. tequilensis, B. velezensis. B. paranthracis*	*B. subtilis*
31	*B. subtilis, B. tequilensis*	*B. subtilis*
32	*B. tequilensis*	*B. subtilis*

Note: Sample No. 1-32 represents 32 different Bacillus based commercial probiotics used in aquaculture purchased for this study corresponding to various bacteria isolated from them and labeled Microorganism.

Table 3
Antibiotic Resistance of isolated bacteria.

Antimicrobial agents	n=*Bacillus sp*					
	Susceptible (S)		Intermediate (I)		Resistant (R)	
	n	(n)%	n	(n)%	n	(n)%
Erythromycin	30	68.2	6	13.6	8	18.2
Minocycline	35	79.5	8	18.2	1	2.3
Tetracycline	27	61.4	15	34.1	2	4.5
Kanamycin	37	84.1	7	15.9	0	0
Penicillin	1	2.3	3	6.8	40	90.9
Ampicillin	16	36.4	7	15.9	21	47.7
Oxacillin	32	72.7	1	2.3	11	25
Deoxycycline	38	86.4	6	13.6	0	0
Cefuroxime	37	84.1	2	4.5	5	11.4
Gentamycin	40	91	2	4.5	2	4.5
Neomycin	36	81.8	7	15.9	1	2.3
Ceftriaxone	40	91	2	4.5	2	4.5

Note: "**n**" represent the number of isolated *Bacillus species* and their respective percentages ("**n%**")for Susceptible (S), Intermediate (I), and Resistance (R).

4. Discussion

4.1. Bacterial composition of probiotic formulations

Studies have addressed the poor microbiological quality of commercial probiotic formulations (Celandroni et al., 2019; Jackson et al., 2019) and the poor correlation in the quality and quantity of the probiotics concerning the specified microorganism stated on their labels, and this raises safety concerns to the consumer (Kesavelu et al., 2020). Proper identification of strains in probiotics is the beginning of its in vitro safety assessment and potential risks (Gueimonde et al., 2013). In this study, some samples did not contain any *Bacillus* species but rather contained different bacteria, of which some are considered pathogenic. (see Table 2: sample no. 6, 19, 26). The continents of commercial probiotic formulations are mostly difficult to be ascertained (Marteau & Shanahan, 2003), and many people depend on the details provided on the labels (Sanders et al., 2018). Most commercial probiotics are wrongly labeled concerning the actual bacterial constituents (Marcobal et al., 2008; Theunissen et al., 2005; Weese, 2003) and their required CFU/mL (Chen et al., 2017). This inaccuracy can range from minor misreporting to major ones, such as misidentifying component bacteria (Weese, 2003). Nonetheless, it cannot be overlooked and makes the safety of the product contentious.

Similarly, this study noticed that not all the commercial probiotics assessed contained the probiotic bacteria labeled on the product. With this, it can be said that the safety of commercial probiotics on the market used in aquaculture is questionable. Safety assessment for commercial probiotics, particularly mixed probiotic formulations for aquaculture, needs to be established, and the constituents of the product are the basis for establishing its safety (FAO/WHO, 2002; Qi et al., 2009).

Table 4
Distribution of Virulence genes in Isolated *Bacillus Species*.

		Virulence Genes								
		nheA	*nheB*	*nheC*	*ces*	*cytK*	*hblA*	*hblC*	*hblD*	*entFM*
Sample*No*.										
1	CMPF 1	–	–	–	–	–	–	–	–	–
2	CMPF 2	–	–	–	–	–	–	–	–	–
3	CMPF 3	–	–	–	–	–	–	–	–	–
3	CMPF 4	+	+	+	–	+	+	+	+	+
4	CMPF 5	–	–	–	–	–	–	–	–	–
5	CMPF 6	+	+	+	–	–	–	–	–	+
20	CMPF 7	–	–	–	–	–	–	–	–	–
22	CMPF 8	–	–	–	–	–	–	–	–	–
22	CMPF 9	–	–	–	–	–	–	–	–	–
7	CMPF 10	–	–	–	–	–	–	–	–	–
8	CMPF 11	–	–	–	–	–	–	–	–	–
8	CMPF 12	–	–	–	–	–	–	–	–	–
9	CMPF 13	–	–	–	–	–	–	–	–	–
9	CMPF 14	–	–	–	–	–	–	–	–	–
11	CMPF 15	–	–	–	–	–	–	–	–	–
12	CMPF 16	–	–	–	–	–	–	–	–	–
13	CMPF 17	–	–	–	–	–	–	–	–	–
13	CMPF 18	–	–	–	–	–	–	–	–	–
14	CMPF 19	–	–	–	–	–	–	–	–	–
14	CMPF 20	–	–	–	–	–	–	–	–	–
14	CMPF 21	–	–	–	–	–	–	–	–	–
15	CMPF 22	–	–	–	–	–	–	–	–	–
15	CMPF 23	–	–	–	–	–	–	–	–	–
16	CMPF 24	–	–	–	–	–	–	–	–	–
17	CMPF 25	–	–	–	–	–	–	–	–	–
17	CMPF 26	–	–	–	–	–	–	–	–	–
18	CMPF 27	–	–	–	–	–	–	–	–	–
18	CMPF 28	–	–	–	–	–	–	–	–	–
21	CMPF 29	–	–	–	–	–	–	–	–	–
21	CMPF 30	–	–	–	–	–	–	–	–	–
23	CMPF 31	–	–	–	–	–	–	–	–	–
23	CMPF 32	–	–	–	–	–	–	–	–	–
24	CMPF 33	–	–	–	–	–	–	–	–	–
25	CMPF 34	–	–	–	–	–	–	–	–	–
25	CMPF 35	–	–	–	–	–	–	–	–	–
27	CMPF 36	–	–	–	–	–	–	–	–	–
28	CMPF 37	–	–	–	–	–	–	–	–	–
29	CMPF 38	–	–	–	–	–	–	–	–	–
30	CMPF 39	–	–	–	–	–	–	–	–	–
30	CMPF 40	–	–	–	–	–	–	–	–	–
30	CMPF 41	+	+	+	–	+	+	+	+	+
31	CMPF 42	–	–	–	–	–	–	–	–	–
31	CMPF 43	–	–	–	–	–	–	–	–	–
32	CMPF 44	–	–	–	–	–	–	–	–	–

Note: (+) and (-) represents the presence and absence of a gene, CMPF represents isolated *Bacillus* species shown in Table 2.

4.2. Resistant to antibiotics

Antibiotics have been used in aquaculture for many decades to aid in dealing with diseases (Vignesh et al., 2011). Even though there are some challenges with using antibiotics in aquaculture (Cabello, 2006; Cabello et al., 2013), they are still being applied with much precaution. (Subasinghe, 2009b; Sun et al., 2020). Resistance to antibiotics is one main safety concerns of probiotics (Courvalin, 2006; Jose et al., 2015; Sharma et al., 2014). This is due to the ability of these organisms to be mediators for the transfer of drug resistance genes to the environment and also to other bacteria living in the same habitat (Gueimonde et al., 2013). Most *Bacillus species* are considered beneficial and used as probiotics (Elshaghabee et al., 2017; Ringø et al., 2020), yet some *Bacillus* species have been identified to be capable of causing severe infections and diseases (Gao et al., 2018). The *Bacillus cereus* group of bacteria have been acclaimed to have the ability to cause disease (Didelot et al., 2009; Ehling-Schulz et al., 2019; Liu et al., 2017b; Rasko et al., 2005). Antibiotic therapy is considered effective in treating and eliminating members of the *Bacillus cereus* group (Turnbull et al., 2004). β-lactam antibiotics are widely used in commercial antibiotics in the treatment of a wide range of infectious diseases (Lingzhi et al., 2018), and infectious diseases have been shown to have a major effect on both marine and aquaculture economics (Lafferty et al., 2015). β-lactam antibiotics are significant ingredients of antibiotics medicaments used in aquaculture to handle infectious diseases (Chowdhury et al., 2015; Lara et al., 2012). Some of the antibiotics, like penicillin, aid against bacterial infections by inhibiting the development of cell walls of pathogens in susceptible organisms (Lara et al., 2012; Yocum et al., 1979). In our study, *Bacillus cereus* group isolates CMPF 4 (*Bacillus cereus*) and CMPF 41 (*Bacillus paranthracis*) were resistant to β-lactam antibiotics; penicillin, ampicillin, oxacillin, cefuroxime, and ceftriaxone used in this study and also to minocycline. Similarly, *B. cereus* isolates were resistant to β-lactam antibiotics (Gao et al., 2018; Owusu-Kwarteng et al., 2017; Yibar et al., 2017). Yu et al. (2019) also observed absolute resistance to β-lactam antibiotics penicillin and ampicillin by *Bacillus cereus* species isolated for vegetables in China. Aside *Bacillus cereus* group isolated, most of the other isolates were resistant to β-lactam antibiotics, penicillin, and ampicillin. Previous studies attribute this to the profuse production of β-lactamases by bacteria, including *Bacillus* species (Majiduddin et al., 2002; Owusu-Kwarteng et al., 2017; Park et al., 2009).

4.3. Prevalence of virulence genes

The presence of non-hemolytic enterotoxins *(nheA, nheB, and nheC),* hemolytic *(hblA, hblC, and hblD)* enterotoxins, enterotoxin complexes including enterotoxin FM (entFM), cereulide (ces), and cytotoxin K (cytK) in some probiotic bacteria have widely studied (Aragon-Alegro et al., 2008; Ehling-Schulz et al., 2006; Gao et al., 2018; Owusu-Kwarteng et al., 2017; Ragul et al., 2020). Disease is one major challenge faced in aquaculture (Li et al., 2011), and maximum efforts are being made to rule out all causes (Flegel, 2019). Therefore, the screening for virulence genes in probiotic bacteria is crucial because of the possibility of gene transfer from the probiotic bacteria into the habitat and the bacteria itself, causing diseases and complications to the host. Probiotics are applied in all spheres, including aquaculture, due to their several benefits (Pérez-Sánchez et al., 2018; Yirga, 2015), and if these known beneficial organisms possess virulence-associated genes, then their safety is uncertain.

The Non-hemolytic enterotoxin (*nhe*) is a complex pore-forming toxin (PFT) that comprises three proteins; *nheA* (41-kDa), *nheB* (39-kDa), and *nheC* (40-kDa) (Ganash et al., 2013). With the *nhe* complex, all three proteins are required to reach maximum cytotoxicity following a specific binding order on cell membranes (Heilkenbrinker et al., 2013; Lindbäck et al., 2010; Liu et al., 2017a). Among the *Bacillus* strains isolated in this study, CMPF 4 (*Bacillus cereus*), CMPF 6 (*Bacillus mojavensis),* and CMPF 41 (*Bacillus paranthracis)* were found to possess all three non-hemolytic enterotoxins genes *(nheA, nheB, and nheC)*. (Lindbäck et al., 2004) observed 100 % cytotoxicity of *Bacillus cereus* associated with the presence of the three *nhe* complex. Maximal (100 %) cytotoxicity is expressed by these *Bacillus* species used in commercial probiotics in aquaculture as they harbor the *nheABC* genes. Also, these three isolated *Bacillus* species were found to have the enterotoxin FM (entFM) gene, whose cytotoxicity is dependent on the bacteria strain (Boonchai et al., 2008). Two isolated *Bacillus* species, CMPF 4 (*Bacillus cereus*) and CMPF 41 (*Bacillus paranthracis*), expressed the hemolytic *(hblA, hblC, and hblD)* genes. None of the samples produced an amplicon for the cereulide *(ces)* gene. The cytotoxin K (*cytK*) is a toxin that belongs to the family of oligomeric β-barrel pore-forming toxins (Menestrina et al., 2001) and is mostly associated with *Bacillus cereus* and is responsible for some severe food poisoning (Fagerlund et al., 2004; Hardy et al., 2001).CMPF 4 (*Bacillus cereus*) and CMPF 41 (*Bacillus paranthracis*) produced amplicon for the cytotoxin K *(cytK)*. These confirm the presence of these toxin-related genes in bacteria present in commercial probiotic formulations sold on the market, which are directly administered to culture organisms. A study conducted by (Cui et al., 2020) in China also reported that *Bacillus* isolates from commercial probiotics expressed high levels of toxicity. Many studies have been conducted and have elaborated on these virulence genes associated with the species *Bacillus* (Boonchai et al., 2008; Hendriksen, 2001; Kim et al., 2020) and this has become a growing concern with the use of probiotics in aquaculture (Wang et al., 2008).

5. Conclusion

The safety of probiotics is very crucial, and thus much attention is needed in safeguarding their usage. The safety of commercial probiotics begins with identifying their constituent bacteria. Therefore much caution should be taken by producers to identify the bacteria being used carefully. Also, labeling is an essential aspect of commercial production. Commercial probiotics should be labeled appropriately with accurate information regarding the composition of the product. *Bacillus species* are well known for their several benefits in the aquaculture industry and several fields, including human medicine and nutrition. Nonetheless, some virulent factors and drug resistance traits are associated with some species. Therefore *Bacillus species* should be judiciously selected to be used in commercial probiotics.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aqrep.2021.100784.

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