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Application of *Bacillus sp.* as a biopreservative for food preservation

*Short Title:**Bacillus sp.* **in Biopreservation of Food**

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Abstract:
Food preservation is enhancing shelf-life and food quality to eliminate food-related illness and product spoilage, especially by the use of food additives.The growing consumer demand for effective preservation of food without altering its nutritional quality and free of potential health risks andto find an attractive and alternative approach to chemical preservatives, have stimulated research in the field of biopreservation by the use of natural or controlled microbiota and/or their antimicrobial compounds including very recent innovation: *Bacillus sp.,* the ubiquitous, Gram positive bacteria, producing inhibitory substances like cyclic peptides and bacteriocins, with a broad antimicrobial spectrum and a history of safe use in food. *Bacillus* spores are also being used extensively as probiotic food supplements where they are used in human as dietary supplements and in feed for livestock and aquaculture as growth promoters.A novel concept multi-target food preservation has emerged in relation to hurdle technology stating the microbial safety, stability, sensorial and nutritional qualities of foods are based on the application of combined preservative factors (called hurdles including *Bacillus sp.*) that microorganisms present in the food are unable to overcome, thus leading to inhibition of microbial growth by disturbing their homeostasis and metabolic exhaustion and avoiding tress reaction by bacteria. Future exploration of the natural preservatives and/or their metabolites, in combination with advanced technologies could result in replacement of chemical preservatives, or could allow less severe processing (e.g. heat) treatments, while still maintaining adequate microbiological safety and quality in foods.
Key words: antimicrobial activity, *Bacillus subtilis*, bacteriocin, biopreservative.

I. INTRODUCTION

Food preservationhas long been an on-going challenge for human with the methods like, drying, salting and fermentation being traditionally done for preservation.Methods such ascanning, freezing and irradiation are relatively recent developments and adopted for preservation of food.Today food preservation is viewed as a 'convenience' of an efficient food system as well as a key to ensuring the availability of food as a vital benefit, particularly in developing countries. Food fermentation was developed by default rather than by design.

An important part of food preservation is the addition of chemical adjuncts to foods which started traditionally by uses of spices. Originally this was done to mask unpleasant flavors of spoiled foods; but it became a way of preserving and imparting flavor as well asvariety to foods. With the industrial revolution and the subsequent development of food industries, food processing moved from kitchen or cottage industries to large scale technological operations with increased need for food preservation. This stimulated the use of food additives, especially for food preservation and enhancing food quality. In recent years the addition of chemical preservatives has fallen into disfavor with consumers whoseek foods that are of high quality, less severely processed (less intensive heating and minimal freezing damage), less heavily preserved, more natural (freer from artificial additives) and safer [1], leading to the emergence of a new generation of chill stored, minimally processed foods.

The shift in consumer preference for minimally processed foods led to the increasing consumption of precooked food, prone to temperature abuse thus increasing the likelihood of food-related illness and product spoilage [2]. Therefore, to harmonize consumer demands with the necessary safety standards, traditional means of controlling microbial spoilage and safety hazards in foods are being replaced by the use of biological antimicrobial compounds, either alone or in combination with mild physicochemical treatments and low concentrations of traditional and natural chemical preservatives, which may be an efficient way of extending shelf life and food safety through the inhibition of spoilage and pathogenic bacteria without altering the nutritional quality of raw materials and food products[3]. Hence, the last two decades have seen intensive investigation on the use of non-pathogenic microorganisms and/or their metabolites to improve microbiological safety and extend the shelf life of foods, which otherwise is defined as biopreservation[4]. One of the most common and traditional form of food biopreservation is fermentation, a process based on the growth of microorganisms in foods, either natural or added. It

employs the breakdown of complex compounds, production of acids and alcohols, synthesis of Vitamin-B12, riboflavin and Vitamin-C precursor, ensures antifungal activity and improvement of organoleptic qualities such as, production of flavor and aroma compounds. Research on biopreservation concentrates on identification and development of protective bacterial cultures with antimicrobial effects against known pathogens and spoilage organisms including *Salmonella*, *Campylobacter jejuni*and *Escherichia coli0157:H7*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium botulinum*. Several compounds such as organic acids, bacteriocins, diacetyl and acetaldehyde, enzymes, CO_2, hydrogen peroxide etc. contributes to antimicrobial activity by microbiota. Among these bacteriocin has received significant attention among the researchers.

Bacteriocins are ribosomally synthesized heterologous group of peptides or complex proteins showing antimicrobial action against other bacteria, principally closely related species [5, 6]. They are normally not termed antibiotics in order to avoid confusion and concern with therapeutic antibiotics, which can potentially illicit allergic reactions in humans and other medical problems [7]. Bacteriocins areproteinaceous agents that are rapidly digested by proteases in the human digestive tract leading to their safe use as natural preservatives in foods [8]. Since, bacteriocins are ribosomally synthesized; there exists a possibility of improving their characteristics to enhance their intensity and spectra of action [8].

Bacteriocin production could be considered as an advantage for food and feed producers since, in sufficient amounts, these peptides can kill or inhibit pathogenic bacteria that compete for the same ecological niche or nutrient pool. This role is supported by the fact that many bacteriocins have a narrow host range, and is likely to be most effective against related bacteria with nutritive demands for the same scarce resources [9].

For these reasons, the use of bacteriocins has, in recent years, attracted considerable interest for use as biopreservatives in food, which has led to the discovery of an ever-increasing potential of these peptides.

Although most research has focused on antimicrobial agents produced by lactic acid bacteria, many bacteriocins of other Gram positive bacteria have been isolated and documented [10, 11]. One area of interest in the use of these bacteriocins is to control the growth of undesirable microorganisms, particularly those of public health concern, e.g., *C. botulinum* and *L. monocytogenes*.

The capacity to produce antimicrobial peptides specifically bacteriocin is widely spread among a variety of Gram-positive bacteria, including *Staphylococcus*, *Clostridium*, and *Bacillus sp*. These substances are directed against competitive microorganisms and thereby generate a selective advantage for their producers [12]. Although the use of these bacteriocins may be precluded from foods because the producer strain may be a pathogen, recent developments in genetic engineering techniques have made the transfer of genes encoding for bacteriocin production from bath Gram positive and Gram negative bacteria to food grade microorganisms possible [13].

II. BENEFICIAL ROLE OF *BACILLUSSP*.

Bacillus species are ubiquitous distribution in the environment and subsequently found as commensal, transient organisms in the gastrointestinal systems of mammals, insects, invertebrates, birds, marine life and even reptiles as well as soil, clays, rocks, dust, aquatic environments, vegetation and even food [14]. Members of the genus *Bacillus* are rod-shaped, Gram-positive, aerobic, endospore-forming bacteria that are characterized by catalase production. *Bacillus* species are phenotypically and genotypicallyheterogenous [15, 16]and consequently, they exhibit quite diverse physiological properties such as the ability to degrade many different substrates derived from plant and animal sources, including cellulose, starch, proteins, agar, hydrocarbons and also biofuels [17]. Furthermore, a number of instances reveal that, some *Bacillus* are heterotrophic nitrifiers, denitrifiers, nitrogen fixers, iron precipitators, selenium oxidizers, oxidizers and reducers of manganese, facultative chemolithotrophs, acidophiles, alkalophiles, psychrophilesand thermophiles [12]. Their ability to survive and grow in different ecosystems by colonizing a wide variety of ecological habitats is based on the production of endospores, diversity in physiological properties and growth requirements.

Observing the nature of the*Bacillus sp.*as a ubiquitous, commensal and transient organisms, the researchers demonstrated that *B. subtilis* was able to survive in space for six years despite the harsh radiation, vacuum, temperatures and other conditions that typically do not support life [18].

B. subtilis have functioned as probiotics since life spawned on this earth and certainly throughout the time that humans evolved. A true probiotic should be formulated with nature's original probiotic strains, *Bacillus*, to support the trillions of beneficial bacteria passed down from mother to child.

B. subtilis plays a key role in mitigating a systemic pro-inflammatory and autoimmune state. It helps in proper digestion and assimilation of food in human and detoxification of the gastrointestinal system. Moreover it produces plenty of the key nutrients (i.e. vitamins, enzymes, carotenoids, lipids, etc.) especially at the sites of absorption.

It was popular worldwide before the introduction of consumer antibiotics, as an immune-stimulatory agent to aid treatment of gastrointestinal and urinary tract diseases. It itself produces more than 24 different antibiotics in vivo that help gastro-intestine from invading foreign species and even over-growth of our own bacteria. It is still widely used in Western Europe and the Middle East as an alternative medicine [19].

USFDA (United States Food and Drug Administration) certified the carbohydrase (amylase) and protease enzymes produced by *B. subtilis*, as GRAS in 1960. According to them, the nontoxigenic and nonpathogenic strains of *B. subtilis* can be safely used in a variety of food applications.

FOSHU (Foods for Specified Health Use), Ministry of Health, Labour and Welfare, Japan approved the Japanese fermented soy bean, *natto* in combination with *B. subtilis*, whichhas been successfully designed for the safety, maintenance and improvement of health by incorporating them into one's diet. Korean food *cheonggukjang* is a very good source of *B. subtilis.*

In context of Veterinary Medicine, the Association of American Feed Control approved *B. subtilis* as a feed ingredient for direct-fed microbial products. The Canadian Food Inspection Agency Animal Health and Production Feed Section has classified *Bacillus* culture dehydrated approved feed ingredients as a silage additive.

Bacillus spores are also being used extensively as probiotic food supplements where they are used in human as dietary supplements and in feed for livestock and aquaculture as growth promoters and competitive exclusion agents. European Food Safety Authority (EFSA, 2008) declared *B. subtilis* as QPS (qualified presumption of safety) status [20], which is further is modified to 'absence of food poisoning toxins, absence of surfactant activities,absence of enterotoxic activities'[12].

Bacillus species are an important source of fine biochemical (such as heterologous proteins, different enzymes like proteases, amylases, rennet substitutes, endonucleases, glucose-dehydrogenase and pullulanase), antibiotics and insecticides. For example, *B. subtilis* produces nucleotides (sold as food flavour enhancers), amino acids (such as tryptophan, histidine and phenylalanine) and vitamins such as biotin, folic acid and riboflavin; *B. thuringensis* produces the proteinaceous metabolites δ-endotoxins etc.[21].

III. *BACILLUS* AS BIOPRESERVATIVE

A diverse array of antimicrobial peptides with several different basic chemical structures, have been produced by different strains of the genus *Bacillus* [22, 23], which are reflected by several reports describing the production, isolation and characterization of these antimicrobial compounds including bacteriocins from these species [24, 25, 26]. Some of these peptides can play a role in competence and in the de-repression of various stationary-phase genes involved in sporulation [21]. Recent studies have revealed that*Bacillus subtilis* can suppress infection against *Escherichia coli* 070:K80, *Salmonella enterica,Chlostrodiumperfringens* and *Citrobacterrodentium*[20]. The growth of two viable food pathogens viz. *L. monocytogenes* and *S. aureus* can be suppressed by a newly isolated bacteriocin from *Bacillus mycoides*[27]. Similarly, paenibacillin (bacteriocin from *Paenibacillus*sp.), was found to be active against many bacteria including *Bacillus* sp., *Clostridium sporogenes*, *Lactobacillus* sp., *Listeria* sp. and *S. aureus* [28]. This proves that bacteriocins secreted from different bacteria behave differently and have their specific inhibition spectra [29]. Many bacteriocins produced by *Bacilli* inhibit Gram-positive bacteria but not Gram-negative bacteria. These include entomocin 9, a bacteriocin produced by *B. thuringiensissp. entomocidus* HD9 [30] and lactosporin, an antimicrobial protein produced by *B. coagulans* ATCC 7050 [31]. On the contrary, a bacteriocin produced by *B. licheniformis*MKU3 did not inhibit *Listeria sp.* but inhibited a Gram negative bacterium, *Escherichia coli*, together with many Gram-positive species [32].

Kindoli et al., [33]reported that, the *B. subtilis* W42, *B. subtilis* SKE 12, *B. subtilis* K21, and *B. subtilis* H27 inhibited most indicators such as some LAB, *B. thuringiensis*, *S. carnosum*, *Enterococcus faecalis* (ATCC 29212)and *S. epidermis* including *B. cereus* and *L.monocytogenes*, the two most important food pathogens, but not Gram-negative bacteria. *B. subtilis* W42 was the most inhibiting as it inhibited 12 out of 20 indicators tested.Sharma et al., [29]claimed that, the culture supernatant of *Bacillus subtilis*R75 expressed strong inhibition against many microorganisms/pathogens that cause serious food spoilage, *viz. L. monocytogenes, L. plantarum, S. aureus, B. subtilis, C. perfringens, B. cereus, E. coli* and *L. mesenteroides* and wide zones of clearance made by crude bacteriocin, ranging up to 5 mm, were observed on the petri dishes, containing indicator bacteria in well diffusion assay. Bacteriocin from *Bacillus subtilis*R75 has been found capable of controlling Listeriosis, caused by *L. monocytogens*in the food items stored even at low temperature in the refrigerator, leading to many deaths throughout the world and thus can meet the serious challenge of controlling the spoilage of refrigerated food. Moreover, this bacteriocin is presumed completely safe for human consumption because of its origin from a food grade bacterium.

The findings of Hammami et al., [34, 35] revealed that, the supernatant of *B. subtilis* 14B showed positive inhibition against different

pathogenic populations such as, *Micrococcus luteus* LB 14110, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 4464, *Bacillus licheniformis*, *Escherichia coli* ATCC 8739, *Pseudomonas savastanoi*pv. *savastanoi*, *Pseudomonas syringae*pv. s*yringae*, *Pseudomonas aeruginosa* CIP 82.118, *Erwiniacarotovora* subsp. *carotovora* , *Salmonella typhimurium*, *Agrobacterium sp*. Biovar1, *Ag. rhizogenes*, *Ag. vitis*, *Ag. rubi*, *Ag. tumefaciens* C58 and an *Agrobacterium* strain isolated from a weeping fig tree. A weaker but still significant activity was obtained with the cell-free supernatant.

Kim et al., [36] claimed that, *Bacillus subtilis*GB-0365 found to inhibit *Botrytis cineria*, *Fusarium*sp.,*Pythium*sp., and *R. solani* and *Bacillus* sp. GB-017 showed inhibition against*Pythium*sp., *Botrytis cineria*, *Fusarium*sp. and *R. solani*.Luis-Villaseñor et al.,[37]reported that, two isolates YC5-2 (*Bacillus tequilensis*) and YC2-a (*Bacillus amyloliquefaciens*) inhibited growth of *Vibrio campbelli* (CAIM 333) and *V. vulnificus* (CAIM 157) with inhibition halos of 5–18 mm diameter, whereas, YC5-2 (*Bacillus tequilensis*), YC2-a (*Bacillus amyloliquefaciens*), YC3-b (*Bacillus endophyticus*), and C2-2 (*Bacillus endophyticus*) were able to inhibit *V. parahaemolyticus*(CAIM 170) and *V. harveyi* (CAIM 1793), with inhibition halos of 11–17.5-mm diameter. *V. alginolyticus* (CAIM 57) showed sensitivity but no inhibition to these probiotic strains.

Awaiset al., [38]reported that the two species *Bacillus subtilis*and *Bacillus pumilus*exhibited better activity against gram-positive *Staphylococcus aureus* and *Micrococcus luteus*. Marahielet al.,[39] isolated a strain of *Bacillus subtilis*C126 from sugar cane fermentation, which produced a polypeptide antibiotic, bacitracin, which inhibited the growth of *Micrococcus flavus*. A *Bacillus licheniformis*strain, 189, isolated from a hot spring environment in the Azores, Portugal, was found to strongly inhibit growth of Gram-positive bacteria by producing peptide antibiotic [40].Joseph et al.,[41]used *Micrococcus luteus*as the indicator organism to detect the antimicrobial activity by producing a clear zone of inhibition around the indicator strain.

There is a wide variation in the inhibition spectrum among the different strains of same genus or even between same species. For example, the BLIS (Bacteriocin Like Inhibitory Substances) of *B. amyloliquefaciens* LBM 5006 showed antibacterial spectrum against *L. monocytogenes*, *B. cereus*, *Serratiamarcescens* and *Pasteurellahaemolytica*, [42] whereas, to that of *B.amyloliquefaciens* CECT 5940 was active against *E. coli* and *Clostridium perfringens* [43]. Same inhibitory bacteriocin having similar chemical composition, designated by same nomenclature can be produced by different species; only the name of strain as suffix made them unique. For example, Bacillocin Bb, produced by *B. brevis* Bb strain, exhibited activityagainst *S. aureus*, *Micrococcus luteus*, *Corynebacteriumdiphtheriae*, *C.xerosis* and *C.hoffmanni* [44]. On the other hand, Bacillocin 490, produced by a thermophilic strain of *B. licheniformis* 490/5 showed antibacterial activity against closely related species such as *G. stearothermophilus*, *B.smithii*, *B. subtilis*, *B. anthracis*, *B. cereus* and *B. licheniformis*, both under aerobic and under anaerobic conditions [45]. The BLIS of *B. licheniformis* P40 exhibited a broad spectrum of activity against pathogenic and spoilage bacteria (*L. monocytogenes*, *B. cereus* and clinical isolates of *Streptococcus sp*.) [46].*B. cereus* produced several BLIS or cereins such as, cerein 8A from*B. cereus* 8A inhibiting *L. monocytogenes* and *B. cereus* apparently by disturbing their membrane function [47, 48, 49], cerein GN105 from *B. cereus* GN105 [26] and the relatively heat stable (75°C far 15 min) bacteriocin Gerein, which is, sensitive to proteolytic enzymes.

The probiotic *B. clausii* O/C strain released a pronase-sensitiveantimicrobial substance showing inhibition against *S. aureus*,*Enterococcus faecium* and *Clostridium difficile*. [50]. The BLIS of *B.firmus* H_2O-1 showed antimicrobial activity against *Desulfovibrioalaskensis* and a group of sulfate-reducing bacteria [51]. The BLIS produced by a foodgrade *B. lentus* NG121 strain was active against *L. monocytogens* and *S. aureus* [52]. Megacinsfrom*B. megaterium* showed wide antimicrobial spectra against food-spoilage bacteria such as *Salmonella typhimurium* and *S. aureus* [53, 54]. The BLIS of *B. mycoides* strain showed activity against food-borne pathogens suchas *L. monocytogenes* and *L. mesenteroides* [27]. A commercial probiotic *B. polyfermenticus* strain showed a narrow spectrum of activityagainst Gram-positive bacteria including all *Bacillus* strains as well as*S. aureus*, *C. perfringens* and *Micrococcus flavus* [55]. Pumilicins from *B. pumilus* are active against *E. faecalis* and some other Gram-positive bacteria [56].

B.subtilis strains are known to produce a wide variety of antibacterial and antifungal compounds [23] including the BLIS Betacin [57]. *B.subtilis* ATCC 6633 produces several lantibiotics [58, 59, 60, 61, 62], rhizocticin [63] and twolipopeptides, surfactin and mycosubtilin [64].The BLIS of *B.subtilis* LFB112 showed activity against both Gram-positive and Gram-negativebacteria involved in domestic animal diseases, including*E. coli*, *Salmonella pullorum*, *P. aeruginosa*, *Pasteurellamultocida*, *C. perfringens*, *M. luteus*, *Streptococcus bovis*and*S. aureus* [65].Subtilin, classified as lantibiotics, a cationic peptide produced by *B. subtilis*, having its molecular mass of 3317 Da., whose structure was determined by Gross et al., [66]. The main spoilage microorganisms of low acid

canned products at high temperature, *B. stearothermophilus*, produce the bacteriocin Thermacin [66, 67, 68]. Shafia et al., [68] reported that 12 out of 22 strains of *B. stearothermophilus*, produced an inhibitory substance against species of the same genus. *Bacillus thuringiensis*ssp. *thuringiensis* HD-2 produces thuricin HD2 [69], showed antimicrobial activity against *B. thuringiensis* strains andsome other Gram-positive species such as, *B. cereus*, *B. megaterium*,*P. polymyxa*, *B. sphaericus*, *C. xerosis*, *S. aureus* and*S. epidermidis*.Thermocin 10 [70], Thermoleovorin-S2, thermoleovorin-N9 [71] and Polyxin [72] are some other examples of bacteriocins produced by *G. stearothermophilus*, *G. thermoleovorans*S-II, *G. thermoleovorans* NR-9 and *P. polymyxa* respectively.

Most of the *Bacillus* antimicrobial peptides are the lantibiotics, included in the Class I of the LAB bacteriocin classification scheme. Furthermore, several other bacteriocins/BLIS produced by *Bacillus* species fall within Class II of LAB bacteriocins [73] which includes both the class IIa and class IIb, i.e., the pediocin-like bacteriocins and the two-peptide bacteriocins respectively [12].

Table: 1 Classification of Bacteriocin Produced by *Bacillus* Isolates [12]

A. Class I: Post-translationally modified peptides

Bacteriocin	**Produced by**	**Molecular wt. (kDa)**	**Special feature**
Subclass-I.I Single-peptide, elongated lantibiotics			
Subtilin	*Bacillus subtilis* group *B. subtilis*	3.34	A-lantibiotic, binds lipid II
Subtilin B		3.42	Succinylatedsubtilin, A-lantibiotic, binds lipid II
ericin S		3.44	Lantibiotic; active against Clavibacter
ericin A		2.98	Lantibiotic
Subclass-I.II Other single-peptide lantibiotics			
Sublancin 168	*Bacillus subtilis* group *B. subtilis*	3.88	AII-lantibiotic, unusual lantibiotic
Mersacidin		1.82	Tetracyclic, B-lantibiotic, binds lipid II
Paenibacillin	*Paenibacilluspolymyxa*	2.98	Lantibiotic
Subclass-I.III Two-peptide lantibiotics			
Haloduracin (A1, A2)	*B. halodurans*	3.04 and 2.33	Two-peptide lantibiotic
Lichenicidin (α, β)	*B. licheniformis*	3.25 and 3.02	Two-peptide lantibiotic
Subclass-I.IV Other post-translationally modified peptides			
Subtilosin A	*Bacillus subtilis* group *B. subtilis*	3.39	Macrocyclicantibiotic
Subtilosin A1		3.41	Macrocyclic antibiotic, variant

B. Class II: Nonmodified peptides

Bacteriocin	**Produced by**	**Molecular wt. (kDa)**	**Special feature**
Subclass-II.I Pediocin-like peptides			
Coagulin	*B. coagulans*	4.6	Pediocin-like bacteriocin
SRCAM 37	*Paenibacilluspolymyxa* *B. circulans*	3.5	Pediocin-like bacteriocin; anti-*Campylobacter*
SRCAM 602		3.5	Pediocin-like bacteriocin; anti-*Campylobacter*
SRCAM 1580		3.5	Pediocin-like bacteriocin; anti-*Campylobacter*
Subclass-II.II Thuricin-like peptides			
Thurincin H	*B. thuringiensis*	3.14	Three structural genes, N-terminal DWTXWSXL
Thuricin S		3.14	N-terminal: DWTXWSXL
Thuricin 17		3.16	N-terminal: DWTXWSXL

Bacthuricin F4		3.16	N-terminal: DWTXWSXL
Cerein MRX1	*B.cereus* group, *B. cereus*	3.14	N-terminal: DWTCWSCLVCAACSVELL
Subclass-II.III Other linear peptides			
Cerein 7A	*B. cereus* group *B.cereus*	3.94	-
Cerein 7B		4.89	Sec-independent leader peptide with GG
Lichenin	*B. licheniformis*	1.4	N-terminal: ISLEICXIFHDN
Thuricin 439	*B. thuringiensis*	2.92 and 2.80	two singly active peptides

C. Class III: Large proteins

Bacteriocin	**Produced by**	**Molecular wt. (kDa)**	**Special feature**
Megacin A-216	*B. megaterium*	32.85; 21.02, 11.85 (Three biologically active fractions, the full-length protein and two cleavage products.)	Phospholipase A activity
Megacin A-19213		39	Phospholipase A activity

Bacteriocins produced by industrially important *Bacillus* species have a history of safe use in food industry [74]. Members of the Bacillus group *sensulato* are considered good producers of antimicrobial substances, including peptide and lipopeptide antibiotics, and bacteriocins [62].

One of the most recent findings of the extensive research regarding biopreservatives and theircommercial value as antimicrobial agents for food preservation is *Bacillus subtilis,*used as a starter culture in thefermentation of various oriental and African seasonings and beverages, has been shown to produce inhibitorysubstances, including cyclic peptides and bacteriocins, with a broadantimicrobial spectrum [75, 76]. The biopreservatives, produced by *B. subtilis*during environmental stress, especially nutrient limitation (such as carbon versus nitrogen), are considered to be secondary metabolites and they prevent further cell growth and division [76].

Bacillus sp. and its bacteriocins could be an acceptable alternative to lactic acid bacteria for several reasons:

i. *Bacillus sp*., like lactic acid bacteria, has been used for hundreds of years in making food including the use of *B. subtilis*as a starter culture in the fermentation of various oriental, African seasonings and beverages. Various enzymes from *Bacillus* have been used intensively in food processing worldwide. Bacteriocins from these microorganisms would be safe for human and no more of a risk than lactic acid bacteria.USFDA (United States Food and Drug Administration) certified the carbohydrase (amylase) and protease enzymes produced by B. subtilis, as GRAS in 1960.
ii. *Bacillus sp*. have a broad antimicrobial spectrum against various Gram positive and Gram negative spoilage and pathogenic bacteria in both food processing and health point of view, as well as fungi, and therefore have a greater antimicrobial spectra than lactic acid bacteria and their bacteriocins.
iii. The metabolic diversities of *Bacillus sp*. may result in bacteriocins with various properties such asthermostability, retention of inhibitory activity at wide pH range, stability after treatment with carbohydrolytic enzymes, different surfectants and organic solvent [35]which make them suitable for food processing.
iv. The physiology/genetics of *Bacillus* are well understood as to that of *Escherichia coli*. So, it's feasible to produce bacteriocins safely for the food industry by applying molecular biological techniques like the production of insulin.
v. The resistant properties of *Bacillus* spores raises the possibilities that they can be incorporated in a number of food products such as beverages, chocolates, baked cake and muffins as probiotic additives, whereas this cannot be considered for more common lactobacilli and bifidobacteria probiotics [20].
vi. Bacteriocins from *Bacillus* sp. especially, bacillocin 490 and cerein 8A, have a potential in preservation of different food substrates like in dairy products such as milk and cheeses. Most of them are heat resistant and stable at wide pH range occurring during food processing, parallely can be degraded by proteases. [12]

IV. CONCEPT OF HURDLE TECHNOLOGY IN RELATION TO BIOPRESERVATION

The hurdle concept was introduced by Leistner in 1978[77]and stated that the microbial safety, stability, sensorial and nutritional qualities of foods are based on the application of combined preservative factors (called hurdles) that microorganisms present in the food are unable to overcome[2], thus leading to inhibition of microbial growth by disturbing their homeostasis and metabolic exhaustion and avoiding tress reaction by bacteria. Using an adequate mix of hurdles is not only economically attractive; it also serves to improve not only microbial stability and safety, but also the sensory and nutritional qualities of a food[78].

A novel concept multi-target food preservation has emerged in relation to hurdle technology, based on the proven fact that, at times, different hurdles in food have not just an additive effect on microbial stability, but a synergistic one [79]. In practical terms, this means that it is more effective to employ different small intensive preservation factor than one large intensity preservation factor because the combine use of several preservation factor may produce a synergistic effect [2].

In industrialized countries, hurdle technology is of great interest in the food industry especially forminimally processed foods with low fat contents and/or salt for extending the shelf life [80]. Whereas, in developing countries, most foods are stabilized by empirical application of hurdle technology as mostly theystored without refrigeration. Several traditional foods have already been optimized by the intentional application ofhurdles for safety and stability enhancement [81].The principle hurdles employed in food safety are temperature (higher or lower), water activity (aw), pH, redox potential (Eh), chemical preservatives, vacuum packaging, modified atmosphere, HHP (High Hydrostatic Pressure), UV and competitive flora (*Bacillus* sp. producing antimicrobial compounds) [2].

Several authors have recommended the use of bacteriocins combined with other preservation methods to create a series of hurdles during the manufacturing process to reduce food spoilage by microorganisms. In fact, it has been proven that the application of chemical preservatives, physical treatments (heat), or new mild non-thermal physical methods (pulsed electric field, HHP, vacuum, or modified atmosphere packaging), which increase the permeability of cell membranes, positively affects the activity of many bacteriocins [82, 83]. Notably, combined treatments of bacteriocins with selected hurdles affecting outer-membrane (OM) permeability increase the effectiveness of some bacteriocins against Gram-negative cells, which are generally resistant. The growth of Gram negative pathogens such as E. coli O157:H7 and *Salmonella* can also be controlled when metal chelators, such as EDTA, sodium tripolyphosphate (STPP) or physical methods such as heat and HHP, are used in combination with bacteriocins [84].

V. CONCLUSION

Food is a complex system and variables, such as the chemical nature of the compound, pH of the food, its solubility and interaction with other food components may all influence its antimicrobial activity. One of the most recent findings of the extensive research regarding biopreservatives and their commercial value as antimicrobial agents for food preservation is *Bacillus subtilis*, as a starter culture in the fermentation of various oriental and African seasoningsand beverages such as, soybeans into the traditional West African condiment dawadawa [85] and African mesquite seeds in the production of the Nigerian food condiment okpehe [86].

The high stability of *B. subtilis* in harsh environmental conditions makes this microorganism a perfect candidate for probiotics applications either in baked and pasteurized foods/beverages or in other forms like tablets, capsules, and powder. Bacteriocins from *Bacillus sp*. especially, bacillocin 490 and cerein 8A, have a potential in preservation of different food substrates like in dairy products such as milk and cheeses [12]. Application of bacteriocin-producing strains in these food substrates may offer new opportunities in food biopreservation.

The use of bacteriocin producing starter cultures as ingredients may not require special considerations in many countries like USA provided the microorganism is GRAS. However, if a purified bacteriocin is used as a food preservative, the substance must be approved as GRAS. Further research is required to gain insight into the molecular mechanisms involved in bacteriocin production, immunity and mode of action, which is necessary for safe and effective exploitation of the bacteriocins. Moreover toxicological data and the fate of the molecule after ingestion are also required to establish the GRAS status.The Surfactin-like-compound produced by *B. subtilis*FN2A has the potential to be a novel biepreservative, though its GRAS status is to be checked before application. If fails, this compound has the potential to be used in animal feedstuff where it may control avian botulism caused by *Clostridium* sp. in chicken [76]. Therefore, further research is required for the broad application of the *Bacillus* and its metabolite in food systems.

Continued study of the physical and chemical properties, mode of action and structure-function relationships of bacteriocins is necessary if their potential in food preservation is to be exploited. Further exploration into the synergistic reactions of these compounds and other natural preservatives, in

combination with advanced technologies could result in replacement of chemical preservatives, or could allow less severe processing (e.g. heat) treatments, while still maintaining adequate microbiological safety and quality in foods.

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