Inhibition of *Clostridium botulinum* and its toxins by probiotic bacteria and their metabolites: An update review

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REVIEW ARTICLE

Abstract

Clostridium (C.) botulinum is the causative agent of foodborne poisoning such as botulism, which includes high mortality rates in animals and humans. Probiotic bacteria play critically functional roles in food matrices, as well as agricultural, clinical and nutritional fields. In this review, potentials of various probiotic bacteria and their metabolites to prevent *C. botulinum* toxicity are reviewed. For this purpose, an introduction about *C. botulinum* and its mechanisms of action is provided. After a short introduction of probiotic bacteria and their beneficial health effects on humans, the bacterial mechanisms of their action are reviewed. Then bacteriocin production by probiotic bacteria is described. After description of *C. botulinum* and its neurotoxins, effects of probiotic bacteria on *C. botulinum* are reviewed with a special focus on effects of the bacterial bacteriocins on this pathogen. Furthermore, physicochemical factors, which show great effects on potential of nisin to prevent growth and toxin production of the bacteria, are introduced. This study has shown that probiotic bacteria and their bacteriocins can be effective on growth, toxin formation and toxicity of *C. botulinum*. In conclusion, probiotic use in food safety studies can be effective in preventing or treating toxicity of *C. botulinum*.

Keywords: bacteriocins; decontamination; prevention; probiotic bacteria

Introduction

Digestive system includes critical roles in digestion and absorption of foods for the production of energy. The gastrointestinal mucosa, which covers a wide surface, is exposed to pathogens and non-pathogen agents (Donaldson *et al.*, 2016). Microorganisms present in the gastrointestinal tract (GIT), especially *Lactobacillus* spp. and *Bifidobacterium* spp., play important roles in health (Sadrizadeh *et al.*, 2018; Zendeboodi *et al.*, 2020). These microorganisms include the greatest effects on immune system function, leading to development of a strong balanced immune system (Butel, 2014; Eslami *et al.*, 2020; Soccol *et al.*, 2010). Because the largest and most complex part of the immune system is associated with the tissues of the GIT, therefore, revival immune

system plays a serious role in protecting humans against various pathogens (Khaneghah *et al*., 2020). If balance of gut microbiota (microorganisms that usually colonize the body) changes due to the use of various drugs such as antibiotics, it can increase the risk of various infectious diseases by adaptable pathogens such as *Clostridium* spp. (Bäckhed *et al.*, 2012; Sánchez *et al.*, 2017). *Clostridium* spp. are Gram-positive, obligate anaerobe endospore-producer bacteria. These bacteria are known as foodborne pathogenic and spoilage bacteria, hazardous to human health (Fooda, 2018). The most important species of this genus include *Clostridium botulinum* (causing botulism), *Clostridium difficile* (causing diarrhea during antibiotic therapy) and *Clostridium perfringens* (causing food poisoning to cellulitis and gas gangrene) (Fooda, 2018). In fact, *C. botulinum* leads to toxin production. Botulism disease affects various individuals, especially infants (transmitted through honey), causing several complications such as paralysis, nausea, vomiting, abdominal cramps, difficult swallowing or speaking, weak cry, irritability, drooping eyelids, tiredness and difficult sucking or feeding (Fooda, 2018). Naturally, *C. botulinum* produces various neurotoxins (A–H) with various effects depending on the target organs. Therefore, control of these pathogens using biological, chemical and physical agents can include effective roles in providing public health.

Nowadays, use of biologic agents in disease control is interested by researchers due to the adverse effects of drug use on general health (Hashempour-Baltork *et al.*, 2019). One of the most important groups of the biological agents are associated with probiotic bacteria and their metabolites (Chugh and Kamal-Eldin, 2020). The World Health Organization (WHO) and Food and Agriculture Organization have considered probiotics as "live microorganisms when administered in adequate amounts confer a health benefit on the host"; hence, their health effects lead to improve or restore gut microbiota. These microorganisms are majorly bacteria of the *Lactobacillus* spp. and *Bifidobacterium* spp. (Verschuere *et al.*, 2000). Recently, these microorganisms and their metabolites (e.g., bacteriocins) have broadly been used in food, pharmaceutical and medical industries due to safety (non-pathogenicity and antibiotic resistance), technological and functional (survival and viability during storage, persistence in gut tract and anti-inflammatory, anti-mutagenic and immunomodulation effects) characteristics (Hashempour-Baltork *et al.*, 2019; Khezri *et al.*, 2016, 2018; Verschuere *et al.*, 2000). In addition, these microorganisms can be used to detoxify various compounds (mycotoxins, heavy metals and bacterial toxins) (Lam *et al.*, 2016; Shetty and Jespersen, 2006; Zoghi *et al.*, 2014). Therefore, the aim of this study is to review studies on inhibition of *C. botulinum* by probiotics bacteria and their metabolites.

Probiotic Bacteria

Probiotic means "for life" from the Greek "pro bios." Probiotics were famous by the Greeks and Romans with cheese and fermented dairy products (Soccol *et al.*, 2010). Moreover, prebiotics are usually known as non-digestible food compounds (e.g., fibers, oligosaccharides, chicory root, garlic, leek, onion and banana) that are selectively used by gut microbiota for fermentation (Han *et al.*, 2015). These compounds stimulate growth or activity of beneficial microorganisms. Furthermore, these bacteria linked to lucrative health after effects can specifically be targeted. Based on the literatures, prebiotics can alter gastrointestinal microbiota. However, it is not quite clear that how changes occur in the microbiota composition and performance by prebiotics, how stable these changes are and how these changes affect human health. Therefore, these characteristics need further investigations (Bäckhed *et al.*, 2012). Nowadays, preparation of probiotics is chiefly based on lactic acid bacteria (LAB) (e.g., lactobacilli, streptococci and bifidobacteria) (Marhamatizadeh and Goosheh, 2016), which produce lactic acids by fermentation and metabolism of carbohydrates (Batra *et al.*, 2019; Gezginc and Kara, 2019; Hayta and Ertop, 2019). Acceptable level of bacteria in probiotic products is up to 10^7 CFU/g (Corcoran *et al.*, 2006). However, the human gut must contain up to 10^{13}–10^{14} cells to ensure they reach the sufficient number caused by withstanding conditions and stresses (Savage, 1977). The major parts of the human GIT include their own distinct microbiota (Dethlefsen *et al.*, 2006; Mills *et al.*, 2011). Aerobic Gram-positive bacteria mostly inhabit stomach (<10^3 CFU/g). *Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Streptococcus* genera inhabit small intestine (10^3–10^4 CFU/g), and *Bacteroides*, *Fusobacterium*, *Lactobacillus*, *Bifidobacterium* and *Eubacterium* genera inhabit large intestine at large numbers (10^{11}–10^{12} CFU/g). The most popular probiotic microorganisms with claimed health benefits for humans and animals are represented in Figure 1 (Bintsis, 2018; Fijan, 2014; Kechagia *et al.*, 2013; Soccol *et al.*, 2010). These microorganisms have been isolated from various sources such as plants, fermented meat and dairy products, pickled fruits and vegetables, beverages, soy sauce, fish products and fermented cereal products.

Recently, researchers have shown that probiotic microorganisms are valuable in prohibition and treatment of various diseases and disorders. Nowadays, use of probiotics has increased intensely by growing awareness of the useful effects of these microorganisms and how these strains act in specific conditions (Gioacchini *et al.*, 2011). It is noteworthy that these definitions are consistent with the definitions provided by WHO (Gioacchini *et al.*, 2011; Postollec *et al.*, 2011). Literatures on probiotics such as *Lactobacillus* usually concentrate on the interactions of

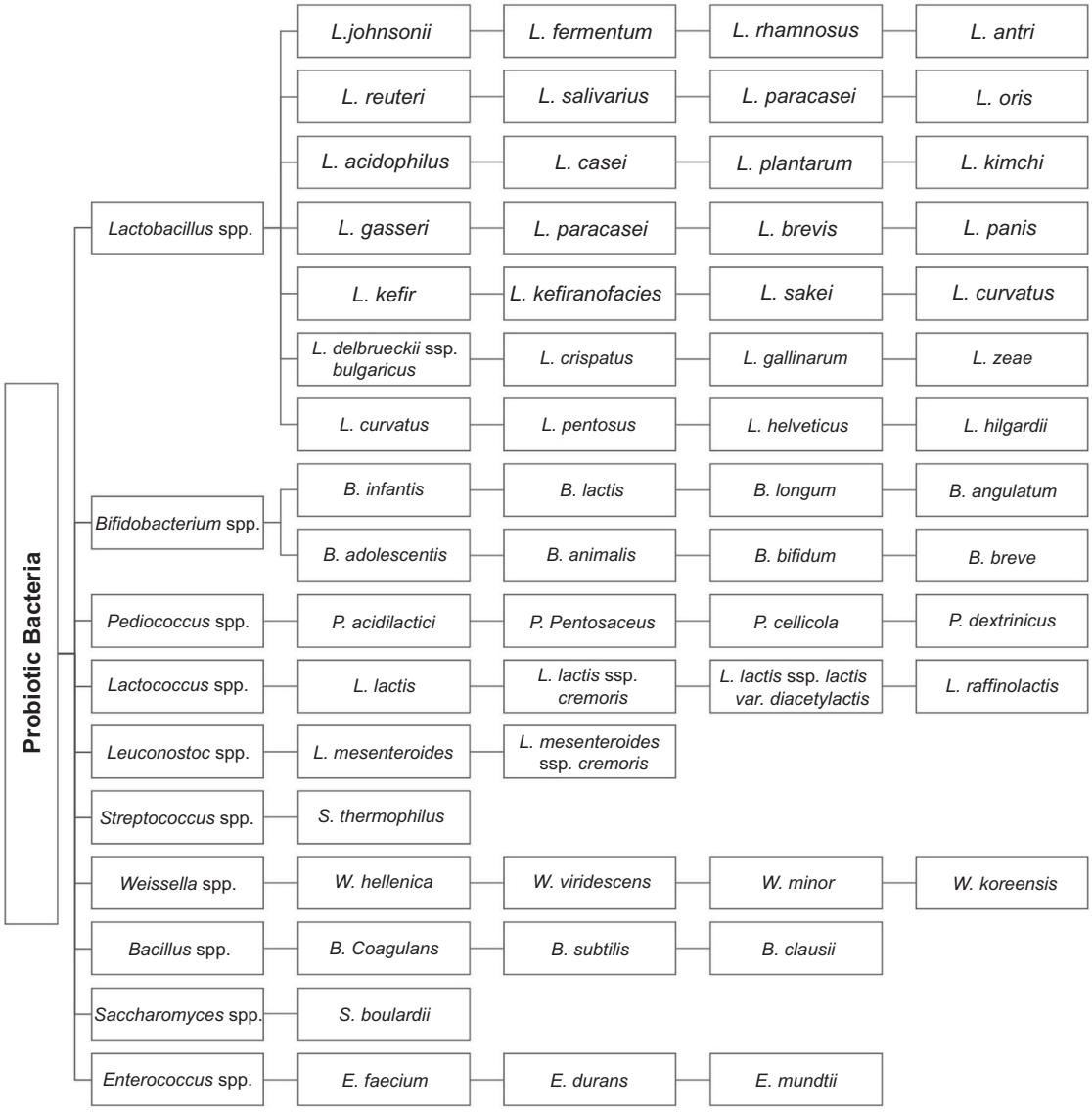

Figure 1. Probiotic microorganisms with claimed health benefits for humans and animals.

these bacteria with immune system (Durlu-Özkaya and Özkaya, 2011) and their effects as anticancer and bio-therapeutic agents. Probiotics include great potentials in treatment of disorders such as *Helicobacter pylori* infection and irritable bowel syndrome, as well as boosting immune systems of healthy individuals (Chapman *et al.*, 2011; Dang *et al.*, 2014; Moayyedi *et al.*, 2010). The LAB such as bifidobacteria include good abilities of removing heavy metals (Bhakta *et al.*, 2012), cyanotoxins (Oelschlaeger, 2010) and mycotoxins from aqueous solutions (Dalié *et al.*, 2010).

Action mechanisms of probiotic bacteria

Probiotics may provide their beneficial health effects in three modes (Markowiak and Slizewska, 2017): (i) adjustment of the host defense system, including inherent and acquired immune systems, (ii) direct or indirect effects on other microorganisms, pathogens and commensals, and (iii) effects on metabolites of microorganisms such as toxins and on host products. Deactivating toxins and detoxifying host products and other food compounds in GIT may be carried out by various activities. Probiotics may use a dual effect for this purpose, preventing or decreasing colonization of pathogen microorganisms in the intestines (Hemarajata and Versalovic, 2013) or interacting with the gut-associated lymphoid tissues to inhibit inflammatory responses and reinforce their own tolerance to foods (Belkaid and Hand, 2014). Overall, major probiotic mechanisms of action include development of the epithelial barrier, enhancement of adhesion to intestinal mucosa and concomitant inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms, production of anti-microorganism substances

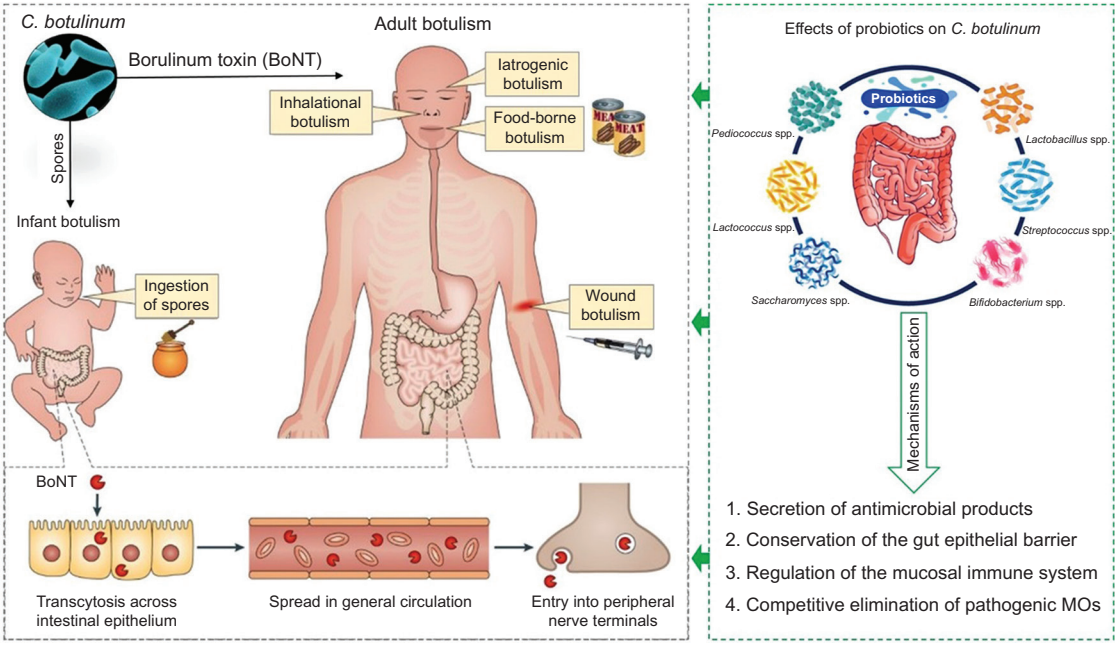

Figure 2. Mechanism action of probiotics bacteria in inhibition of *Clostridium botulinum*.

and modulation of the immune system (Plaza-Diaz *et al.*, 2019) (Figure 2).

Bacteriocins produced by probiotic bacteria

Bacteriocins or probiotic metabolites are classified into four major groups based on their molecular mass, thermo-stability, enzymatic sensitivity, modified amino acids and mechanism of action (Chugh and Kamal-Eldin, 2020). As presented in Table 1, several bacteriocins are described, which can be achieved by probiotic bacteria. Nisin, one of the most important bacteriocins, is the prototype lantibiotic (an amphipathic antibiotic peptide) from *Lactococcus lactis* and *Streptococcus lactis* (Figure 3a). As shown in Figure 3b, two inhibition or killing mechanisms of nisin are usually seen in bacterial cells. Nisin can bind to lipid II (located in the cell membrane and plays a fundamental role in cell wall synthesis), causing pore formation. The second mechanism is interfering and preventing the production of cell wall (Breukink and de Kruijff, 2006; Raybaudi-Massilia *et al.*, 2009; Zhou *et al.*, 2015).

Clostridium botulinum and Its Neurotoxins

Clostridium spp., anaerobic spore-forming bacteria, are widely found in nature, intestines of humans and animals and foodstuffs, especially fresh meats, drinks, milks and canned foods (Fooda, 2018). The *C. botulinum* spores are often found on the surfaces of fruits and vegetables and in seafood. The bacteria grow best under low-oxygen conditions, producing spores and toxins. The toxin is most commonly formed when foods are improperly processed (canned) at homes. These bacteria produce spores and are very resistant to heat. Of the various bacterial species, *C. botulinum* causes serious food poisoning disorders associated with meats, fishes and vegetables (Fooda, 2018). The *C. botulinum* strains are categorized into four major groups, depending on their toxin types and proteolytic abilities (Table 2). Group I consists of Type A and Type B proteolytic strains and is characterized as highly proteolytic, and Group II are non-proteolytic Type B and all Type E strains (Nadjafi and Hamzeh, 2017). The optimum and minimum temperature of growth for proteolytic strains include 37 and 10 °C, respectively. Sodium chloride and nitrite at refrigeration temperatures synergistically affect *C. botulinum* spores, especially in cured meats (Alahakoon *et al.*, 2015). The neurotoxin blocks releasing acetylcholine from the motor nerve endings, resulting in flaccid paralysis in humans and animals (Rossetto *et al.*, 2014). The *C. botulinum* causes three major infections in humans, including foodborne botulism, infant botulism and wound botulism. The toxin is usually demolished by heating (80 °C/20 min or 85 °C/5 min). In other hand, in a recent study conducted by Guo *et al.* (2020), they summarized the researches involved in benefits and potential risks of *Clostridium* species to our health, to develop *Clostridium* species as novel probiotics for human health and animal production. Up to now, *Clostridium* species have been reported to attenuate inflammation and allergic diseases effectively owing to their distinctive biological activities. Their cellular components and metabolites, such as butyrate, secondary bile acids and indolepropionic acid, play a probiotic role primarily through energizing

Table 1. Some important bacteriocins produced by probiotic bacteria

Producing Organism	Bacteriocins	References
Lactobacillus curvatus	Curvalicin a; Curvalicin b; Curvalicin c; Curvaticin FS47; Curvacin-A; Curvaticin L442	Ghalfi *et al.*, 2010; Hammami *et al.*, 2010; Nishant *et al.*, 2011
Lactobacillus acidophilus	Acidocin J1132 β; Acidocin B (AcdB); Acidocin 8912; Acidocin A	Gillor *et al.*, 2008; Hammami *et al.*, 2010; Nishant *et al.*, 2011
Lactococcus lactis	Bacteriocin J46; Nisin Q; Nisin F; Lacticin Z; Lacticin Q	Hammami *et al.*, 2010; Nishant *et al.*, 2011
Lactococcus lactis subsp (*Streptococcus lactis*)	Lacticin 481 (Lactococcin DR); Nisin A; Lactococcin MMFII; Lacticin 3147 A2; Lacticin 3147 A1; Nisin Z; Lactococcin-G β; Lactococcin-G α; Lactococcin 972; LsbB	Hammami *et al.*, 2010; Nishant *et al.*, 2011
Lactococcus lactis subsp (*Streptococcus cremoris*)	Lactococcin-B; Lactococcin-A	Hammami *et al.*, 2010; Nishant *et al.*, 2011
Lactobacillus paracasei	Lactocin-705	Hammami *et al.*, 2010; Nishant *et al.*, 2011
Lactobacillus plantarum	Plantaricin S β; Plantaricin C19; Plantaricin W α; Plantaricin 1.25 β; Plantaricin 163; Plantaricin-A; Plantaricin J; Plantaricin S α; Plantaricin K; Plantaricin E; Plantaricin F; Plantaricin NC8 α; Plantaricin NC8 β; Plantaricin 423; Plantaricin W β; Plantaricin ASM1; Glycocin F	Hammami *et al.*, 2010; Nishant *et al.*, 2011
Lactobacillus sakei	Lactocin-S; Bavaricin-A; Sakacin-A; Sakacin-P (Sakacin 674); Bavaricin-MN; Sakacin G	Hammami *et al.*, 2010; Nishant *et al.*, 2011
Lactobacillus amylovorus	Lactobin-A (Amylovorin-L471)	Hammami *et al.*, 2010; Nishant *et al.*, 2011
Lactobacillus johnsonii	Lactacin-F (lafA); Lactacin-F (lafX)	Gillor *et al.*, 2008; Nishant *et al.*, 2011
Lactobacillus reuteri	Reutericin	Hammami *et al.*, 2010; Nishant *et al.*, 2011
Lactobacillus salivarius	Bactofencin A; Bacteriocin L-1077	Gillor *et al.*, 2008; Nishant *et al.*, 2011
Lactobacillus rhamnosus	Rhamnosin A	Hammami *et al.*, 2010
Leuconostoc mesenteroides	Leucocin-B; Mesentericin Y105; Leucocin C; Leucocyclicin Q	Hammami *et al.*, 2010; Nishant *et al.*, 2011
Pediococcus acidilactici	Pediocin PA-1 (Pediocin ACH)	Gillor *et al.*, 2008; Hammami *et al.*, 2010
Weissella cibaria	Weissellicin 110	Hammami *et al.*, 2010
Escherichia coli	Microcin J25; Microcin B17 (MccB17); Microcin H47; Colicin-V (Microcin-V); Colicin-E1; Colicin-10; Colicin-N; Colicin-M; Colicin-Ia; Colicin-Ib; Microcin-24; Microcin C7	Gillor *et al.*, 2008; Hammami *et al.*, 2010
Enterococcus mundtii	Mundticin; Mundticin KS; Enterocin CRL35 (Mundticin KS); Mundticin L	Hammami *et al.*, 2010
Enterococcus faecium	Enterocin Q; Enterocin P; Enterocin 7A (Enterocin L50A); Enterocin A; Enterocin B; Bacteriocin E50-52; Enterocin HF; Enterocin Xalpha; Enterocin Xbeta; Enterocin K1; Bacteriocin T8	Gillor *et al.*, 2008; Hammami *et al.*, 2010
Bacillus subtilis	Subpeptin JM4-B; Subtilin; Subtilosin-A; Sublancin 168; Subtilosin; LCI	Gillor *et al.*, 2008; Hammami *et al.*, 2010
Bacillus cereus	Thiocillin (Micrococcin P1) (Micrococcin P2) (Thiocillin I) (Thiocillin II) (Thiocillin III) (Thiocillin IV) (Antibiotic YM-266183) (Antibiotic YM-266184); Cerein 7B	Gillor *et al.*, 2008; Hammami *et al.*, 2010

intestinal epithelial cells, strengthening intestinal barrier and interacting with immune system. In turn, our diets and physical state of body can shape unique pattern of *Clostridium* species in gut. In view of their salutary performances, *Clostridium* species have a huge potential as probiotics. However, there are still some nonnegligible risks and challenges in approaching application of them (Guo *et al*., 2020).

Effects of Probiotic Bacteria on *Clostridium botulinum*

There are at least 7–8 various serotypes of *C. botulinum* neurotoxins; from which, four serotypes of A, B, E and F cause botulism in humans (Gonzalez-Escalona *et al.*, 2014). The neurotoxins of *C. botulinum* are very dangerous for humans with the oral and parenteral and lethal

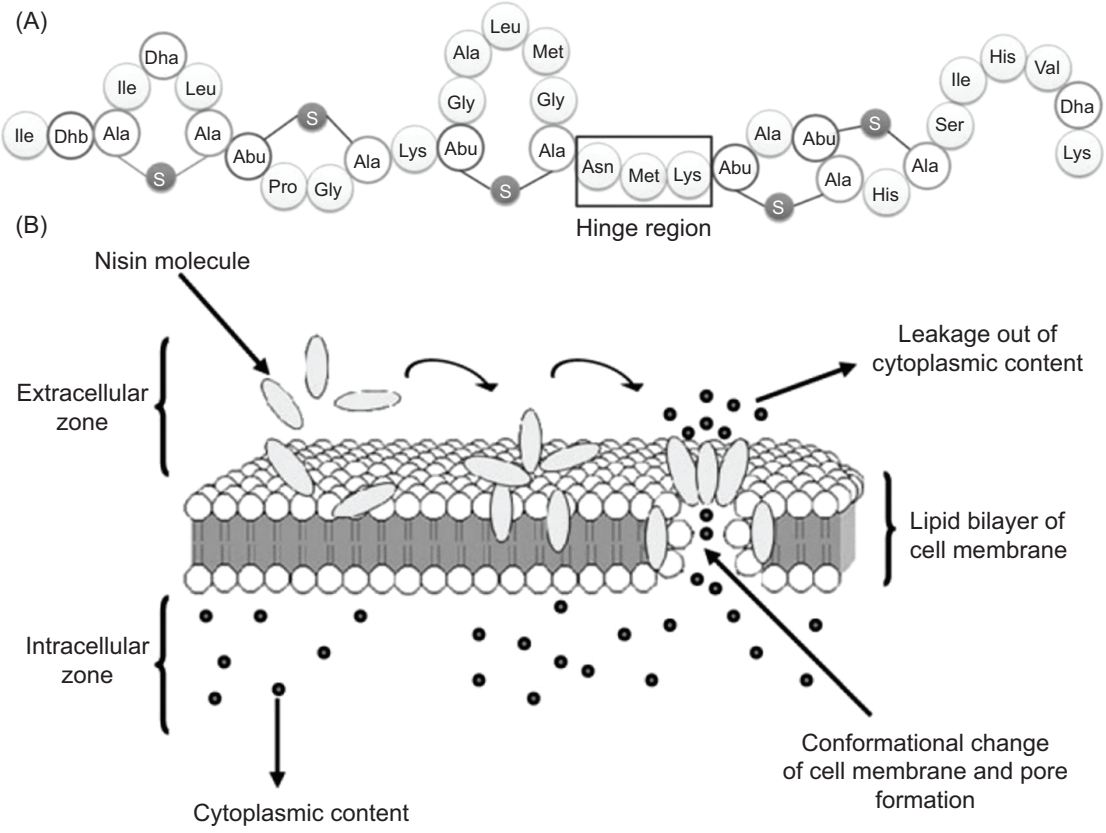

Figure 3. (A) Structure of nisin A. Dhb, dehydrobutyrine; Dha, dehydroalanine; Abu-S-Ala, β-methyllanthionine; Ala-S-Ala, lanthionine; Hinge region (Asn-Met-Lys) (42). (B) Mechanism of action of nisin in the cell of bacteria (45).

(other than intestinal canal) doses of 0.1–1 ng/kg and 1000 ng/kg, respectively. Because of the toxin lethality, there is a considerable economic burden linked to the management of intoxication (Rummel, 2015; Tighe and Schiavo, 2013). The first function in *botulinum* neurotoxin defection and foodborne illnesses is surviving in GIT. Then, toxin should bind and insert to the intestinal epithelia to spread into the bloodstream. The probiotic mechanisms against pathogens include the following: (i) conservation of the gut–epithelial barrier, (ii) competitive elimination of pathogenic microorganisms, (iii) emission of antimicrobial metabolites and (iv) regulation of the mucosal immune system (Oelschlaeger, 2010; Salminen *et al.*, 2010).

Effects of Bacteriocins on *Clostridium botulinum*

Bacteriocins include bacterial peptide toxins, which inhibit growth of similar or closely linked bacterial species. They vary structurally, functionally and biologically (Han *et al.*, 2015). Activity of bacteriocins depends on the properties of food systems (Noordiana *et al.*, 2013). Generally, pH less than 6.0 in low-fat and low-protein foods include the most effects on nisin (Chikindas *et al.*, 2018). Use of bacteriocins as pathogen prevention agents is particularly interesting in components such as processed refrigerated meats. The *C. botulinum* is a great problem in minimally processed refrigerated meats because of its heat-resistant spores, which lead to production of toxins in foods at inappropriate temperatures (Bonsaglia *et al.*, 2014; Kasalica *et al.*, 2011). Several LAB include excellent abilities to produce bacteriocins for the inhibition of *Listeria monocytogenes*, *C. botulinum* and a wide range of foodborne pathogens (Gálvez *et al.*, 2010; Reis *et al.*, 2012). Indeed, *C. botulinum* is a nisin-resistant clostridial species (Zhou *et al.*, 2014). In a relevant study, 23 LAB strains were assessed for bacteriocin-like activity against Types A and B spores from *C. botulinum* strains (Cizeikiene *et al.*, 2013). Overall, *Pediococcus pentosaceus* ATCC 43200, *P. pentosaceus* ATCC 43201, *L. lactic subsp. lactic* ATCC 11454, *L. acidophilus* N2, *L. plantarum* Lb75, *L. plantarum* Lb592 and *L. plantarum* BN demonstrated bacteriocin-like prevention to all *C. botulinum* strains. Based on the minimum inhibitory concentration (MIC) assay, *P. pentosaceus* 43200 included the maximum inhibitory effects on *C. botulinum* (Dobson *et al.*, 2012). Nisin is one of the most common bacteriocins, showing wide effects against Gram-positive bacteria

Table 2. Abstract of *C. botulinum* strains and their toxins (Jay *et al.*, 2005)

Property	A	B	B	E	F	F	G
Year discovered	1904	1896	1960	1936	1960	1965	1969
P or NP	P	P	NP	NP	P	NP	P (weak)
Group	I	I	II	II	I	II	IV
Primary habitat	Terrestrial	Terrestrial	Aquatic	Aquatic	Aquatic	Aquatic	Terrestrial
Minimum growth temp. (°C)	~10	~10	3.3	3.3	~10	3.3	~12
Maximum growth temp. (°C)	~50	~50	~45	~45	~50	~45	n.d.
Minimum pH for growth	4.7	4.7	4.7	4.8	4.8	4.8	4.8
Minimum aw for growth	0.94	0.94	~0.97	~0.97	0.94?	~0.97	n.d.
Thermal D values for endospores (°C)	D110 = 2.72–2.89	D110 = 1.34–1.37	n.d.	D80 = 0.80	D110 = 1.45–1.82	D82.2 = 0.25–0.84	D110 = 0.45–0.54
Radiation D values of spores (kGy)	1.2–1.5	1.1–1.3	n.d.	1.2	1.1; 2.5	1.5	n.d.
Maximum NaCl for growth (%)	~10	~10	5–6	5–6	8–10	5–6	n.d.
Relative frequency of food outbreaks	High	High	n.d.	High (seafood)	1 outbreak	1 outbreak	None
H_2S production	+	+	–	–	+	–	++
Casein hydrolysis	+	+	–	–	+	–	+
Lipase production	+	+	+	+	+	+	–
Glucose fermentation	+	+	+	+	+	+	–
Mannose fermentation	–	–	+	+	–	+	–
Propionic acid produced	+	+	n.d.	n.d.	+	n.d.	n.d.

Note: P= proteolytic; NP= non-proteolytic; + = positive; ++ = strongly positive; – = negative; n.d. = no data.

(Fernández-Pérez *et al.*, 2018). Nisin is "generally recognized as safe" (GRAS) to prevent *C. botulinum* spores. Synergistic effects of nisin and heating on *C. botulinum* have previously been demonstrated by Gao and Ju (2008). Literatures have described that various types of *C. botulinum* strains include various resistances to nisin; however, results may be controversial (Chung and Yousef, 2007). Furthermore, growth condition and food components affect nisin efficiency. Low-acid environments, short heat-shocking periods, high spore loads, high protein and phospholipid concentrations and increased incubation temperatures can decrease nisin ability to inhibit *C. botulinum* growth (Gharsallaoui *et al.*, 2016).

Conclusion

Considering the importance of pathogenicity and the mortality rate of *C. botulinum*, control of the bacterial growth and its neurotoxin production is critically important due to food safety aspects. Probiotics and their bacteriocins, as biological control agents, play significant roles in detoxification and decrease of the risk by these pathogens. The antagonism between *C. botulinum* and bacterial members of the ecosystems is well known. Reports have revealed that use of probiotics and their metabolites can help inhibit *C. botulinum* colonization and decrease its neurotoxin production. Proteolytic and non-proteolytic types of *C. botulinum* show similar reactions to inhibitory effects of probiotics in foodstuffs. However, matrices of the foods include great effects on action mechanisms of nisin. The major affecting factors on potency of nisin to prevent *C. botulinum* growth include pH, heat-shocking periods, spore loads, protein and phospholipid contents and environmental temperatures.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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