PROPERTIES AND SAFETY EVALUATION OF *BACILLUS VELEZENSIS* BUU004 AS PROBIOTIC AND BIOPRESERVATIVE IN SEAFOOD PRODUCTS

Namphueng Butkhot[1], Pornpimon Soodsawaeng[2], Traimat Boonthai[2], Verapong Vuthiphandchai[3] and Subuntith Nimrat[1,4]

[1]Environmental Science Program, [2]Biological Science Program, [3]Department of Aquatic Science, [4]Department of Microbiology, Faculty of Science, Burapha University, Chon Buri, Thailand

Abstract. *Bacillus* probiotics have been proposed for inhibition of several foodborne pathogens, which cause considerable public health burden and pose a challenge to control measures. Selection of probiotics requires close scrutiny in terms of biosafety and probiotic characteristics prior to incorporation into human food products. *Bacillus velezensis* BUU004, previously used as an aquaculture probiotic, showed strong antagonistic effect on seafood spoilage bacteria, namely, *Aeromonas schubertii*, *Proteus mirabilis*, *Sphingobacterium spiritivorum, Staphylococcus hominis*, *Staph. mucilaginosus*, *Staph. saprophyticus*, and *Staph. warneri*, and on foodborne pathogens, *Bacillus cereus* and *Staph. aureus*. *B. velezensis* BUU004 was susceptible to several classes of antibiotics used in human and veterinary clinical therapy, was α-hemolytic with absence of hemolysin genes: *hlyI*/*clo*, *hlyII* and *hlyIII* homolog, did not carry enterotoxin genes: *cytK*, *hblACD* and *nheABC*, harbored genes encoding proteins associated with cell adhesion activity, namely, fibronectin-binding, collagen-binding surface and Gram-positive anchor proteins, and exhibited robust survivability under gastric and bile conditions. In conclusion, *B. velezensis* BUU004 was non-pathogenic with the characteristics of a potential probiotic safe for use as a source of biopreservative to prevent foodborne infections of seafood products.

Keywords: *Bacillus velezensis* BUU004, antibacterial activity, biopreservative, probiotic, safety, seafood

INTRODUCTION

Probiotics are defined as live microorganisms conferring beneficial health effects on host when administrated in adequate amounts (FAO/WHO, 2006). Potential benefits of probiotics are the prevention of gastrointestinal disorders, enhancement of gut immunity, reduction in anti-allergy effects, and decrease in serum cholesterol level (Guo *et al*, 2010; Lefevre *et al*, 2017). An increase in foodborne antibiotic-resistant pathogens has prompted a search for alternate therapeutic agents (Achi and Halami, 2016; Elshaghabee *et al*, 2017). Probiotics provide an attractive viable solution owing to their efficacy, cost-effectiveness, non-invasive application, long history of safe use, and promotion of gut natural

Correspondence: Subuntith Nimrat, Department of Microbiology and Environmental Science Program, Faculty of Science, Burapha University, Chon Buri 20131, Thailand.
Tel: +66 (0) 38 103120; Fax: +66 (0) 38 393490
E-mail: subunti@buu.ac.th; subunttbt@gmail.com

flora (Elshaghabee *et al*, 2017). To date, probiotics, mainly Gram-positive bacteria belonging to genera *Bifidobacterium*, *Enterococcus*, *Lactobacillus*, *Streptococcus*, and yeast (*Saccharomyces cerevisiae*), have been marketed as novel food products, dietary supplements and therapy for several intestinal infectious diseases in humans (Saarela *et al,* 2000; Elshaghabee *et al,* 2017; Lefevre *et al,* 2017).

Among probiotics, spore-forming *Bacillus* spp have gained large interest due to their several advantages over other non-spore formers, *eg* better stability under harsh conditions during food processing, higher survival in gastrointestinal tract conditions, long shelf-life, higher viability at ambient temperature and refrigerated conditions, and low effective dose required for use as food supplement (Cutting, 2011; Nithya and Halami, 2013). Probiotic preparations containing *Bacillus* strains have been reported to prevent gastrointestinal illnesses, such as irritable bowel syndrome (Tompkins *et al*, 2009), reduce pathogenic *Helicobacter pylori* infection (Lesbros-Pantoflickova *et al*, 2007), and promote intestinal and immunological health (Elshaghabee *et al*, 2017). Among more than 100 species known, *Bacillus* spp included in *B. subtilis* complex, *eg B. amyloliquefaciens*, *B. atrophaeus*, *B. licheniformis*, *B. methylotrophicus*, *B. mojavensis*, *B. siamensis*, *B. subtilis*, *B. tequilensis*, and *B. vallismortis*, are considered as generally regarded as safe (GRAS) for human consumption (Lefevre *et al*, 2017; Chen *et al*, 2018). However, if *Bacillus* spp, such as *B. cereus* and its relatives, harbor transmissible antibiotic resistance determinants and/or virulent factors, they can pose serious health risks (Chon *et al*, 2012). In addition, a small number of species within *B. subtilis* group have occasionally been reported to be causative agents of food poisoning (European Food Safety Authority, 2005). Thus, it deems necessary to evaluate the biosafety features of potential probiotics on a strain-by-strain basis.

A recent study on bacteriological quality of dried seafood products sold in Chon Buri Province, Thailand, revealed nearly half of the samples contained viable bacteria above allowable limits (Nimrat *et al*, 2019). Pathogenic bacteria, such as *B. cereus*, *Staphylococcus aureus* and *Salmonella* spp, have been isolated from dried seafood products in Thailand (Thungkao and Muangharm, 2008; Butkhot *et al*, 2019), indicating a need for intervention measures to control bacterial contamination and reduce a potential hazard of foodborne infection. As several *Bacillus* spp synthesize a wide range of antimicrobial agents, *eg* bacteriocins, these bacteria species could be selected as sources of biopreservatives in food products (AlGburi *et al*, 2016). However, any potential candidate *Bacillus* spp are required to undergo a rigorous assessment of their possible toxigenic and probiotic properties to ensure safe use in humans.

Hence, the study aimed to assess antibacterial activity against seafood spoilage and pathogenic bacteria of five *Bacillus* isolates previously used as probiotics in shrimp marine culture (Nimrat *et al*, 2012) and ascertain virulent characteristics and probiotic attributes of a candidate *Bacillus* isolate with potential for future application in dried seafood industry.

MATERIALS AND METHODS

Bacteria and culture conditions

Five *Bacillus* spp, namely, *B. megaterium* BUU002, *B. polymyxa* BUU003, *B. subtilis*

BUU005, *B. thuringiensis* BUU001, and *B. velezensis* BUU004, previously isolated from black tiger shrimp *Penaeus monodon* and shrimp pond sediment, were selected due to their beneficial effects on black tiger and whiteleg shrimp *Litopenaeus vannamei* and their antagonistic properties against shrimp pathogenic *Vibrio* spp and *Pseudomonas* spp (Nimrat *et al*, 2008; Nimrat *et al*, 2012). Each *Bacillus* strain was grown in Trypticase Soy Broth (TSB) (Becton BD, Sparks, MD, USA) at 30°C for 24 hours with shaking, sedimented at 8,000*g* at 4ºC for 10 minutes, and filtered through a 0.45 μm filter (Sartorius, Goettingen, Germany) and then, cell-free supernatants (CFSs) were stored at -20ºC until used.

Spoilage and pathogenic bacteria strains isolated from dried seafood products used for evaluation of antibacterial activity of the five *Bacillus* spp were obtained from the Environmental Microbiology Laboratory, Department of Microbiology, Faculty of Science, Burapha University, and cultured in TSB (Becton BD) at 35°C for 24 hours.

Bacteriostatic activity assay

Bacteriostatic activity of CFS from *Bacillus* BUU001, BUU002, BUU003, BUU004 and BUU005 strains was analyzed using an agar well diffusion method (Yilmaz *et al*, 2006). In brief, a 24-hour culture of each spoilage and pathogenic bacteria strain was adjusted to 10^6 CFU/ml and then swabbed onto Mueller Hinton Agar (MHA) (Difco BD, Sparks, MD, USA). Then, 50-μl aliquot of CFS was added into a well of 6 mm in diameter bored into the MHA (Difco BD) plate. All petri dishes were incubated at 35°C for 24 hours and the diameter of a clear zone around well was recorded. Each experiment was carried out in triplicate.

Antibiogram profiling

Antibiogram profile of each *Bacillus* strain was conducted using a Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standards Institute protocols (CLSI, 2016). Cell suspension of each strain was adjusted to a 0.5 McFarland standard unit (equivalent to 10^8 CFU/ml) and seeded onto MHA (Difco BD) plate. Antibiotic-impregnated disks (Oxoid, Basingstoke, UK) were placed on the seeded plate and diameter of inhibition zones was recorded following incubation at 37°C for 20 hours. Each experiment was performed in triplicate.

Blood hemolysis assay and hemolysin gene detection

B. velezensis BUU004 was streaked onto Trypticase Soy Agar (TSA) (Difco BD) plate containing 5% human blood, incubated at 37°C for 24 hours, and examined to check for pattern of hemolytic activity: complete hemolysis (β-hemolysis), partial hemolysis (α-hemolysis) and no change (γ-hemolysis). Presence of hemolysin (*hlyI*/*clo*, *hlyII* and *hlyIII* homologs) and surfactin lipopeptide (*srfA*) genes were detected by examination of genome sequence of *B. velezensis* BUU004 (GenBank accession no. SJCZ01000000) (http://www.mgc.ac.cn/VFs/) (Liu *et al*, 2018) using antiSMASH v.5.0 software (Blin *et al*, 2017).

Detection of enterotoxin genes

B. velezensis BUU004 enterotoxin genes [*cytK*, *nhe* (A, B, C) and *hbl* (A, C, D)] were detected by PCR (Chon *et al*, 2012). In short, a loopful of bacteria was inoculated in TSB (Becton BD), incubated overnight at 30°C, and centrifuged at 10,000*g* for one minute at 4°C. DNA was extracted from the cell pellet using a DNA extraction kit (Bio-Helix, Keelung City, Taiwan). Reaction mixture (25 µl)

contained 100 ng of DNA, 2.5 µl of 10X PCR buffer (Vivantis, Selangor Darul Ehsan, Malaysia), 2.5 µl of 2.5 mM dNTPs, 2.5 µl of 25 mM $MgCl_2$, 1 µl of each primer (10 pmol/µl), 1 U of *Taq* DNA polymerase (5 U/µl) (Vivantis), and 14 µl of sterile nuclease-free water. Thermocycling was performed in a Mastercycler nexus (Eppendorf, Hamburg, Germany) as follows: 94°C for 2 minutes, followed by 35 cycles of 94°C for 1 minute, annealing temperature for 1 minute and 72°C for 2 minutes; and a final step of 72°C for 5 minutes. Amplicons were visualized using 1.5% agarose gel electrophoresis containing ethidium bromide (0.5 mg/ml) and recorded under UV transillumination. *B. cereus* ATCC 14579 DNA was used as positive control. PCR primers, amplicon size and annealing temperatures used in detecting enterotoxin genes are summarized in Table 1.

Detection of genes encoding proteins related to adhesion

The annotated genome sequence of *B. velezensis* BUU004 described above were examined for *pp1-Y1* (encoding mucin-binding protein), *fbpA* (encoding fibronectin-binding protein), *cna* (encoding collagen-binding surface protein), *fha* (encoding filamentous adhesin), and *srtA* (encoding Gram-positive anchor LPXTG sortase) using an online HMMER webserver (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) (Finn *et al*, 2011).

Acid and bile-salt tolerance

Tolerance of *B. velezensis* BUU004 to gastric acid and bile salts was evaluated as previously described by Jeon *et al* (2016). In brief, one-ml aliquot of a 24-hour culture suspension was diluted 1:10 in artificial gastric juice (0.3% (w/v) pepsin (Sigma-Aldrich, St Louis, MO) in TSB pH 2.5) and incubated at 37°C for 3 hours with shaking. Tolerance to bile salts was assessed by inoculation of one-ml aliquot of cell culture in 9 ml of TSB containing 0.3% (w/v) oxgall (Sigma-Aldrich), and incubation at 37°C for 24 hours with shaking. Afterwards, viable *B. velezensis* BUU004 cells were counted using a spread plating method and presented as percent viable cells.

Data analysis

All experiments were performed in triplicate and data were expressed as mean ± SD.

RESULTS

Bacteriostatic activity of CFS from the tested *Bacillus* spp

Seafood spoilage and pathogenic bacteria (n = 20) were tested for their susceptibilities to CFS from the five tested *Bacillus* spp, namely, *B. megaterium* BUU002, *B. polymyxa* BUU003, *B. subtilis* BUU005, *B. thuringiensis* BUU001, and *B. velezensis* BUU004. All seafood spoilage and pathogenic bacteria were susceptible to *B. velezensis* BUU004 CFS with inhibition zones ranging from 7.2 ± 0.1 to 17.3 ± 0.6 mm. The most susceptible strains were *B. cereus* 001 and *Staph. saprophyticus* (Table 2). *B. subtilis* BUU005 CFS was able to inhibit only *B. cereus* 001 and *B. cereus* 004, while the other three *Bacillus* CFSs did not express antibacterial activity against the 20 spoilage and pathogenic bacteria. Hence, *B. velezensis* BUU004 was selected for subsequent experiments.

Antibiogram profile

B. velezensis BUU004 exhibited susceptibility to 14/16 antibiotics tested, with only intermediate resistance to the polypeptide family of antibiotics: bacitracin and polymyxin B (Table 3).

Table 1
PCR primers used in detection of enterotoxin genes associated with gastrointestinal ailments.

Enterotoxin	Primer	Sequence (5′-3′)	Amplicon size (bp)	Annealing temperature (°C)	Reference
Hemolytic enterotoxin	*hblA*-F	AAGCAATGGAATACAATGGG	1,154	56	Guinebretière *et al* (2002)
	hblA-R	AGAATCTAAATCATGCCACTGC			
	hblC-F	GATACTCAATGTGGCAACTGC	740	58	Guinebretière *et al* (2002)
	hblC-R	TTGAGACTGCTCGTCTAGTTG			
	hblD-F	ACCGGTAACACTATTCATGC	829	58	Guinebretière *et al* (2002)
	hblD-R	GAGTCCATATGCTTAGATGC			
Non-hemolytic enterotoxin	*nheA*-F	GTTAGGATCACAATCACCGC	755	56	Guinebretière *et al* (2002)
	nheA-R	ACGAATGTAATTTGAGTCGC			
	nheB-F	TTTAGTAGTGGATCTGTAGC	743	54	Guinebretière *et al* (2002)
	nheB-R	TTAATGTTCGTTAATCCTGC			
	nheC-F	TGGATTCCAAGATGTAACG	683	58	Guinebretière *et al* (2002)
	nheC-R	ATTACGACTTCTGCTTGTGC			
Enterotoxin- T	*CytK*-R	GAATACTAAATAATTGGTTTCC	505	48	Stenfors and Granum (2001)
	CytK-F	GTAACTTTCATTGATGATCC			

Table 2
Bacteriostatic activity against dried seafood spoilage and pathogenic bacteria tested using *Bacillus* spp cell-free supernatant.

Spoilage and pathogenic bacteria	Inhibition zone (mm) (mean ± SD)[a,b]	
	Bacillus velezensis BUU004	*B. subtilis* BUU005
Staphylococcus hominis	15.6 ± 0.4	-
Staph. warneri	11.0 ± 0.2	-
Staph. mucilaginosus	8.0 ± 0.0	-
Staph. saprophyticus	17.3 ± 0.6	-
Proteus mirabilis	7.1 ± 0.1	-
Aeromonas schubertii	7.2 ± 0.0	-
Sphingobacterium spiritivorum	14.9 ± 3.6	-
B. cereus 001	17.1 ± 0.9	9.3 ± 0.14
B. cereus 002	14.2 ± 0.7	-
B. cereus 003	16.0 ± 0.5	-
B. cereus 004	8.7 ± 0.78	8.3 ± 1.1
Staph. aureus D 4	9.2 ± 0.3	-
Staph. aureus H 8	9.2 ± 0.2	-
Staph. aureus L12	10.8 ± 0.14	-
Staph. aureus O15	10.6 ± 0.1	-
Staph. aureus P16	12.1 ± 0.1	-
Staph. aureus R18	8.92 ± 0.14	-
Staph. aureus S19	11.7 ± 0.4	-
Staph. aureus BB28	11.2 ± 0.2	-
Staph. aureus DD30	12.4 ± 0.3	-

[a]Agar well diffusion method; [b]No bacteriostatic activity from *B. megaterium* BUU002, *B. polymyxa* BUU003 and *B. thuringiensis* BUU001 was observed after cell-free supernatants.

***B. velezensis* BUU004 hemolytic activity and carriage of hemolysis-related genes**

Based on the observation of partial hemolysis on blood agar plate, *B. velezensis* BUU004 was identified as an α-hemolytic strain (data not shown). Examination of the annotated whole genome (GenBank accession no. SJCZ010000000) showed that *B. velezensis* BUU004 was devoid of hemolysin genes: *hlyI/clo*, *hlyII* and *hlyIII* homologs, but contained *srfA* (GenBank accession no. TCJ49361.1), encoding surfactin non-ribosomal peptide synthetase, responsible for surfactin-lipopeptide production.

***B. velezensis* BUU004 enterotoxin genes associated with gastrointestinal diseases**

PCR amplification using a set of enterotoxin gene-specific primers demonstrated the absence of enterotoxin

Table 3
Antibiogram profile of *Bacillus velezensis* BUU004.

Class of antibiotics	Antibiotic	Zone of inhibition (mm) (mean ± SD)[a]	Interpretation[b]
B-lactam	Penicillin (10 U)	32.5 ± 0.5	S
	Ampicillin (10 μg)	26.7 ± 0.6	S
	Cephalothin (30 μg)	45.0 ± 1.0	S
	Cefotaxime (30 μg)	25.8 ± 0.8	S
	Cefoxitin (30 μg)	28.0 ± 0.0	S
	Imipenem (10 μg)	44.7 ± 0.6	S
Glycopeptide	Vancomycin (30 μg)	23.0 ± 0.0	S
Polypeptide	Bacitracin (10 μg)	10.8 ± 0.8	I
	Polymyxin B (300 U)	11.0 ± 0.0	I
Aminoglycoside	Gentamicin (10 μg)	25.6 ± 0.8	S
	Kanamycin (30 μg)	29.0 ± 1.0	S
Fluoroquinolone	Ciprofloxacin (30 μg)	42.3 ± 0.3	S
Aminocoumarin	Novobiocin (30 μg)	23.8 ± 0.8	S
Tetracycline	Tetracycline (30 μg)	20.7 ± 0.6	S
Phenicol	Chloramphenicol (30 μg)	26.0 ± 1.0	S
Folate pathway antagonist	Trimethoprim (1.25 μg)/ Sulfamethoxazole (23.75 μg)	34.5 ± 1.5	S

[a]Disk diffusion assay; [b]CLSI (2016); I, intermediate resistance; S, susceptible.

genes in *B. velezensis* BUU004 including hemolytic enterotoxin (*hblA*, *hblC*, *hblD*), non-hemolytic enterotoxin (*nheA*, *nheB*, *nheC*) and cytotoxin K (*cytK*) genes, which were present in positive control *B. cereus* ATCC 14579 (data not shown).

***B. velezensis* BUU004 genes related to cell adhesion**

Three genes encoding proteins involved in cell adhesion, *ie fbpA* (Pfam no. PF05833; GenBank accession no. TCJ52445.1), *cna* (Pfam no. PF05737; GenBank accession no. TCJ48793.1) and *srtA* (Pfam no. PF00746; GenBank accession no. TCJ49076.1) were present in *B. velezensis* BUU004 genome.

***B. velezensis* BUU004 tolerance to artificial gastric juice and bile salts**

Percentages of viable *B. velezensis* BUU004 following a 3-hour exposure to artificial gastric juice (pH 2.5) and a 24-hour exposure to 0.3% (w/v) oxgall were 73 ± 2.8% and 98 ± 5.2% respectively.

DISCUSSION

Bacillus spp are able to synthesize a diversity of antimicrobial substances considered as part of their defense mechanisms to survive and inhibit competing microflora (Achi and Halami, 2016). Based on the beneficial effects of the five *Bacillus* spp previously used as a

putative probiotic consortium in shrimp cultivation (Nimrat *et al*, 2012), this study showed that *B. velezensis* BUU004 exhibited properties of a candidate biopreservative in dried seafood products, i.e. bacteriostatic activity against spoilage and pathogenic bacteria. Recently, Chen *et al* (2018) reported that a crude extract of *B. velezensis* 157 had broad inhibitory spectrum against several human and aquatic animal pathogens, such as *Aeromonas caviae*, *A. hydrophila*, *A. veronii*, *Clostridium perfringens*, *Escherichia coli*, *Proteus hauseri*, *Salmonella choleraesuis*, *Sal. enterica* Typhimurium, *Sal. Enteritidis*, *Staph. aureus*, and *Strep. agalactiae.* In accordance with Yi *et al* (2018), *B. velezensis* JW was reported to inhibit growth of fish pathogens, e.g. *A. hydrophila*, *A. salmonicida* and *Vibrio parahemolyticus*. Bacteriostatic effects of *B. velezensis* BUU004 may due to the release alone or in combination of antimicrobial substances, such as polyketides, non-ribosomal peptides and bacteriocins (Chen *et al*, 2018).

Safety evaluation of potential probiotics is of importance and need to be verified prior to being recommended for use in human and animal nutrition, either directly as a feed additive or indirectly as a source of such additives. A novel potential probiotic should be non-pathogenic to the host and not be resistant to any human and veterinary antibiotics (Kavitha *et al*, 2018). This study clearly demonstrated that *B. velezensis* BUU004 possessed such probiotic properties, including sensitivity to a broad range of antibiotics, lack of genes associated with gastrointestinal diseases, carriage of some cell adhesion genes, and tolerance to low pH and bile salts. With regard to the latter property, Kavitha *et al* (2018) observed that *Bacillus* spp were able to withstand a wide range of acidic conditions (pH 1-6) and up to 5% (w/v) bile concentrations, and Borah *et al* (2019) reported strong tolerance of *B. velezensis* DU14 after exposure to low (2-6) pH and 1% (w/v) bile salts.

B. velezensis BUU004 was of intermediate resistance to bacitracin and polymyxin B. Reva *et al* (1995) examined antibiotic susceptibility of *B. subtilis* (30 isolates) and *B. licheniformis* (10 isolates) using a disk diffusion method and observed complete resistance to polymyxin in all strains and low level of susceptibility to polymyxin in strains related to *B. megaterium. B. subtilis* strains resistant to bacitracin and polymyxin B were also identified by others (Galizzi *et al*, 1975; Bernard *et al*, 2007). In addition, *B. velezensis* BUU004 was α-hemolytic on human blood agar, although the bacterium did not carry homologs of the well-known hemolysin genes present in pathogenic *B. cereus* (Ramarao and Sanchis, 2013). The partial hemolytic activity might be associated with presence of active surface molecules as *srfA* that codes a surfactin non-ribosomal peptide synthetase was present in *B. velezensis* BUU004 genome. *B. velezensis* H3 exhibited an ability to lyse human blood cells (Seydlová and Svobodová, 2008). In order to obtain safety approval, hemolytic mechanisms of *B. velezensis* BUU004 should be further studied.

Another important component of screening for potential probiotics is the adhesion ability to intestinal epithelial cells, mucus, or constituents of the extracellular matrix (Dunne *et al*, 2001). Host attachment allows probiotic bacteria to access nutrients, grow in suitable environmental conditions and display the beneficial actions effectively. Colonization and persistence of beneficial bacteria in intestinal canals depend, at least in part, on their surface proteins that play a key

role in adherence of the bacteria to host tissues. Although no adhesion capability to intestinal cells was evaluated, several genes encoding proteins related to adhesion characteristics were present in *B. velezensis* BUU004 genome. A probiotic strain of *Lactobacillus acidophilus* NCFM displayed ability to attach to intestinal epithelial Caco-2 cells by means of multiple cell surface proteins, *ie* fibronectin-binding protein A, Gram-positive cell wall anchor domain, surface-layer protein A and mucin-binding protein (Buck *et al*, 2005). Likewise, Ayala *et al* (2017) reported adhesive properties to extracellular matrix proteins of probiotic *B. subtilus* natto RG436 through fibronectin-binding protein YloA containing two Pfam domains, namely, a FbpA domain predicted to be involved in fibronectin binding and Duf814 domain associated with FbpA domain. *In vivo* evaluation of adherent ability of *B. velezensis* BUU004 strain and its endospores to intestinal epithelial cells should be further established to determine its ability to colonize intestinal tracts.

In summary, the study provided evidence that *B. velezensis* BUU004 exhibited strong antibacterial activity against seafood spoilage and pathogenic bacteria, and properties of a probiotic, namely, non-toxigenicity, vulnerability to a wide range of human and veterinary antibiotics, absence of hemolysin (*hlyI*/*clo*, *II*, and *III* homologs) and enterotoxin (*cytK*, *hblACD* and *nheABC*) genes, presence of genes encoding proteins involving cell adhesion (fibronectin-binding, collagen-binding surface and Gram-positive anchor proteins) and high tolerance to gastric and bile salt conditions. Thus, *B. velezensis* BUU004 is a potential probiotic and has a promising potential as safe biopreservative in seafood products.

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