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Review

Antimicrobial compounds of the genus Bacillus: A review

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Abstract

Antibiotics are low molecular-weight molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. Microbial secondary metabolites have been a major source of clinically useful antibiotics. Members of the genus *Bacillus* are known to produce a wide arsenal of antimicrobial substances, including peptide and lipopeptide antibiotics. These peptides can be classified into two different groups based on whether they are ribosomally (bacteriocins) or nonribosomally (polymyxins and iturins) synthesized. The present paper provides a general overview of antimicrobial peptides derived from *Bacillus* species, including their structure and classification, as well as several extraction and purification methods used to obtain these bioactive compounds.

Keywords Antibiotics, *Bacillus* species, extraction, purification.

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Introduction

Antimicrobial resistance gains more and more public attention as one of the biggest threats to prevention and treatment of an increasing number of infections. Thus, there is a great demand for a continuous supply of novel antibiotics to combat such diseases (J.J. HUG & al [1]).

Microorganisms are the most potential source for production of natural therapeutic agents (A. BHARDWAJ & al [2]). The discovery and use of antibiotics, which have been produced by several microorganisms through secondary metabolic pathways has been one of the major scientific achievements in the earliest of 20th century (M. RUKMINI & al [3]).

The study of antibiotics began with the discovery of penicillin by A. Fleming and its subsequent introduction into therapy (S.L. DERDERIAN & al [4]).

Antibiotics are chemotherapeutic agents that can be extracted from plants or obtained from microbial sources as secondary metabolites in the late logarithmic or early stationary phase of growth of batch cultures. Over 4000 antibiotics have been identified from different microorganisms, but only 50 have been commercially used to treat human, animal and plant diseases (R.A. SLEPECKY & al [5]).

Secondary metabolites are synthesized by a wide variety of pathways, and both the specific genetic makeup of the producing strains and different environmental conditions can affect their activity. These molecules are primarily produced by microorganisms that live in soil. There are a few classes of microorganisms that can be used as sources for clinically useable antibiotics belonging to the genera *Streptomyces*, *Cephalosporium*, *Penicillium*, *Micromonospora* and *Bacillus* (S.A. MUHAMMAD & al [6], A. BHARDWAJ & al [2]).

Bacillus species are one of the largest sources of bioactive natural products, which exhibit a wide range of antibiotic activities and are produced as low molecular weight polypeptides, by ribosomal or non-ribosomal mechanisms. The amount of antibiotics produced by bacilli was approaching 167, being 66 derived from *B. subtilis*, 23 from *B. brevis* and the remaining peptide antibiotics are produced by other species of *Bacillus* genus (M. AWAIS & al [7]).

In the scientific literature, already hundreds of different pathogenic strains have been described as test organisms in the direct activity-based screenings. The most frequent test organisms were *Staphylococcus aureus*, *Micrococcus (Sarcina) lutea*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans* and others (J. BERDY & al [8]).

According to their biosynthetic pathway, the first class of antibiotics comprises ribosomally synthesized peptides, including bacteriocins, whereas the second class comprises small microbial peptides synthesized enzymatically by non-ribosomal pathways (A.G. CHALASANI & al [9], F. BARUZZI & al [10]).

The ribosomal peptide antibiotics are produced during active cell multiplication, and non-ribosomal ones are produced during sporulation or after growth.

Ribosomally synthesized peptides

Bacteriocins and bacteriocin-like inhibitory substances (BLIS)

Bacteriocins comprise a huge family of ribosomally synthesized peptides that usually show antimicrobial activity against strains that are closely-related to the producer strain (narrow-spectrum bioactivity) and sometimes against strains across genera (broad-spectrum bioactivity) (R.H. PEREZ & al [11]).

Bacillus has been investigated for their ability to produce so called bacteriocin-like inhibitory substance BLIS. It has been reported that strains of *B. thuringiensis*, *B. subtilis*, *B. stearothermophilus*, *B. licheniformis*, *B. megaterium* and *B. cereus* produce BLIS (E.A. ABADA & al [12]).

The classification of bacteriocins was previously established by KLAENHAMMER [13], NES & al [14], and recently by ABRIQUEL & al [15], which consist in three groups: class I includes antimicrobial peptides that undergo different kinds of post-translational modifications; class II presents nonmodified and linear peptides, and class III, which includes large proteins (>30 kDa).

Class I. Post-translationally modified peptides

This class can be subdivided into four subclasses: Subclasses I.1-I.3 include peptides with typical modifications of lantibiotics (e.g. formation of lanthionine and β-methyl lanthionine residues), while subclass I.4 includes other unique modifications (ABRIQUEL & al [15]).

Subclass I.1. Single-peptide, elongated lantibiotics

This group is represented by lantibiotics, which contain unusual amino acids, lanthionine and methylanthionine (<5 kDa). Type A lantibiotics (2100-3500 Da; 21-38 amino acid residues) includes subtilin, a 32-aminoacid pentacyclic lantibiotic (3320 Da) produced by *Bacillus subtilis*.

Subtilin has the bactericidal effect on many Gram positive and certain Gram negative bacteria (A. GÁLVEZ & al [16]). Subtilin production in *B. subtilis* is regulated in a growth-phase-dependent manner, starting in the mid-exponential growth phase and increasing to reach maximal level at the beginning of the stationary phase (P. KAUR & al [17]). Structurally, it is similar to nisin from *Lactococcus lactis* (A.L. DEMAIN & al [18]), epidermin and pep5 from *Staphylococcus epidermidis*, and to ericins from *B. subtilis* A1/3. Recently, Fuchs & al [19] isolated entianin, which is a novel subtilin produced by *B. subtilis* subsp. *spizizenii* DSM 15029 with a strong antibacterial activity against *S. aureus*, *E. faecalis*, and other Gram-positive pathogens.

Subclass I.2. Other single-peptide lantibiotics

Subclass I.2 includes the type B globular lantibiotic mersacidin, and other lantibiotics, such as sublancin 168 and paenibacilllin.

Mersacidin (1824 Da) produced by *Bacillus sp.* strain HIL Y-85.54728, exhibits a more globular structure due to the formation of four intermolecular thioether bridges (type B). Mersacidin exerts its antibacterial activity by the inhibition of cell wall biosynthesis; this compound forms a complex with the peptidoglycan precursor lipid II as demonstrated for subtilin (J. PARISOT & al [20]). Several works showed that this peptide successfully inhibited *in vitro* and *in vivo* the growth of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* strains (MRSA) (D. KRUSZEWSKA & al [21]).

Sublancin 168 (3877.78 Da), produced by *B. subtilis* 168, contains a single lanthionine linkage and two unusual disulfide bridges; this compound exhibits bactericidal activity mainly against Gram-positive bacteria including important pathogens such as *B. cereus*, *Streptococcus pyogenes* and *Staphylococcus aureus* (J.Y.F. DUBOIS & al [22]).

Paenibacillin is a lantibiotic of 2983.5 Da produced by *Paenibacillus (Bacillus) polymyxa* OSY-DF, which also produces the peptide antibiotic polymyxin E1 (Z. HE & al [23]). Due to their high affinity for the lipid moiety of lipopolysaccharide and disruptive effect on membrane integrity, polimixins are used against Gram-negative bacteria infections (D. LANDMAN & al [24], F. BARUZZI & al [10]) produced by *Clostridium sporogenes*, *Listeria spp.*, *S. aureus* and *Streptococcus agalactiae* species.

A.A. ARIAS & al [25] identified amylolysin, a putative type B-lantibiotic, that was isolated from *B. amyloliquefaciens* GA1 strain. Pure amylolysin was found to have an antibacterial activity toward Gram-positive bacteria, including methicillin resistant *Staphylococcus aureus*, and *Listeria monocytogenes* strains.

Subclass I.3. Two-peptide lantibiotics

Subclass I.3 includes the two-component lantibiotics, such as haloduracin and lichenicidin.

Haloduracin, produced by the alkaliphile isolate *Bacillus halodurans* C-125, which provides bactericidal activity against a wide range of Gram-positive bacteria, including *Listeria*, *Streptococcus*, *Enterococcus*, *Bacillus* and *Pediococcus* species (A.L. McCLEREN & al [26]).

Also, *Bacillus licheniformis* ATCC 14580 and *Bacillus licheniformis* DSM 13 produce lichenicidin, a “putative lantibiotic mersacidin precursor” (J. DISCHINGER & al [27]). It exhibited antimicrobial activity against all *Listeria monocytogenes* strains tested, as well as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant enterococci strains (M. BEGLEY & al [28]).

Subclass I.4. Other post-translationally modified peptides

Subclass I.4 includes the unique cyclic peptide subtilosin A, ribosomally synthesized by *B. subtilis* ATCC 6633 and other *B. subtilis* strains, as well as *Bacillus amyloliquefaciens*, with a strong bactericidal activity

against *L. monocytogenes* and Gram-negative bacteria (C.E. SHELBYNE & al [29]).

Class II. Nonmodified peptides

Class II of bacteriocins consists of heat-stable, membrane-active peptides without modified residues and can be subdivided into three subclasses.

Subclass II.1. - Pediocin-like bacteriocins

Pediocin-like bacteriocins, include coagulin, a protease-sensitive peptide produced by *Bacillus coagulans* I4 (C. LE MARREC & al [30]), and proved bactericidal activity against pathogens like *Leuconostoc*, *Pediococcus*, *Enterococcus* and *Listeria*.

Subclass II.2. - Peptide- like thuricin

B. thuringiensis is also able to produce BLIS, such as tochicin (10.5 kDa) and the large family of thuricins, (J.W. JIN & al [31]) with a broad inhibitory spectrum, including *L. monocytogenes*, *Salmonella enterica* and *Pseudomonas aeruginosa*. Thuricin 17 (3160 Da) inhibits *B. cereus* strains, *E. coli* MM294, thuricin H (3139.51 Da) is active against a wide range of Gram-positive bacteria, such as *Bacillus spp.*, *Listeria spp.* and *Staphylococcus aureus*. *Bacillus cereus* produces bacteriocins as cerein MXR1 (3137.93 Da), which shows a wide spectrum of inhibition mostly against Gram positive bacteria (S. SEBEI & al [32]).

Subclass II.3. include other linear peptides.

Bacillus licheniformis 26L-10/3RA produces lichenin (1344 Da), a chromosomally encoded hydrophobic BLIS, with antibacterial effect against *Streptococcus bovis* and *Eubacterium ruminantium* (F. BARUZZI & al [10]). *Bacillus cereus* produce cerein 7A (3940 Da), 7B (4893 Da), which exhibits a strong antibacterial activity against Gram positive bacteria. Thuricin 439 produced by *B. thuringiensis* B439 consists of two active peptides (A and B) with slightly different molecular masses (2919.9 and 2803.8 Da, respectively). Thuricin 439 shows a narrow spectrum of activity against *B. cereus*, *B. thuringiensis* strains and *L. innocua*, but not against Gram-negative bacteria (H. ABRIQUEL & al [15]).

Recently, J. WANG & al [33] identified the cerecids, including cerecidin A1 and A7, a novel class of lantibiotics that are produced by *Bacillus cereus*. They exert antimicrobial activity against a broad spectrum of Gram-positive bacteria and exhibit high efficacy against some drug-resistant pathogens, implying potential applications as peptide antibiotics in food preservation or pharmaceuticals (L. ZHANG & al [33]).

Class III. Large proteins

The A-group megacins include the inducible megacins A-216 produced by *B. megaterium* 216 and A-19213 produced by *B. megaterium* ATCC 19213. *Bacillus cereus* produces cereins, such as cerein 8A produced by *B. cereus* 8A, which inhibits several pathogenic and food-spoilage microorganisms such as *L. monocytogenes* (D. BIZANI & al [34]).

Non-ribosomally biosynthesized peptides

Non-ribosomally generated amphipathic lipopeptide antibiotics with condensed β -hydroxyl or β -amino fatty acids are widespread in *B. subtilis* (T. STEIN & al [35]; E.A. ABADA & al [36]). These are synthesized by large multienzyme systems that have a modular structure. Lipopeptide antibiotics identified so far have been divided into three main groups according to their structure: iturin group and macrolactones, like surfactin and fengycin groups T. STEIN & al [35]. All these agents occur as families of closely related isoforms which differ in length and branching of the fatty acid side chains and in amino-acid substitutions in the peptide rings. These agents are natural compounds with a high potential for biotechnological and pharmaceutical applications (M. ALAJLANI & al [37]).

The iturin group is a large family of cyclic heptapeptides with a C14-C17 aliphatic β -amino fatty acid. The group includes iturin (variants A, C, D and E), bacillomycin (D, F, L and Lc) and mycosubtilin, as well as other variants with names that reflect their bacterial source (e.g. mojavensin (C.R. HARWOOD & al [38])). Iturin production is strongly associated with *B. amyloliquefaciens* and closely related species, such as *B. velezensis*. All members of the iturins group have strong antifungal activity against a number of important fungal pathogens (e.g. *Rhizoctonia*, *Penicillium*, *Aspergillus*, *Fusarium* and *Pyricularia*) (Y. TOURE & al [39]) and, consequently, strains producing these compounds are being developed as potential biocontrol and plant growth promotion agents (C.R. HARWOOD & al [40]).

Lipopeptides of the surfactin family (surfactin, lichenysins and pumilacidins) are produced by several *B. subtilis*, *B. licheniformis*, *B. natto* and *B. pumilus* strains and contain a cyclic heptapeptide that forms a lactone bridge with β - hydroxy fatty acids, and show antibacterial (against *S. aureus*) and also, antifungal activities (M.H.M. ISA & al [41]).

Q. GOAFU et al [42] reported the antifungal mechanism of action of a new member of the surfactin family, named WH1 fungin, produced by *B. amyloliquefaciens* WH1.

Among small peptides, bacilysin produced by *B. subtilis* is also included, which contains an N-terminal alanine residue and L-anticapsin; the release of L-anticapsin irreversibly inhibits glucosamine synthase, involved in the synthesis of nucleotides, aminoacids and coenzymes, and resulting in the lysis of microbial cells such as *S. aureus* and *Candida albicans* (M. ONGENA et al [43]). Some strains of *B. subtilis* also produce chlorotetaine, a chlorinated derivative of bacilysin with similar antibacterial activity (T.G. PHISTER & al [44]).

Also, gramicidin S, a small cyclic peptide, synthesized by *Bacillus brevis*, is an important and powerful antibiotic, effective against a variety of Gram-positive and Gram-negative bacteria (M. BERDITSCH & al [45]; J.W. LIOU & al [46]).

Tyrocidine, a cyclic decapeptide produced by *B. brevis* (M.A. MARQUES & al [47], E.A. FELNAGLE & al [48]) is active against several important pathogens, including the Gram-positive bacterium *Listeria monocytogenes*, the pathogenic fungus *Aspergillus fumigatus*, the yeast *Candida albicans*, and the human malaria parasite *Plasmodium falciparum* (M. WENZEL & al [49]).

The non-ribosomal dodecapeptide bacitracin (1486 Da), released by some *B. licheniformis* and *B. subtilis* strains (O.A. SICUIA & al [50]), proved to be an inhibitor of cell wall biosynthesis of Gram positive bacteria (E.C. AZEVEDO, [51], A. AMIN & al [52]). Bacitracin is a mixture of at least 5 polypeptides, and consists of 3 separate compounds, bacitracin A, B and C. Bacitracin A is the major constituent. It is active against many Gram positive organisms, such as *Staphylococcus*, *Streptococcus*, anaerobic cocci, *Corynebacter* and *Clostridia*, but not against most other Gram negative organisms (A.A.H.S. AL-JANABI & al [53]) (see Figure 1).

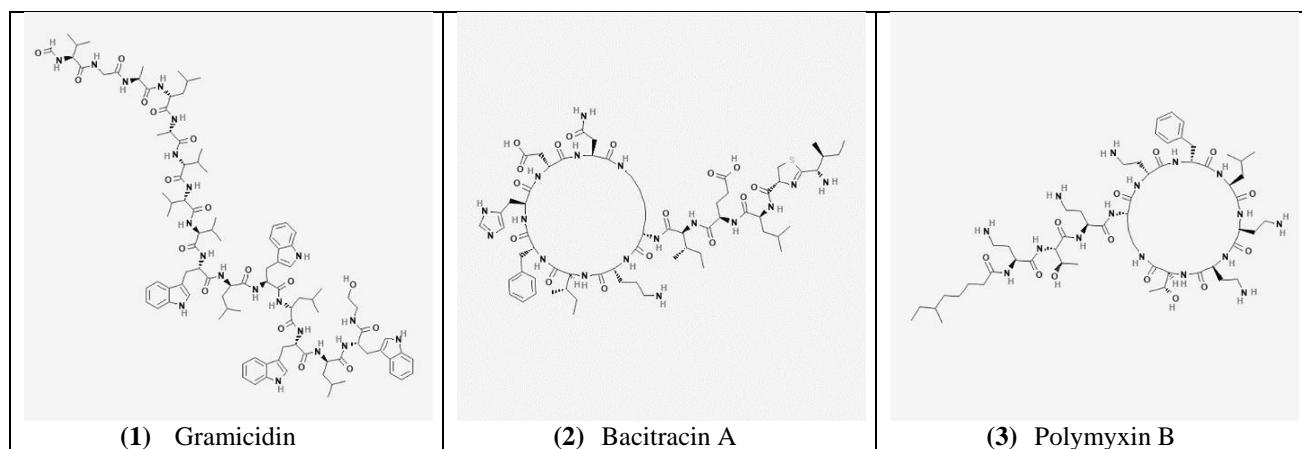


Figure 1. Chemical structure of some *Bacillus* antibiotics: **1)** Gramicidin; **2)** Bacitracin A; **3)** Polymyxin B

Non-peptide-based antibiotics

Bacillus strains also produce a variety of non-peptide antibiotics with different chemical structures.

I.V. PINCHUK et al [54] investigated 51 *Bacillus* strains isolated from different habitats, from which 47 have been identified as *B. subtilis*. Among them, there are 11 amicoumacins producers, a class of compounds responsible for antagonistic activity against *S. aureus* and *Helicobacter pylori*. Recently, a new member of the amicoumacin group, Hetiamacin B-D, was discovered from the fermentation broth of an endophytic bacterium, *Bacillus subtilis* PJS. It contained a special hexahydroimidazole ring in the side chain of its chemical structure, and exhibited outstanding activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (S. LIU & al [55]). Other antibiotics are represented by macrolactins and their derivatives, succinyl or glycosylated macrolactin, containing three separate diene structure elements in a 24-membered lactone ring. Over 18 macrolactins from *Bacillus* spp. have been chemically described, which are considered to have antibacterial activity against *S. aureus* (M. ROMERO-TABAREZ & al [56]). Difficidin and oxydifficidin, isolated from fermentation of *B. subtilis* ATCC 39320, represent a class of antibiotics characterized by highly unsaturated 22-membered macrolide phosphates and exhibit a good antibacterial activity against both aerobic and anaerobic organisms (L.B. ZIMMERMAN & al [57]).

B. subtilis produces rhizoictins, phosphonate oligopeptide antibiotics, which exhibit antifungal and antibacterial activity (S.A. BORISOVA & al [58]).

The increasing demands for new lead compounds in pharmaceutical and agrochemical industries have driven scientists to search for new bioactive natural products in extreme environments (L.S. SANTHI & al [59]).

LU et al [60] isolated a novel macrolactin antibiotic from a culture broth of marine *Bacillus* sp., macrolactin S, which exhibited antibacterial activity against *E. coli* and *S. aureus*. Novel thiopeptide antibiotics YM-266183 and YM266184 were found in the culture broth of *Bacillus cereus* QNO3223 which was isolated from the marine sponge *Halichondria japonica*. These antibiotics exhibited potent antibacterial activities against Staphylococci and Enterococci including multiple drug resistant strains whereas they were inactive against Gram-negative bacteria (K. NAGAI & al [61]). A. AMIN et al [62] investigated different soil samples from extreme conditions (saline soil) and screened for production of antibiotics against *Micrococcus luteus* and *Staphylococcus aureus*. The identified antibiotic was bacitracin produced by *Bacillus* strain GU057.

Z. NASFI et al [63] studied the potential for antimicrobial compounds production of different bacterial strains, isolated from rhizospheric soils obtained from Tunisian arid areas. Fungicides of the bacillomycin and fengycin group were identified. The study concluded that the isolated strain (*Bacillus* sp.) has a potential for the discovery of compounds with antibacterial and antifungal activities and highlights the high potential of the arid strains for the biosynthesis of specialized metabolites.

Table 1 summarizes the main *Bacillus* antibiotics.

Table 1. Some *Bacillus* antibiotics

No.	Producer Organism	Antibiotics	Sensitive organisms	References
1.	<i>Bacillus subtilis</i>	Subtilin, Ericin, Entianin, Subtilosin A, Bacilysin, Mersacidin, Sublancin 168, Bacitracin, Polymyxin, Difficidin	Gram positive bacteria, certain Gram negative bacteria	[16], [14], [19], [27], [41], [20], [22], [47], [8]
2.	<i>Bacillus brevis</i>	Gramicidin S, Tyrocidine	Gram positive bacteria, certain Gram negative bacteria	[45], [46] [47], [48]
3.	<i>Bacillus cereus</i>	Cerexin, Cerein	Gram positive bacteria	[69]
4.	<i>Bacillus licheniformis</i>	Lichenicidin, Bacitracin	Gram positive bacteria	[27], [48]
5.	<i>Bacillus thuringiensis</i>	Thuricin	Gram positive bacteria	[31]
6.	<i>Bacillus coagulans</i>	Coagulin	Gram positive bacteria	[29]
7.	<i>Bacillus polymyxa</i>	Paenibacillin, Polymyxin	Gram positive bacteria, certain Gram negative bacteria	[69]
8.	<i>Bacillus halodurans</i>	Haloduracin	Gram positive bacteria	[25]
9.	<i>Bacillus amyloliquefaciens</i>	Bacillomycin, Mycobacillin, Fungistatin	Molds and Yeast	[68]

Several extraction and purification methods of bacteriocins produced by *Bacillus* spp.

Bacteriocins are often extracted from culture broths by classical methods, including acidic precipitation (HCl), recrystallization, and extraction by organic solvents. Unfortunately, impurities are co-extracted and the extraction must be completed by chromatographic procedures (F. PEYPOUX & al [64]).

J.C. OSCARIZ et al [65] isolated and identified the bacteriocin cerein 7 produced by *B. cereus* strain. The bacteriocin was purified by a two-step procedure: first, ammonium sulfate precipitation of culture supernatants, and then, butanol extraction step, followed by the recovery of antibiotic from the organic phase. The compound was active against most Gram-positive, but not Gram-negative bacteria.

A series of new antibiotics have been isolated from well-known *B. subtilis* strains. These include bacilysocin, an anti-microbial phospholipid, that was isolated from *B. subtilis* 168 cells by extraction with n-butanol and purification by Thin-layer chromatography (TLC). Bacilysocin demonstrated antimicrobial activity, especially against certain fungi (N. TAMEHIRO & al [66]). Subtilin can be isolated from submerged culture fermentation broth by solvents extraction, (e.g. butanol, ethanol, n-hexane, ethyl acetate) and purified by counter-current distribution in a butanol-water system, ultrafiltration or nanofiltration processes (N.S. SHALIGRAM & al [67]). QINGWEI G. et

al [68] have purified bacillomycin D from *Bacillus subtilis* fmbJ using RP-HPLC method. Also, in the study of S. WU et al [69] antimicrobial peptides designated as Subpeptin JM4-A and Subpeptin JM4-B, produced by *Bacillus subtilis* JM4, were purified to homogeneity by ammonium sulfate precipitation, sequential SP-Sepharose Fast Flow, Sephadex G-25 and C18 reverse-phase chromatography. In another study, R.W. PHELA et al [70] have determined the purification of subtilomycin, a new lantibiotic from *Bacillus subtilis* strain MMA7 isolated from the marine sponge *Haliclona simulans*. The purification method involved an initial ammonium sulphate precipitation step (40% saturation), followed by Reverse Phase High Performance Liquid Chromatography (RP-HPLC). P.K. DAS et al [71] extracted from the production medium polymyxin B (*Bacillus polymyxa*) and cerexin A (*Bacillus cereus*) and purified by comparing various methods, like adsorption through activated charcoal, acetone precipitation, dialysis, ion exchange and Sephadex column chromatography, with the best results on an Ion exchange column.

Therefore, the methods used to obtain antibiotics are relatively similar, including extraction with different organic solvents, and completed by chromatographic procedures, depending on each bioactive compound in part. Currently, of the several hundred naturally produced antibiotics that have been purified, only a few have been sufficiently non-toxic to be used in medical practice (Table 2).

Table 2. Different antibiotics clinically used

No.	Producer Organism	Antibiotics	Clinical uses
1	<i>Bacillus subtilis</i> ; <i>Bacillus licheniformis</i>	bacitracin	topically and orally in the form of creams, ointments, and aerosol preparation, against staphylococci and streptococci
2	<i>Bacillus brevis</i>	gramicidin	topical cream, ophthalmic solution, against bacterial infections
3	<i>Bacillus polymyxa</i>	polymyxin	topical therapy against otitis caused by <i>Pseudomonas aeruginosa</i>

Conclusions and Perspectives

Bacillus represent an important source of bioactive compounds as a result of numerous antimicrobial substances discovered for more than half a century. The identification of compounds with antimicrobial activity involves extraction from the fermentation broths, followed by purification and analysis methods in order to characterize their structure and chemical composition.

Nowadays, the discovery of new drugs from microbial sources represents a major challenge among the researches, because it is absolutely necessary to combat human

pathogens that are resistant to antibiotics that have controlled them so far.

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Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

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