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# Antimicrobial substances produced by *Bacillus* spp.: innovations for food application

## Substâncias antimicrobianas produzidas por *Bacillus* spp.: inovações para aplicação em alimentos

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## ABSTRACT

One of the main challenges faced in the production of food is microbial contamination and spoilage. Due to the increasing consumer demand for safe foods and with low addition of synthetic preservatives, industry is looking for natural and safe alternatives. In this scenario, antimicrobial peptides stand out. *Bacillus* spp. are considered excellent producers of antimicrobial substances such as bacteriocins, bacteriocin-like substances (BLIS), and lipopeptides. However, these substances face practical and economic obstacles that make their definitive use in the food industry difficult. The present work aims to develop a systematic literature review focused on antimicrobial substances produced by *Bacillus* spp., as well as new technologies developed with potential use in the food industry and the difficulties faced in their development and application. The search resulted in 175 papers and these were refined based on the selection criteria, resulting in 28 studies included in this systematic review. Nanotechnology and hurdle technology proved to be potential strategies to promote controlled release of active substances, increase the stability of antimicrobial compounds in contact with food matrices and increase the spectrum of action compared to the antimicrobial used alone. In conclusion, the use of antimicrobial peptides produced by *Bacillus* spp. as natural food preservatives shows great potential, however, it is necessary to optimize production processes when thinking about scaling up for industrial production.

**Keywords:** bacteriocins, lipopeptides, antimicrobial activity, hurdle technology, nanotechnology.

## RESUMO

Um dos principais desafios enfrentados na produção dos alimentos é a contaminação e deterioração microbiana. Com o aumento da demanda por alimentos seguros e com baixa adição de conservantes sintéticos, a indústria busca alternativas naturais e seguras para auxiliar na manutenção da qualidade destes alimentos. Neste cenário destacam-se os peptídeos antimicrobianos. As bactérias do gênero *Bacillus* spp. são consideradas excelentes produtoras de substâncias antimicrobianas como bacteriocinas, substâncias tipo bacteriocinas (BLIS) e lipopeptídeos. No entanto, estas substâncias enfrentam obstáculos práticos e econômicos que impedem seu emprego definitivo na indústria alimentícia. O presente trabalho visa desenvolver uma revisão sistemática da literatura com foco em substâncias antimicrobianas produzidas por *Bacillus* spp., bem como abordar novas tecnologias desenvolvidas com potencial de uso na indústria de alimentos e as dificuldades enfrentadas em seu desenvolvimento e aplicações. A busca resultou em 175 artigos e estes foram refinados com base nos critérios de seleção, resultando em 28 estudos incluídos nesta revisão sistemática. Novas tecnologias como a nanotecnologia e o emprego de técnicas integradas no combate a patógenos, apresentam-se como potenciais estratégias para garantir uma liberação controlada das substâncias, estabilidade em contato com as matrizes alimentares, amplo espectro de ação contra patógenos alimentares do que quando usado de forma isolada. Em conclusão, o uso de peptídeos antimicrobianos produzidos por *Bacillus* spp. como conservantes naturais de alimentos apresenta grande potencial, porém é necessário otimizar os processos produtivos quando se pensa em escalonamento para a produção industrial.

**Palavras-chave:** bacteriocinas, lipopeptídeos, atividade antimicrobiana, tecnologia de barreira, nanotecnologia.

## INTRODUCTION

Quality assurance and food safety are one of the main objectives and challenges of the food industry. Foods are exposed to several factors that can compromise their integrity, such as humidity, temperature and pH variations, presence of contaminating microorganisms, among others. In this context, the industry focuses on economically viable strategies, such as the use of active packaging and the addition of chemical preservatives.

Bacteria of the genus *Bacillus* spp. are known for their adaptability to adverse environments, which is reflected in their ability to produce diverse antimicrobial substances, including bacteriocins, bacteriocin-like substances (BLIS), ando lipopeptides (Woldemariamyohannes et al., 2020). *Bacillus subtilis* (Hyun et al., 2021), *B. thuringiensis* (Su et al., 2020), and *B. amyloliquefaciens* (Perez et al., 2017) are species of great importance and reported as producers of these antimicrobial substances. However, the practical challenges associated with using antimicrobial substances produced by *Bacillus* spp. as natural food preservatives need to be addressed before its widespread implementation in industry. New technologies, such as the combination of these substances and nanotechnology, present themselves as promising alternatives to overcome such challenges.

In this context, this study aims to review antimicrobial substances produced by *Bacillus* spp. and new technologies with potential use in the food industry, as well as the challenges faced in their development and application. We systematically developed this study through the search for two sets of keywords in the Google Scholar database. We selected publications in the time frame from 2016 to 2021 and we adopted selection criteria in the analysis of studies that will be explained in the methods section.

## METHODOLOGY

For the development of this literature review, the following database was used: Google Scholar (https://scholar.google.com.br/). The choice of Google's school platform is due to its free and comprehensiveness, unlike traditional scientific databases, such as Scopus and Web of Science (WoS), which require a subscription. We performed two searches with the following sets of words: 1st search (food technology AND safety, BLIS, lipopeptides, *Bacillus*); 2nd research (nanoencapsulation technology AND food safety, Bacteriocins as substances, Hurdle technology, *Bacillus*). We carried the searches out in June and July 2021 and the selected articles were the time frame of 2016-2021.

The search resulted in 175 works and these were refined based on the following selection criteria: 1st identification of duplicates; 2nd adaptation to the theme based on the title and abstract readings; and 3rd Selection of articles published in journals with Qualis/CAPES A and B, based on the Qualis-Periódicos of 2013-2016. We show the flowchart used in the literature review of this work in Figure 1. However, it was necessary to include studies that were relevant to the research that did not fit the adopted system.

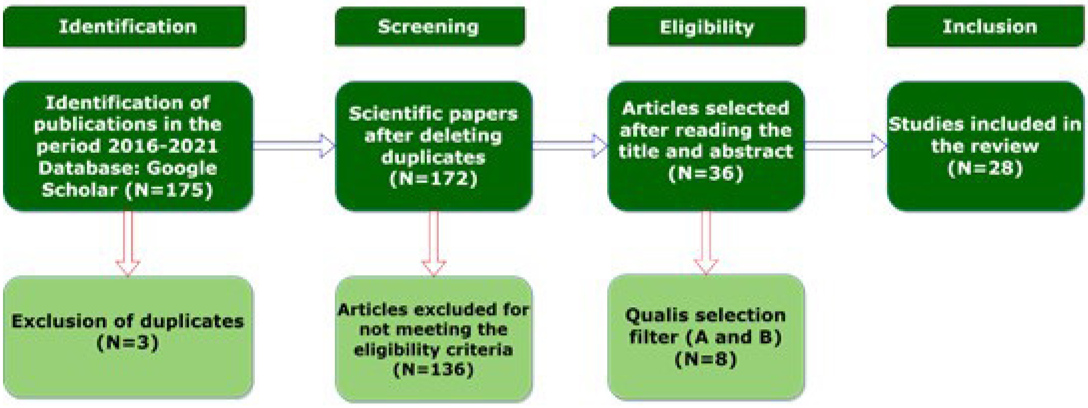

**Figure 1.** Systematics adopted in this bibliographic research.
Source: Authors (2021).

## RESULTS AND DISCUSSION

The search resulted in 185 papers and these were refined based on the selection criteria, 3 articles were excluded because they were duplicates, 136 of the remaining studies were excluded because they were not related to the theme and 8 were excluded because they were not published in Qualis A and B journals, resulting in 28 studies included in this systematic review.

### Antimicrobials Produced by *Bacillus* spp. for Food Safety

The potential presented by the genus *Bacillus* to produce substances with antimicrobial action is historically recognized. Bacteria of this genus can produce a diverse range of substances that are recognized as safe, besides forming endospores, which allow them to survive in extreme environments. These characteristics arouse great industrial and economic interest, especially by the food industry (Woldemariamyohannes et al., 2020).

Among the substances produced by *Bacillus* spp., bacteriocins and BLIS stand out. Bacteriocins are low molecular weight peptides of ribosomal origin that are secreted into the extracellular medium and undergo a post-transcriptional maturation process, which has not yet been fully explained. The main target of bacteriocins is the plasma membrane, where they cause permanent changes in its structure, leading to the death of the target microorganism (Ogaki et al., 2015). BLIS have a broader spectrum of action than bacteriocins acting against Gram-positive (Huang et al., 2016) and having antifungal activity (Salazar et al., 2017).

The interactions between antimicrobial peptídeos and food components also is a challenge in the development of new substances. Leite et al. (2016) report that antimicrobial substances can interact with food components so that they only inhibit the target pathogens, a behavior called bacteriostatic. A promising behavior of the BLIS produced by *B. cereus* LFB-FIOCRUZ 1640 was observed, which showed a satisfactory spectrum of action *in vitro* against indicator microorganisms, which often contaminate fruits, such as *Listeria monocytogenes,* and also showed satisfactory activities when applied in the pineapple pulp.

In fact, bacteriocins and BLIS are promising substances for ensuring food safety and innocuity. Table 1 shows some substances with potential for applications in food. However, one cannot ignore the fact that there are many challenges that make it difficult to implement new compounds on the market, which leads the food industry to bet on already well-known substances, such as nisin and pediocin. Nevertheless, it is known that certain microorganisms responsible for food contamination have developed resistance to nisin and pediocin, highlighting the need to explore alternative inhibitory substances. As will be discussed in another topic of this review, the integration of new technologies with these active substances shows promising potential in terms of offering a broad spectrum of action, enhanced stability, and controlled release of substances.

Currently, the use of microbial surfactants, also called biosurfactants, has increased. That occurs due to their environmental compatibility. Furthermore, a relevant

**Table 1.** Substances with potential application in food.

| Substance | Producing strain | Sensitive pathogens | Reference |
|---|---|---|---|
| BLIS | *Bacillus thuringiensis* BRC-ZYR2 | *Bacillus cereus* | Huang et al., 2016 |
| BLIS | *B. cereus* LFB-FIOCRUZ 1640 | *Listeria monocytogenes* | Leite et al., 2016 |
| Bacteriocin | *B.amyloliquefaciens* RX7 | *L. monocytogenes, B. cereus, Staphylococcus aureus* and *Pseudomonas aeruginosa* | Lim et al., 2016 |
| BLIS | *B. amyloliquefaciens* ELI149 | *Fusarium oxysporum, F. avenaceum* and *Mucor sp.* | Salazar et al., 2017 |
| Thuricin 4AJ1 (bacteriocin) | *B. thuringiensis* 4AJ1 | *B. cereus* | Su et al., 2020 |
| BLIS | *B. subtilis* BSC35 | *Clostridium perfringens* | Hyun et al., 2021 |

feature is their ability to interact with interfaces, promoting interfacial and superficial strain reduction. They also prevent the adhesion of biofilms and microorganisms on surfaces and have high biodegradability, in addition to low toxicity to living beings (Perez et al., 2017).

From another perspective, Perez et al. (2017) identified two bacterial strains isolated from Puba, *Bacillus sp.* P5 and *Bacillus sp.* C3, which had genes for the production of lipopeptides, such as iturin A, surfactin and subtilosin A for the first isolate and subtilosin A and subtilin for the second one. Both acted against the tested microorganisms, however, the P5 species showed more toxic activity against pathogenic fungi and bacteria and, specially on bacteria that cause food spoilage in the industry, such as *L. monocytogenes* and *B. cereus*.

According to the study of Kourmentza et al. (2021), the strains *B. subtilis* BBG131 and *B. subtilis* Bs2504 were able to produce antimicrobial lipopeptides, however, there was low stability during the purification process. The combination of surfactin, produced by *B. subtilis*, with nisin and mycosubtilin, a bacteriocin and a lipopeptide respectively, produces interesting antimicrobial activities that may lead to future research related to the combination of antimicrobial substances.

Despite the great potential of lipopeptides, they may show unwanted effects such as hemolysis, nephrotoxicity or even susceptibility to proteolysis by proteases, which may result in their non-commercialization. In addition, the scarcity of products based on these antimicrobial substances is also due to the high production costs (Kourmentza et al., 2021; Biswaro et al., 2018).

Therefore, it is worthwhile to explore new technologies that aim to achieve enhanced antimicrobial efficacy while utilizing smaller quantities of antimicrobial substances produced by *Bacillus* sp. In this sense, two approaches with great potential for application in the food industry stand out, which will be discussed below.

## Hurdle Technologies

The decrease in the use of chemical preservatives and replacement of conservation processes (or stages) that present a high cost for the food industry, as well as the search for new natural alternatives capable of guaranteeing food quality and safety, can be achieved with antimicrobial substances produced by bacteria of the genus *Bacillus*. The biopreservation can be leveraged using barrier technologies that combine methods that separately do not present the same preservation efficiency, but that combined increase safety of foods and ensure their organoleptic characteristics (Devi and Babar, 2018).

The use of antimicrobial substances in biopreservation can be studied from several perspectives: i) direct application (purified or semi-purified form); ii) the combination of other barrier or hurdle technologies producing an effective synergistic effect; and iii) inoculation of the food with the bacteriocinogenic strain (producing the antimicrobial substance *in situ*) (Silva et al., 2018). In this topic, the focus will be on the analysis of the most cited barrier technologies today.

Barrier technologies provide synergistic effects owing to the combination of antimicrobials with other substances or physical treatments. The combination of some bacteriocins with chelating agents, such as EDTA (ethylenediamine tetra-acetic acid), may allow the inhibition of Gram-negative bacteria by facilitating the antimicrobial reaching the microorganism's plasma membrane, since the complexed metal ions can interact with the lipopolysaccharides of the outer membrane making it more permeable (Borges and Teixeira, 2018). The same authors also mention that the combination of different bacteriocins or bacteriocins with another natural antimicrobial compound can improve the antimicrobial effect and broaden the inhibition spectrum. Or even that it is possible to make use of “non-thermal” physical treatments such as high intensity pulsed electric field applications, for inactivation by high voltage and high hydrostatic pressure, to potentiate the antibacterial activity.

Regarding the combination of antimicrobials and heat treatment methods, other studies report that the application of these methods (alone), such as the pasteurization process, is widespread in the food industry to eliminate pathogenic microorganisms. However, some bacterial pathogens have heat resistance – a characteristic that may allow the survival of some spores during food processing (Patrovský, 2016). It was demonstrated that the pre-exposure of heat-activated *Geobacillus stearothermophilus* spores to nisin (50 µg/mL) at 4 °C in milk for 15 h and 24 h resulted in a reduction below the level of detection for these spores when compared to untreated spores (Egan et al., 2016).

### Nanotechnology

Nanotechnology has great potential to be explored in the food field, and nanoencapsulation is a strategy that offers protection to antimicrobial compounds, such as those produced by *Bacillus* spp.. Nanoencapsulation is a process by which an active agent is coated by carrier material of nanometric dimensions, providing protection, controlled release and possible cost reduction, since a lower concentration of the antimicrobial compound can be used (Campo et al., 2017; Stefani et al., 2019). Several technologies can be employed for nanoencapsulation, highlighting for applications in food: nanoemulsions, liposomes, polymeric nanoparticles and solid lipid nanoparticles (Martins et al., 2016).

Nanoemulsions are dispersions of two immiscible liquids stabilized by a surfactant(s) with a size range between 10 and 1000 nm. In general, the use of surfactants in formulations and the reduction of the average diameter of the droplets contribute to increase stability of nanoemulsions (Stefani et al., 2019). Liposomes are colloidal vesicular structures composed of one or more lipid bilayers surrounding an equal number of aqueous compartments. Liposomes can be produced from natural substances, and therefore, are biocompatible, biodegradable, non-toxic and non-immunogenic (Maja et al., 2020). As liposomes contain lipid and aqueous phases, they can be used for entrapment, delivery and release of hydrophilic, hydrophobic and amphiphilic materials (Malheiros et al., 2012a). Polymeric nanoparticles (NPs) are colloidal systems comprised of natural or synthetic polymers. Within these nanoparticles, the encapsulated compound can be dispersed, dissolved, or adsorbed. Notably, NPs offer the advantage of easily modifying their surface chemistry, as well as the composition and size of the nanocarriers (Isaia et al., 2021). Solid lipid nanoparticle (SLN) are a colloidal delivery system made from solid (under room temperature) lipids. SLNs were developed as an alternative carrier system to other traditional colloidal carriers, namely oil-in-water emulsions, liposomes, and polymeric micro or nanoparticles. SLN are attractive for food applications due to their capability to encapsulate both hydrophilic and hydrophobic compounds. They offer additional benefits such as low toxicity, excellent bioavailability, and biodegradability.

Although several studies show that the application of nanoencapsulated antimicrobials has an advantage over the use of the free (non-encapsulated) compound, this technique is still barely used for encapsulation of antimicrobials produced by *Bacillus spp.* (Malheiros et al., 2012b; Isaia et al., 2021; Lin et al., 2020). Table 2 provides information on the nanoencapsulation of antimicrobial substances produced by *Bacillus* spp., including studies about the specific type of nanoencapsulation employed and its corresponding food applications.

Studies with BLS P34, an antimicrobial peptide produced by *Bacillus sp* P34 isolated from Piau-com-pinta fish (*Leporinus sp.)* found in the Amazon basin (Brasil), showed that this compound inhibits a wide range of bacterial species, including *L. monocytogenes.* Purified BLS P34 showed low toxicity for eukaryotic cells with an effect similar to that observed for nisin, showing great potential for application in food (Malheiros et al., 2012a; Isaia et al., 2021; Malheiros et al., 2012b). Malheiros et al. (2012a) encapsulated BLS P34 in soy phosphatidylcholine liposomes to inhibit

**Table 2.** Technologies utilized for nanoencapsulation of antimicrobial substances produced by *Bacillus* spp. and food applications.

| **Antimicrobial substance produced by *Bacillus spp*** | **Technologies for nanoencapsulation** | **Food applications** | **References** |
|---|---|---|---|
| BLS P34 | Liposomes | Reduction of *L. monocytogenes* in skim milk | Malheiros et al., 2012a |
| BLS P34 | Liposomes | Inhibition of *L. monocytogenes* in Minas Frescal cheese | Malheiros et al., 2012b |
| BLS P34 | Polymeric nanocapsules | Inhibition of *L. monocytogenes* in BHI broth | Isaia et al., 2021 |
| Cyclic lipopeptides | Solid lipid nanoparticles | Inhibition of *Aspergillus niger* and *A. carbonarius* | Lin et al., 2020 |
| CAMT2 | Nanovesicles | Inhibition of *L. monocytogenes* in skim and whole milk | Jial et al., 2020 |

the multiplication of *L. monocytogenes* in skim milk, whole milk and during the production of Minas Frescal cheese. The authors showed that this antimicrobial, both free and nanoencapsulated, inhibited the growth of *L. monocytogenes* inoculated in skim milk stored at 7 °C, between days 5 and 8, but after this period the pathogen kept its growth, maintaining levels below control (without antimicrobial). On the other hand, encapsulated BLS P34 was able to reduce *L. monocytogenes* in Minas frescal cheese after ten days of storage, being efficient in this food (Malheiros et al., 2012b).

BLS P34 was also encapsulated in polymeric nanocapsules using Eudragit RS-100 (EUD) (Isaia et al., 2021). The authors evaluated the antimicrobial effects of nanocapsules containing BLS P34 in relation to the growth of *L. monocytogene*s in BHI (Brain Heart Infusion) culture medium and milk. Interestingly, in skim milk, the BLS P34-loaded EUD nanocapsules did not inhibit the growth of *L. monocytogenes*. This was attributed to a potential interaction between the casein proteins present in milk and the surface of the nanocapsules. However, in BHI broth, the P34-EUD nanocapsules successfully inhibited the growth of *L. monocytogenes*.

Lin et al. (2020) conducted a study on the stability of cyclic lipopeptides extracted from the culture of *B. subtilis* during a 20-day storage period. The authors discovered that these cyclic lipopeptides underwent rapid oxidation, resulting in a loss of their antifungal activity over time. To address this issue, one strategy employed was the encapsulation of the lipopeptides in SLNs. The analysis performed in this study revealed that the hydrophobic interactions between the fatty acid portions of the cyclic lipopeptides and the SLN became stronger, leading to improved storage stability of the encapsulated lipopeptides. Moreover, the encapsulated cyclic lipopeptides demonstrated enhanced antifungal activity compared to their free form. They effectively hindered the development of hyphae and spores, as well as reduced levels of ochratoxin A (important mycotoxin). Hence, the utilization of SLN proved to be a successful approach for protecting and enhancing the functionality of cyclic lipopeptide.

The encapsulation of CAMT2, a bacteriocin produced by *Bacillus amyloliquefaciens,* was studied by Jiao et al. (2020) using nanovesicles prepared from soybean phosphatidylcholine. The authors demonstrated that when CAMT2 was encapsulated, it significantly reduced the counts of *L. monocytogenes* to zero after 8 days of storage at 4 °C in both skim and whole milk. In contrast, when free CAMT2 was used, the number of *L. monocytogenes* reached a minimum at 4 days (~ 2 log CFU/ml) and then recovered to the original level at 10 days in whole milk. These findings highlight the superior efficacy of encapsulated CAMT2 in maintaining antimicrobial activity over an extended period. The results suggest that the encapsulation of CAMT2 holds promise as an effective approach for enhancing its stability and anti-listerial efficacy. This encapsulation method could contribute to the efficient control of food-borne spoilage and pathogenic bacteria, particularly in fatty food matrices.

Therefore, the use of nanotechnology to protect and increase antibacterial and antifungal efficacy should be evaluated for each type of antimicrobial produced by *Bacillus* spp. In addition, the selection of an appropriate nanomaterial should consider various factors, including cost, release kinetics of the nanoencapsulated active compound, stability within the desired food matrix, and target pathogens. This comprehensive evaluation is necessary to ensure optimal outcomes in terms of protection, efficacy, and practical application.

## CONCLUSION

Based on this literature review, the species *B. amyloliquefaciens, B. subtilis* and *B. thuringiensis* of the genus *Bacillus* spp. stand out as producers of antimicrobial substances with potential application in the food industry. At this stage, the main challenge to large-scale use of antimicrobial peptides are the costs involved in the production, purification and characterization processes. In this context, the development of new hurdle technologies directly supports the needs of the industry, in which it is possible to contribute together with the use of antimicrobial peptides produced by *Bacillus* spp.

Studies related to the nanoencapsulation of antimicrobial substances produced by *Bacillus* sp. are scarce. However, this review has shown satisfactory results in increasing the stability of the compounds in contact with food matrices and in controlling their release. The use of antimicrobial peptides as natural food preservatives shows great potential. Such substances, combined with barrier technologies and nanotechnology, have potential application to guarantee food safety and quality.

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