Nanoformulations Based on *Bacillus subtilis* Lipopeptides: The Future of Agriculture

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5.1 Introduction

One-third all agriculture and food production would be lost if pesticides were not used. On the other hand, pesticides are hazardous – endocrine disruptors, neurodevelopmental toxicants, immunotoxicants, and carcinogens, among others (Jiang et al. 2018; Kalliora et al. 2018). Pesticides can be classified into insecticides, fungicides, nematicides, herbicides, and plant growth regulators. At industrial scale, these compounds are diluted into suitable solvent or colloidal dispersion – solid (granules or powders), liquid (true and colloid solutions and emulsions), or gas (vapors). In this sense, the (nano)formulation systems, particularly hydrophobic pesticides, are essential to achieve high performance. Solvents such as N,N-dimethylformamide and methanol are often used to enhance the solubility of hydrophobic pesticides; nevertheless, this strategy is unsafe (flammable) and has an impact on the environment (ecotoxicology). Usually, surfactants (dodecyl benzene sulfonate and calcium linear alkylbenzene sulfonate) and cosurfactants (n-propanol and n-butanol, ≈4%) improve the (nano)formulation system – pesticides (Feng et al. 2018). Thus, there is a strong trend to replace, at least partially, pesticides by eco-friendly alternatives. One of the most promising eco-friendly formulations in agriculture – a biosurfactant – is based on *Bacillus subtilis* lipopeptides.

Since the early nineteenth century, the production of antibiotic biomolecules, in particular those biosynthesized by *B. subtilis*, has been under research, in which *B. subtilis* lipopeptides appear to be an active substance. Chemically, *B. subtilis* lipopeptides are composed of a cyclic amino acid sequence linked to a fatty acid chain.

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Thus, *B. subtilis* lipopeptides can be classified into three families: iturin, surfactin, and fengycin (Ramirez 2017).

It is well known that *B. subtilis* lipopeptides affect plant pathogenic microorganisms such as *Penicillium* sp., *Rhizopus* sp., *Botrytis* sp., and *Aspergillus* sp., among others (Soberón-Chávez 2011). Therefore, based on the natural role of *B. subtilis* lipopeptides, nanoformulations that contain *B. subtilis* lipopeptides are considered to be the future of agriculture.

5.2 Conventional Pesticide Formulations

According to Feng et al. (2018) ideal characteristics of pesticide formulations are: (I) easy pesticide handling and application, (II) longer shelf life, (III) low toxicity, (IV) high release control, (V) high bioactivity, and (VI) broad-spectrum activity.

Regarding conventional pesticide formulations, they can be classified as (I), *emulsifiable concentrate* systems, such as organochlorine and organophosphorus insecticides, that are usually composed of pesticides that were solubilized in organic solvents (benzene, toluene, and xylene) and surfactants. Prior to pesticide application, the emulsifiable concentrates are diluted in water. As a result, an oil-in-water emulsion with pesticides (active ingredient) within the oil droplets (droplet dimension – molecular dimension) is established, whereas (II) *microemulsion* systems have advantages over emulsifiable concentrates such as lower flammability; cheaper; higher permeability – fine oil droplets (10–100 nm) (III) *emulsion* systems (oil-in-water) are produced by colloid mills, high-pressure valve homogenizers, microfluidizers, and sonicators. The main advantage of emulsion systems is related to low level of organic solvents and surfactants, and thus, it does not have an impact on the environment (drop size – 2–5 μm). Nanoemulsion formulations, when compared to emulsion, exhibit higher stability and higher pesticidal activity (lower drop size <200 nm). However, it is a promising technology (Feng et al. 2018).

5.3 History of *Bacillus* spp. Lipopeptides

Bacillus spp. are the best-characterized Gram-positive bacteria (Gu et al. 2018). They produce biocompounds with strong antimicrobial properties such as bacitracin (cyclic peptide) (Landy et al. 1948), bacilysin (dipeptide) (Vanittanakom et al. 1986), and iturin, surfactin, and fengycin (lipopeptides) (Soberón-Chávez 2011). Thus, these biocompounds, particularly, lipopeptides, stand out as the most promising alternatives to replace pesticides – nanoformulations that are aligned to the principle of green chemistry.

5.3.1 Iturin Family

In 1947, it was already known that subtilin and bacitracin, peptides synthesized by *Bacillus subtilis*, present antifungal activity (Landy et al. 1948).

Johnson and Burdon (1946) produced a new antibiotic "eumycin" from *Bacillus subtilis* strain. The authors did not give details about the chemical structure of eumycin; nevertheless, it was inferred that eumycin significantly affected the growth of *Trichophyton mentagrophytes*, *Microsporum gypseum*, and *Epidermophyton floccosum*.

In 1948, Landy et al. (1948) were doing research on antibiotics active against *Torula histolytica* infections. The authors realized that the antibiotic the *B. subtilis* strain produced was different from eumycin. Thus, they designated it as bacillomycin. In addition, bacillomycin showed strong antifungal properties against dermatophyte disease caused by *Epidermophyton floccosum* and *Trichophyton rubrum* and also against systemic fungi, in particular *Blastomyces dermatitidis*.

In 1950, Decambre (1950) described a novel antibiotic "iturin" that was produced by *B. subtilis* that was isolated in Belgian Congo, Africa. Iturin inhibited the growth of *Escherichia coli*, *Serratia marcescens*, *Micrococcus pyogenes* var. *aureus*, *Sarcina lutea*, and *Corynebacterium diphtheriae gravis*. On the other hand, Walton and Woodruff (1949) were the very first researchers to report the mycosubtilin, bioproduced by *B. subtilis* 370 – obtained from the Western Regional Research Laboratory of the United States Department of Agriculture – using beet molasses-based culture medium. The authors identified the presence of amino acids such as aspartic acid, glutamic acid, serine, alanine, tyrosine, arginine, valine, norvaline, isoleucine, leucine, norleucine, phenylalanine, and proline in the mycosubtilin structure. Walton and Woodruff (1949) also found the mycosubtilin antimicrobial activities against yeasts and fungi. Therefore, since the discovery of mycosubtilin, there is a strong interest in its use as antimicrobial agent – eco-friendly controller of plant diseases.

In the early 1970s, a much more accurate determination of the chemical structures of iturin family was carried out. Peypoux et al. (1973, 1974, 1976) elucidated first the iturin structure, in which iturin was separated in three fractions, A, B, and C, by thin-layer chromatography. Iturin A fraction was composed of seven amino acids, cyclic heptapeptide, Asp (3x), Glu, Tyr, Ser, and Pro, linked to fatty acid chain C_{14} (40%) and C_{15} (60%) (Peypoux et al. 1973, 1974). Peypoux et al. (1976) also identified the chemical structure of mycosubtilin, which was mainly composed of two homologous lipopeptides that differed due to the fatty acid moiety. In this sense (determination of the chemical structures), Besson et al. (1976) suggested that a unique active compound was responsible for antimicrobial properties of *B. subtilis* compounds. As already mentioned, at that time, even with the possibility of performing amino acids and fatty acid analyses, the chemical structure of most *B. subtilis* lipopeptides remained indeterminate.

Besson et al. (1976) showed that iturin A was found to be the active component to bacillomycin B, bacillomycin R, and eumycin. It was also proved that iturin A has strong antimicrobial activity against *Penicillium notatum* and *Penicillium chrysogenum*.

5.3.2 Surfactin Family

In May 1968, Arima et al. (1968) were the very first to report about surfactin, an extracellular biocompound (lipopeptide), which was synthetized by *B. subtilis* sp. The authors described a lipopeptide composed of L-aspartic acid, L-glutamic acid, L-valine, L-leucine, and D-leucine (1:1:1:2:2) linked to unidentified fatty acids. The authors chose the name "surfactin" due to the higher surface activity (when compared to sodium lauryl sulfate) and prospect its use as inhibitor of fibrin clot formation. Then, later in 1968, the same research group reported antifibrinolytic property of surfactin by fibrin plate method (Kakinuma et al. 1968).

Thomas and Ito (1969) described the reinvestigation (chemical structure by mass spectrometry) of esperin (surfactin family – see Table 5.1). They indicated (more accurately) that esperin is composed of seven amino acids Glu.Leu.Leu.Val.Asp.Leu.Leu(Val) and the fatty acid chain C_{10}–C_{12} rather than Glu.Asp.Val.Leu.Leu and C_{10} (fatty acid chain).

Kameda et al. (1974) were doing research on cytolytic activity on Ehrlich ascites carcinoma cells and isolated 113 *Bacillus natto* strains. The *B. natto* strain that showed the highest cytolytic activity on Ehrlich ascites carcinoma cells was selected. The active compound (surfactin) obtained from the selected strain was first extracted from the culture medium, and then, its chemical structure was identified by elementary analysis, as well as by infrared, nuclear magnetic

Table 5.1 *Bacillus subtilis* lipopeptides – amino acid sequences

Iturin family									
Asn	Tyr	Asn		Pro	Glu		Ser	[a] Thr	
Asn	Tyr	Asn		Gln	Pro		Asn	[a] Thr	
Asn	Tyr	Asn		[b] Ser	[b] Glu		[b] Ser	[a] Thr	
Asn	Tyr	Asn		Gln	Pro		Asn	[a] Ser	
Asn	Tyr	Asn		Gln	Pro		Asn	[a] Ser	
[b] asp	Tyr	Asn		Gln	Pro		Asn	[a] Ser	
Asn	Tyr	Asn		Gln	Pro		[b] Ser	[b] Asn	
Surfactin family									
Glu	Leu	Met		Leu	[b] pro		Leu	[a] Leu	
Glu	Leu	Leu		[b] Val	Asp		Leu	[a] Leu	
Glu	Leu	Leu		[b] Val	Asp		Leu	[a] Val	
Glu	Leu	Leu		Leu	Asp		Leu	[a] Val	
Glu	Leu	Leu		Leu	Asp		Leu	[b] Ile	
Fengycin family									
Glu	Orn	Tyr	Thr	Glu	[a]Ala	Pro	Gln	Tyr	Ile
Glu	Orn	Tyr	Thr	Glu	[a]Val	Pro	Gln	Tyr	Ile
Glu	Orn	Tyr	Thr	Glu	[a]Ala	Pro	Gln	Tyr	Ile
Glu	Orn	Tyr	Thr	Glu	[a]Val	Pro	Gln	Tyr	Ile

[a]Often found
[b]Unconventional
Adapted from Soberón-Chávez 2011

resonance, and mass spectrometry. The authors concluded that the active compound was found to be identical to surfactin.

In the 1980s, research on surfactin gradually increased. Cooper et al. (1981) reported a large-scale surfactin production, in which the biosurfactant was recovered by foam overflow strategy. Synthetic culture medium composed of 4% glucose as carbon source showed the best surfactin production, which was enhanced by the addition of either iron or manganese salts. The culture medium, named Cooper's medium, developed in this study is now well-known for research on surfactin production.

Regarding surfactin production, in 1981 it was already known that some peptide antibiotics are produced by non-ribosomal biosynthesis, including surfactin (Kurahashi 1981). In this sense, Kluge et al. (1988) used ^{14}C-labeled Leu, Val, and Asp that were found directly into the surfactin structure. The ^{14}C-acetate was identified in fatty acid portion of surfactin and also converted into Leu – via acetyl-CoA + α-ketoisovaleric acid. The author inferred also that the biosynthesis of surfactin production occurs by peptide synthesizing system (aminoacyl phosphates), non-ribosomally, which is different from glutathione or mycobacillin biosynthesis. Later, Menkhaus et al. (1993) proved the surfactin production by cell-free extracts of *B. subtilis* ATCC 21332, which indicated that surfactin biosynthesis is through multienzyme system instead of ribosomally. To the best of our knowledge, Nakano et al. (1988) were the first to identify the two genetic loci related to surfactin production – genetic engineering. One year later, Nakano and Zuber (1989) suggested that surfactin production is positively regulated at the transcriptional level by the *srfB* (*comA*) gene product.

After the development of the surfactin production techniques (culture medium, genetic engineering, purification by solvent extraction, etc.), Mulligan and Gibbs (1990) reported the first study on surfactin membrane-based purification process, in which ultrafiltration was used to purify surfactin from collapsed foam. This strategy takes advantages of the ability of surfactin to form micelles that can be separated from lower molecular weight impurities such as salts, free amino acids, peptides, and small proteins. The ultrafiltration using 50 kDa membrane showed the best retention, and 160-fold purification was achieved.

Sheppard and Cooper (1990) investigated the effects of surfactin on oxygen transfer. The authors concluded that the presence of surfactin makes the oxygen transfer rate independent of gas-liquid interfacial area; consequently, lower oxygen transfer rates lead to lower microbial growth.

In 1994, a new cyclic acylpeptide was reported – halobacillin. It was composed of seven amino acids: Gln.Leu.Leu.Val.Asp.Leu.Ile and C_{15} (fatty acid chain) (Triscbman et al. 1994). One year later, Yakimov et al. (1995) described the chemical identification of lichenysin – new lipopeptide surfactant – which was composed of Glu.Leu.Leu.Val.Asn.Leu.Ile and C_{12}–C_{17} (fatty acid chain). However, as indicated by Soberón-Chávez (2011), halobacillin and lichenysin belong to the same surfactin family sub-class.

5.3.3 Fengycin Family

In 1986, almost simultaneously, Nishikiori et al. (June 1986) of Japan and Vanittanakom et al. (July 1986) of German published the very first reports on the fengycin family.

Nishikiori et al. (1986) studied fengycin production by *Bacillus cereus* BMG302-fF67. The authors named fengycin as plipastatins, because they were researching phospholipase A_2 inhibitors. Thus, fengycins are also known as plipastatins. Plipastatins were purified by Amberlite XAD-7 column (adsorption). Then, the crude powder was solubilized in propanol and filtered in a silica gel chromatography column to obtain plipastatin fractions A1, A2, B1, and B2. Vanittanakom et al. (1986) studied fengycin production by *Bacillus subtilis* F-29-3. Fengycin was produced using nHA as culture medium and purified by HCl precipitation followed by solvent extraction, adsorption (Sephadex LH-20), and silica gel column. Vanittanakom et al. (1986) also verified the antimicrobial activity against many bacteria and fungi (*Oomycota*, *Zygomycota*, and *Ascomycota*). Fengycin did not show antimicrobial effect on bacteria; nevertheless, it did show antimicrobial effect on fungi, particularly on *Basidiobolus microsporus* Til 174, *Conidiobolus coronatus* Til 256, *Pyricularia oryzae* Til 692, *Alternaria kikuchiana* Tii 169, *Curvularia lunata* TO 627, *Stemphylium* sp. Tii 609, and *Rhizoctonia solani* Tii 8104.

5.4 Production and Chemical Structures of *Bacillus* spp. Lipopeptides

Bacillus spp. lipopeptides are amphipathic molecules – cyclic peptide (hydrophilic group) linked to a fatty acid chain (hydrophobic group). These lipopeptides can be classified into three families: iturin, surfactin, and fengycin. Surfactin and iturin have similar structures (Fig. 5.1), in which both have seven α-amino acids; however, their amino acid sequences are different (Table 5.1). Furthermore, the heptapeptide of surfactin is closed by a lactone ring and linked to one β-OH fatty acid, whereas the heptapeptide of iturin is closed by a lactam ring and linked to β-NH_2 fatty acid chain. When compared to surfactin and iturin families, fengycin has higher amino acid sequence – ten α-amino acids (decapeptide). Like surfactin, the peptide of fengycin is closed by a lactone ring and linked to β-OH fatty acid chain (Soberón-Chávez, 2011).

Bacillus spp. lipopeptides are biosynthesized by a large multienzymatic complexes designated as non-ribosomal peptide synthetases. The active form of non-ribosomal peptide synthetases depends upon transcriptional induction, translation, and also posttranslational modification and assemblage (Das et al. 2008). The surfactin (>15 kb) and iturin (>38 kb) operons are composed of four and five genes, respectively: *srfA-A*, *srfA-B*, *srfA-C*, and *srfA-D* and; *fenA*, *fenB*, *fenC*, *fenD*, and *fenE*. Whereas the fengycin operon (>37 kb) is composed of five genes: *FenA*, *FenB*, *FenC*, *FenD*, and *FenE* (Jacques 2011; Cosmina et al. 1993).

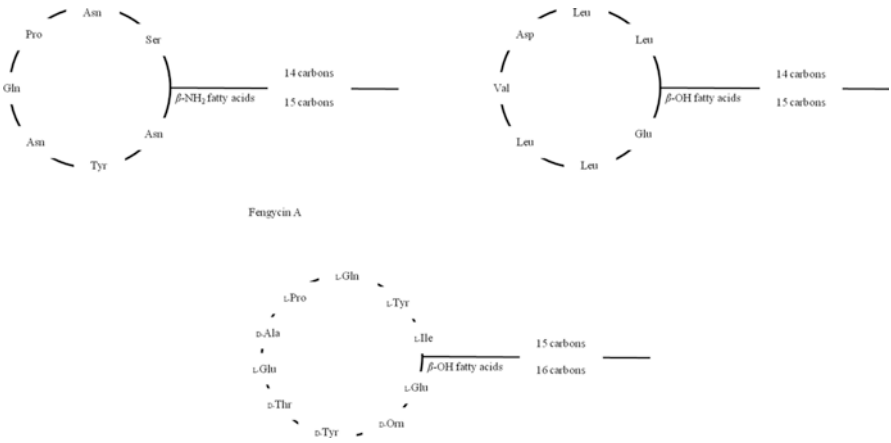

Fig. 5.1 Examples of chemical structures of iturin, surfactin, and fengycin families: iturin A, surfactin, and fengycin A, respectively

Regarding production yield of *Bacillus* spp. lipopeptides, Zhang et al. (2017) showed that the simultaneous overexpressing of *comA* and *sigA* genes (sporulation and antibiotic production) in *B. subtilis* ZK0 enhanced significantly (43-fold) the production of iturin A, which reached 215 mg of iturin A per liter of culture medium: 16 g of glucose, 0.1 g of yeast extract, 3.7 g of KH_2PO_4, 52.1 g of soy peptone, and 7.3 g of $MgSO_4 \cdot 7H_2O$ per liter of water, pH 8, and IPTG at 1 mmol. Thus, the authors have drawn attention to the *quorum sensing* system that competes to the production of *Bacillus* spp. lipopeptides. Jiao et al. (2017) developed the *Pg1-Pg3* promoters that were used to engineer the *B. subtilis* THY-7. The metabolic engineered *B. subtilis* THY-7 reached 9.74 g per liter of culture medium: 70 g/L sucrose, 1 g/L yeast extract, 25 g/L $NaNO_3$, 0.333 g/L KH_2PO_4, 1 g/L $Na_2HPO_4.12.H_2O$, 0.15 g/L $MgSO_4 \cdot 7H_2O$, 7.5 mg/L $CaCl_2$, 6 mg/L $MnSO_4.H_2O$, and 6 mg/L $FeSO_4.7H_2O$ (pH 7). Yaseen et al. (2016) studied the fengycin promoter (P_{fen}) of *B. subtilis* BBG111 and different strategies of bioprocesses (carbon source and oxygenation rate). The authors indicated low oxygen rate (10%), ammonium sulfate (nitrogen source), and mannitol (carbon source) – Landy culture medium, in which very high fengycin yields were obtained at a rate of ≈ 480 mg/L.

5.5 Self-Assembly of *Bacillus* ssp. Lipopeptides in Aqueous Solution

In the past 25 years, the self-assembly properties of *Bacillus* ssp. lipopeptides and their potential applications have been drawing attention to the scientific community. It is well-known that surface-activity compounds form aggregates. In this sense, biological surface-activity molecules have a very a unique structures, in which

subtle differences affect significantly the self-assembly properties, for instance, the relative abundance of surfactin isoforms can affect the critical micelle concentration value and the concentration of surfactin influences the size of micelles, among others (Andrade et al. 2016).

Knoblich et al. (1995), one of the earliest studies on self-assembly of surfactin, evaluated the effect of pH, $CaCl_2$, and NaCl on micelle forms. They described six types of self-assembly of surfactin: (I) spherical 4–5 nm (diameter), (II) spherical 7–8 nm, (III) small ellipsoidal 9 nm (length) × 6 nm (width), (IV) large globular 9–20 nm, (V) ellipsoidal 19 nm × 11 nm, and (VI) cylindrical 40–160 nm (length) × 10–14 nm (width). In addition, $CaCl_2$ (20 mM) and NaCl (100 mM) changed surfactin micelles from cylindrical to spherical or ellipsoidal forms. These results are aligned to Arutchelvi et al. (2014) that showed the relation of four divalent counterions Ni^{2+}, Zn^{2+}, Cd^{2+}, and Ca^{2+} on the self-assembly of the surfactin. It was observed that the higher the concentration of four divalent counterions, the lower the critical micelle concentration of surfactin. Very likely, the divalent counterion decreases the electrostatic repulsion by neutralizing the negative charges of aspartate and glutamate. In addition, they identified the mean aggregation number (critical micelle concentration of surfactin), when surfactin was at 200 μM and the divalent counterion was at 500 μM. The experiment absence of divalent counterion (control) showed ≈100 monomers of surfactin, whereas the presence of divalent counterion decreased the mean aggregation number: 58 (Ni^{2+}), 42 (Zn^{2+}), 62 (Cd^{2+}), and 70 (Ca^{2+}). Obviously divalent counterion affects the shape of surfactant micelles and critical micelle concentration value of surfactin, very likely due to the chances on the secondary structure of surfactin as β-turn → β-sheet (Han et al. 2008).

Regarding the relation between micelle of surfactin (size) and surfactin concentration, Han et al. (2008) used two surfactin solutions 103.6 and 310.8 mg/L, both at pH 7.4 (phosphate buffer). They observed a bimodal distribution (hydronamic radius) one very small peak at 4–6 nm (for both concentrations) and another broad peak centered at 85 nm (103.6 mg/L) and ≈ 108 nm (310.8 mg/L). In addition, they identified the secondary structure of surfactin (micelles), in which low concentrations as 103.6 mg/L and 310.8 mg/L (pH 7.4) lead to β-turn conformation, whereas higher concentrations such as 518 mg/L result in β-sheet conformation. Similarly, Jauregi et al. (2013) studied also the relation between micelle of *B. subtilis* lipopeptides (surfactin and mycosubtilin) and their concentrations, nevertheless at higher range of concentration (10, 50, 100, and 500 mg/ L – Tris buffer 50 mM at pH 8.5). Higher concentration of surfactin (500 mg/L) formed small-sized micelles (5 nm), whereas lower concentrations of surfactin (50 mg/L and 100 mg/L) formed large-sized micelles (≈ 100 nm) – unimodal distribution. The lowest surfactin concentration (10 mg/L) resulted in bimodal distribution: 68 nm and 342 nm. The authors also reported that the micelle size of mycosubtilin decreases proportionally with increasing of mycosubtilin concentration (8 nm at 500 mg/L).

Therefore, the shape and size of micelles (lipopeptide) are dependent on lipopeptide concentration, pH, and divalent counterions.

5.6 Nanoformulations Based on *Bacillus subtilis* Lipopeptides as Controllers of Plant Pathogens: Fruits

In North America, Australia, and New Zealand, the percentage losses of fruits and vegetables at various stages are follows: 20% (production), 3% (post-harvest), 1% (processing and packaging), 12% (distribution and retail marketing), and 28% (consumption) (Porat et al. 2018). It is worth noting that production (20%) represents a parameter that can be improved by more efficient (nano)formulations, in particular due to the microbial contamination, specially fungal (Baños et al. 2013; Panebianco et al. 2015). *Penicillium expansum* (blue mold) is mainly related to apple losses. *Colletotrichum gloeosporioides* is responsible for losses in the production of mango, avocado, and papaya, among others (Baños et al. 2013). *Botrytis cinerea* can significantly affect the production of strawberry, which leads up to 50% strawberry losses (Panebianco et al. 2015). In this sense, in order to minimize fruit and vegetable losses, pesticides are very often overused in agriculture. Thus, it increases the consumer's risk associated with exposure to pesticide residues, in particular residues in food and water. These pesticides can act as endocrine disruptors, neurodevelopmental toxicants, immunotoxicants, and carcinogens, in which the nervous system is especially affected (Kalliora et al. 2018).

Thus, less hazardous antimicrobial molecules have to be researched and then produced at industrial scale (agriculture). *Bacillus* spp. lipopeptides have remarkable antimicrobial properties, in which surfactin family has strong antibacterial activity, and iturin and fengycin families have mainly antifungal activity. Therefore, *Bacillus* spp. lipopeptides are promising alternatives (nanoformulations) to the current pesticides – sustainable chemistry (Palazzini et al. 2016; Soberón-Chávez 2011). Using FAO (2011) data, the NRDC (2012) report indicated that in North America (i.e., the USA and Canada), Australia, and New Zealand, F&V losses totaled 20% during production, 3% during postharvest handling and storage, 1% during processing and packaging, 12% during distribution and retail marketing, and 28% consumption.

5.6.1 Mango, Avocado, and Papaya

C. gloeosporioides is often related to quality losses in mango, avocado, and papaya (anthracnose). In this sense, Il et al. (2010) isolated strains of *B. subtilis* (over 200 bacterial strains) that were grown using M9-broth and then tested cell-free medium against *C. gloeosporioides* (petri dishes on PDA medium). The authors separated the *B. subtilis* lipopeptides in fractions (HPLC) and tested them against *C. gloeosporioides*. Unfortunately it was not described which fluid was used to dissolve the lipopeptide fractions. The fluid used is critical, since it will result in lipopeptide micelles (aqueous solutions) or lipopeptide monomers (solvent solutions) (Jauregi et al. 2013). They concluded that all fractions of fengycin and iturin families are more effective than fraction of surfactin family (Il et al. 2010).

5.6.2 Apple

Dimkic et al. (2013) isolated (I) fungi and (II) bacteria from decayed walnut fruit and cabbage leafs: (I) *Alternaria alternata*, *Aspergillus flavus*, *Botryosphaeria obtusa*, *Mucor* sp., *Colletotrichum acutatum*, *Fusarium oxysporum*, and *Penicillium expansum* and (II) *Xanthomonas arboricola* and *Pectobacterium carotovorum*. Then, they used Luria-Bertani broth for the growth of two *Bacillus subtilis* subspecies. The cell-free supernatants were filtered (0.45 μm) and applied into well-diffusion assay on agar plates against two Gram-negative phytopathogenic bacteria, in which *X. arboricola* showed higher sensitivity to *B. subtilis* compounds, whereas the radial growth inhibition assay was used to evaluate the antifungal property of *B. subtilis* metabolites. However, the *B. subtilis* metabolites were extracted using ethyl acetate and then applied as antimicrobial. After the in vitro experiments, the antimicrobial activity of *B. subtilis* metabolites in vivo was evaluated using apples. The authors concluded that *B. subtilis* metabolites significantly affected all fungi. It is worth noting that both these nanoformulations cell-free supernatant and *B. subtilis* metabolites solubilized in ethyl acetate had, probably, very different solubilization systems. Considering that active substances were based on *B. subtilis* lipopeptides, the cell-free supernatant was composed of micelles of *B. subtilis* lipopeptides, whereas in the nanoformulation based on ethyl acetate, the micelles of *B. subtilis* lipopeptides were destabilized (Jauregi et al. 2013).

Touré et al. (2004) showed that *B. subtilis* (isolated from strawberry) could be efficient in disease control and inhibition of *B. cinerea* proliferation in apple fruits. The authors first tested (in vitro – plate count on PCA medium) the *B. subtilis* strain (GA1) against wide variety of plant pathogenic fungi, *Fusarium graminearum*, *Fusarium oxysporum*, *Pythium ultimum*, *Rhizoctonia solani*, *Rhizopus* sp., *Alternaria* sp., *Aspergillus flavus*, *Aspergillus niger*, *Botrytis cinerea*, *Gaeumannomyces* sp., *Mucor* sp., *Penicillium expansum*, *Trichoderma harzianum*, and *Trichoderma reesei*, in which the range of mycelium growth inhibition was from 24% (*Penicillium expansum*) to 70% (*Botrytis cinerea*). Then, based on these results, they carried out in vivo tests (apples). It is worth noting that, very likely, the antifungal property was related to *B. subtilis* lipopeptides, in which they acted by diffusion against plant pathogenic fungi, instead of nanoformulations.

5.6.3 Strawberry and Grapes

The productivity of strawberry and grape plants is significantly affected by *Botrytis cinerea*, which can lead up to 50% losses (Panebianco et al. 2015). Recently, Toral et al. (2018) studied, first in vitro and then in vivo relations between *Bacillus velezensis* lipopeptides (fengycin, surfactin, and iturin families) and *B. cinerea* (gray mold). The in vitro tests were performed in both solid (mycelium inhibition rate) and liquid media (multiwell culture plates). The in vitro results were very promising (inhibition rates of 60% and 100%, respectively), in which significant inhibitory effect of lipopeptides was observed at concentrations as low as 8 mg/

mL – minimum inhibitory concentration. The in vivo assays were performed in strawberries and grapes, in which *B. velezensis* lipopeptides were solubilized in sterile distilled water (20 mg/mL). It was observed by transmission electron microscopy images that *B. velezensis* lipopeptides led to morphological changes in *B. cinerea*. In addition, the *B. velezensis* lipopeptides increased the antioxidant activity in grapes. These results indicate a close metabolic relation between the antimicrobial effect of *B. velezensis* lipopeptides and the (bio)synthesis of antioxidants in fruits.

Bacillus spp. lipopeptides act effectively against phytopathogens (fruits) such *C. gloeosporioides*, *P. expansum*, *X. arboricola*, *P. expansum*, and *B. cinerea*, among others. Therefore, *Bacillus* spp. lipopeptides are one of the most promising alternatives (nanoformulations) to the current pesticides.

5.7 Perspectives

- Reduction (%) of fruit and vegetable losses (production) by more efficient and sustainable pesticides.
- Partial replacement of chemical pesticides by *Bacillus* spp. lipopeptides.
- Evaluation of emulsifiable concentrate system using *Bacillus* spp. lipopeptides.
- Investigation of the relationship between antimicrobial properties and nanoformulations.

5.8 Conclusions

Currently, chemical pesticides are essential for agriculture and food production. Nevertheless, they are potential hazards to humans and environment (carcinogens and neurodevelopmental toxins, among others). When compared to emulsion (pesticides), nanoemulsion formulations show higher stability and higher bioactivity; however, preparation of nanoemulsion formulations is not cost-effective. *Bacillus* spp. lipopeptides can be classified into three families: iturin, surfactin, and fengycin. They are amphiphilic molecules and consists of a cyclic peptide (hydrophilic group) linked to a fatty acid chain (hydrophobic group), which shows antimicrobial activity against phytopathogens (e.g., phytopathogens that attack fruits such as mango, avocado, and papaya, apple, and strawberry and grapes) such as *C. gloeosporioides*, *P. expansum*, *X. arboricola*, *P. expansum*, and *B. cinerea*. Thus, *Bacillus* spp. lipopeptides could be used to, at least partially, replace chemical pesticides in nanoformulations. In this sense, there is not much information about synergistic or antagonistic effects of chemical pesticides and *Bacillus* spp. lipopeptides, as well as the relationship between antimicrobial properties and nanoformulations (shape and size of micelles).

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