Chapter 1

Nanoprobiotics: when technology meets gut health

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Abstract. Nanotechnology is a fast-rising industry not defined by a single field of research, but as the convergence of disciplines, such as chemistry, biology, physics, mathematics and engineering, which exploits the benefits of nanoscale dimensions and characteristics for application in the macroworld. Current applications vary widely from nanorobotic industry to simple household items. But the combination of such phenomena with probiotic science, another emerging and potentially promising area for the prevention and treatment of several human gastrointestinal and extraintestinal disorders using beneficial microorganisms, gives birth to "Nanoprobiotics", a field that focuses on the application of nanoscience into the probiotic-related world. In this chapter we will navigate through the basic nanotech and probiotic knowledge, the current technologies employed with success for probiotic delivery and ultimately, discussing what possibilities lie ahead in the nanoprobiotic future.

1.1 Nanotechnology: the who, what, why, how and where

1.1.1. Who

The first definition describing technological processes that allowed the achievement of higher precision and ultra-small dimensions (on the nanometer scale, 10^{-9} m) was attributed by Norio Taniguichi, of Tokyo Science University, where he stated that "Nano-technology mainly consists of the processing of separation, consolidation, and deformation of materials by one atom or one molecule" (Taniguchi, 1974). Born from the Greek word "nanos", meaning "dwarf", the prefix nano- refers to the factor of 10^{-9}, in the international system for units of weights and measures. Interestingly, the concept of the engineer operating at a molecular level is not novel. In the year of 1959, during a lecture titled "There's plenty of room at the bottom" given at the American Physical Society meeting at Caltech, Nobel laureate physicist Richard Feynman suggested that laws of physics allowed the controlled arrangement at an atomic level, envisioning the technology for material manipulation and control at a nanoscale, without ever naming it as such (Feynman, 1960).

1.1.2. What

Following decades of breakthrough in this ever-modernizing era, despite the many definitions that can be encountered (Theis et al., 2006), the consensus lies on the criteria that nanotechnology is about the design, characterization, production and application of materials and systems by controlling the shape and size at the nanoscale, more specifically below 100 nm (Bhushan, 2010). It is, however, important to differentiate it from nanoscience which is,in essence, an extension of the existing science areas to the study of the phenomena and manipulation of living and non-living matter into the nanometer (Hornyak, 2009; Jeevanandam et al., 2018). In fact, another criterion that should be included in the definition is that a nanoparticle (NP) or nanomaterial must be engineered, or synthetically produced (Bhushan, 2010). Is important to note that, NPs are not a human invention, and the deliberately manufactured NPs are in fact a minority. They exist widely in nature in the form of photochemical and volcanic activity products, mineral composites (such as oxides and carbonates) and magnetotactic bacteria (Griffin et al., 2017; Jeevanandam et al., 2018). Additionally, incidental NPs have been created as the byproducts of processes such as

combustion of diesel fuels (Sioutas et al., 2005). Regarding the engineered NPs, they can be further classified based on the number of dimensions that fall outside of the nanometer range, meaning that a zero-dimensional nanostructure would have all its dimensions fitted into the nanoscale whereas a two-dimensional (2D) nanostructure would have one dimension within the nanometric diameter and two in the macrometric diameter (Table 1).

Table 1- Nanostructures dimensional classification. Adapted from Ngô and Van de Voorde, (2014).

Nanoscale Dimensions	Examples
0D "zero-dimensional"	Quantum dots.
1D "one-dimensional"	Nanofibers, nanotubes, nanorods, and nanowires.
2D "two-dimensional"	Nano-coatings, nanofilms, nanolayers, and graphene.
3D "three-dimensional"	Bulk powders, dispersions of nanoparticles, bundles of nanowires, and nanotubes as well as multi-nanolayers.

1.1.3. Why

The nanoscience application phenomenon is an effort that requires the involvement of various research fields and different areas of technology and, lies on the premise that fabrication of materials, devices and systems at this scale will allow the enhancement of the design and production of materials with subsequent applications in traditional industries (Abdullaeva, 2017a). The improvement in the bulk properties of materials is based on the following rationale: nanostructures affect the mechanical, chemical and electrical properties when employed, by increasing the main material surface area. Secondly, when the matter is at the nanoscale its behavior is ruled by quantum effects, which affect the electrical, optical and magnetic performance of materials (Davies, 2007).

1.1.4. How

Irrespective of the type of materials utilized and the purpose of the fabrication, there are two main techniques to manipulate matter into the nanoscale: top-down and bottom-up approaches (Fig. 1).

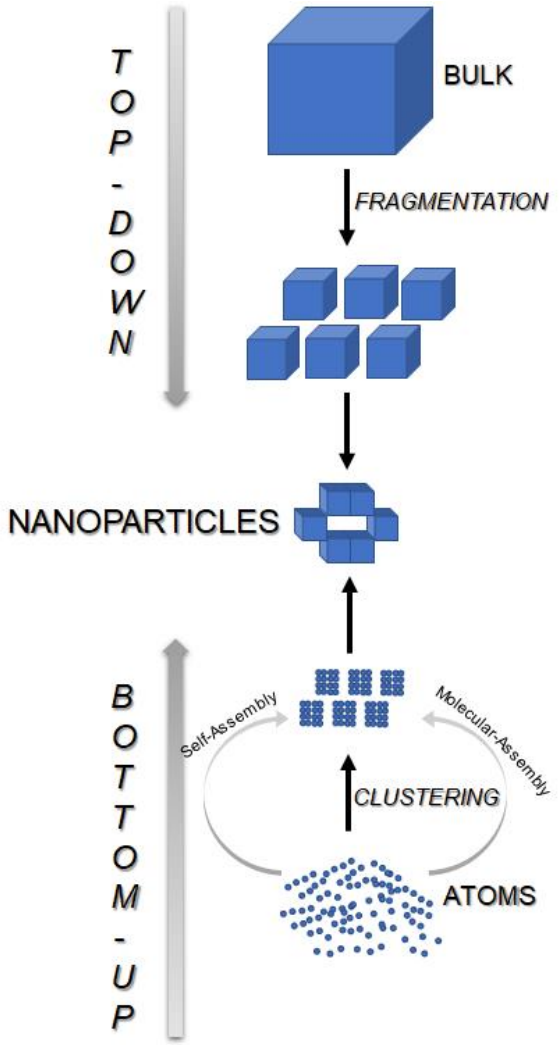

Fig. 1- Top-down" and "bottom-up" approaches for the synthesis of nanomaterials (Adapted from Galstyan et al, 2018; Rawat et al., 2015).

In the top-down approach, the bulk material undergoes a restructuration producing smaller components, the nanomaterials (e.g. lithography) (Liddle and Gallatin, 2016). This usually requires laborious processes, such as milling, that entail more costs, are energy expensive and involve the usage of toxic reagents, and sometimes the resulting product is not reproducible. In contrast, in bottom-up approaches, nanomaterials are assembled from

their basic building blocks (atoms, molecules), which are usually nanoobjects with the appropriate features for the bulk material. This can further occur via 'self-assembly', in which there is a natural structural rearrangement determined by the interactions among the individual components, requiring little intervention as possible (e.g. protein folding, chemical synthesis) (Whitesides and Grzybowski, 2002); and via 'positional assembly' (or molecular assembly), in which atoms and molecules are purposefully manipulated. Overall, positional assembly, offers greater control over fabrication, but is still viewed more as a visionary concept, not yet suitable for industrial applications (e.g. robotic molecular manufacturing systems) (Trobe and Burke, 2018). Bottom-up 'self-assembly' occurrences are abundant in nature (e.g. protein folding, lipid bilayer formation, colloid crystallization, bacterial colonies formation, weather patterns, solar systems) (Whitesides and Grzybowski, 2002) and, chemists have been exploiting for centuries the self-organizational physicochemical principles for the design of molecular structures with specific properties (Wong et al., 2009). Some of the most important bottom-up processes in nanomaterial production that lead to the manufacturing of complex structures with controlled dimensions and morphology are, among others, precipitation reactions (microemulsions, micelles and liposomes) (Silva et al., 2015); and sol-gel processes (production of a gel from powder-shaped materials) (Gonçalves, 2018).

1.1.5. Where

For all the reasons previously highlighted, it should be clear by now that "nanotech" products have found their way into several areas of industry. Indeed, the technology has already become industrially relevant (e.g. chemical and automobile sector), such as in the form of nanocomposites, nanoclays, nanocoatings, nanopaints, among many others (Stark et al., 2015). Granted, nanomaterials are not exclusively used for industrial purposes or even "sci-fi" type scenarios, but also incorporated into many commercially available products with the purpose of exploiting the benefits of the nanoscale at lower costs (Iavicoli et al., 2014). The application of nanotechnology into common products such as UV sunscreen filters, containing titanium dioxide and zinc oxide (Dastjerdi and Montazer, 2010); textiles, containing nanosilver particles with antimicrobial properties (Morais et al., 2016); and microelectronics (nanochips, nanobatteries, touch screens, sensors) (Abdullaeva, 2017b) is relatively well-known and becoming ubiquitous in our daily lives.

Notwithstanding the great impact that facilitates our daily lives, the extremely diverse facets of nanotechnology research and development also revolutionized biological technologies, which benefited, among others, the food and biomedical sectors.

1.2 Nanotechnology in human health

In 1996, forty years after Feynman's famous speech, experimental chemist Richard Smalley received a Nobel Prize for the discovery of fullerene, a C60 spherical molecule. He recognized the significance of nanosized materials in medicine and related areas stating that "human health has always been determined on the nanometer scale; this is where the structure and properties of the machines of life work in every one of the cells in every living being. The practical impact of nanoscience on human health will be huge" (Burgess, 2012).

Indeed, nanobiotechnology, cross-links the concepts of nanotechnology into biological systems, enabling the control over biological processes that occur at the nanoscale (including the microbial physiology). This emergent field benefits areas intimately connected to human health, such as food-related products and medicine, by improving and developing new analytical tools, diagnostic techniques and therapeutic protocols (Boulaiz et al., 2011). In nanomedicine, the employment of "nanotech" knowledge and tools for the diagnostics, treatment, and prevention of diseases and traumatic injuries, two distinct but interconnected areas have been positively impacted: diagnostics/prevention and therapeutics (Freitas, 1999). Nanoparticle-enabled diagnostics is an increasingly emerging area, with NPs being used as contrast media for imaging technologies such as magnetic resonance and computed tomography, enhancing both the state-of-the-art sensitivity and accuracy, translating into high-resolution cellular imaging (Li et al., 2016), which has a major impact specially in early detection diseases like cancer (Kumagai et al., 2013) and Alzheimer's (Keating, 2005). In terms of nanomedicine therapeutics, regenerative medicine (e.g. tissue engineering) is still in early development, with only existing concepts and prototypes (Xavier et al., 2015; Krueger et al., 2016). In fact, most nano-applications are focused on drug delivery vehicles, seeking improved substance-targeting, controlled release and bioavailability (Alvarez et al., 2017). These include (i) polymeric micelles, (ii) dendrimers, (iii) polymeric nanoparticles, (iv) polyplexes, and (v) liposomes, all possessing different chemical structures and biological characteristics (Blanco et al., 2009).

Nanotechnology impact in food science and food microbiology is evident in operations such as agricultural productivity enhancement (Sekhon, 2014), water treatment and (Singh et al., 2017), which can benefit the quality of available foods and drinking water. Furthermore, the multidisciplinary facets of nanotechnology are also expected to significantly enhance functional foods and nutraceuticals development. The improved delivery of bioactive compounds and micronutrients (better encapsulation agents generate optimized protection, targeting and integration in food matrices), increased bioavailability thereof and food contaminants detection (e.g. rapid and sensitive isolation and detection of foodborne pathogens such as *Escherichia coli* or *Staphylococcus aureus*; and of chemicals) are some of the practical applications of nanotechnology that rapidly translate into better human health (Singh et al., 2017). Alongside, with the more recent emergence of probiotics as an exciting and promising strategy to prevent and treat inflammatory/metabolic dysbiosis related conditions (Almeida et al., 2019; Markowiak and Slizewska, 2017), this "miniaturization" can add value to a wide spectrum of immobilization techniques such as encapsulation. As a result, "Nanoprobiotics" arises as a term standing for the integration of probiotic organisms in nanotechnology. In fact, the nanoprobiotics concept might overlap with nanomedicine, since it relates to the production of targeted delivery and release of specific probiotics and bioactive food ingredients (Sekhon 2010). Nanomaterials for encapsulation comprehend traditional agents, such as alginate-Na, implemented in nanoemulsions, liposomes, micelles (Silva et al., 2015) which can be integrated into food matrices (functional foods) or delivered as therapeutic formulations. Thus, inspired by nanotechnology, both food and pharmaceutical industries have achieved great advances in the development of novel delivery systems into a probiotic related field. As discussed later, this has contributed to enhancement of the effectiveness and efficiency of these bioactives to improve human health.

1.3 Probiotics

1.3.1 Concepts, health benefits and requirements

The word "probiotic" comes from the Greek, meaning "for life" and it is a term widely used in this last century mainly in nutrition and health contexts (Markowiak and Slizewska 2017). Historically, the concept of probiotics was first put onto a scientific basis by the work of Elie Metchnikoff

performed at Pasteur Institute. In fact, Metchnikoff hypothesized that the intake of fermented dairy products with lactic acid bacteria, such as yogurt, was linked with enhanced health and longevity in the elderly Bulgarian population (Metchnikoff 1907). Thereafter, the definition of probiotics has been modified and evolved over time. The current definition of probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" is widely accepted and it was generated in 2001 by Food and Agriculture Organization of the United Nations and World Health Organization (FAO and WHO 2001) and recently maintained and reinforced by International Scientific Association for Probiotics and Prebiotics (Hill et al. 2014).

In the last decades, the use of probiotics as a way to prevent and treat a panoply of human gastrointestinal and extraintestinal disorders has gained a growing number of supporters, among clinicians and researchers (Figueroa-González et al. 2011; Pintado et al. 2014). Indeed, the consumption of probiotics has been linked with several health benefits including improvement of intestinal health and alleviation of symptoms associated with inflammatory enteral conditions (Saez-Lara et al. 2015), enhancement of lactose tolerance (Oak and Jha 2019), enhancement of the immune response and prevention of allergic disease (Wang et al. 2019), hypocholesterolemic (Ishimwe et al. 2015) and anticancer effects (Marinelli et al. 2017). As depicted in Fig. 2, several mechanisms of action have been associated with probiotic benefits, including production of antimicrobial substances like hydrogen peroxide, bacteriocins or organic acids (Vandenbergh 1993); competition with pathogens for adhesion sites (Collado et al. 2007) and nutrients (Deriu et al. 2013), degradation of toxins and blocking of toxin receptors (Castagliuolo et al. 1996) as well as modulation of immune responses (Ashraf and Shah 2014).

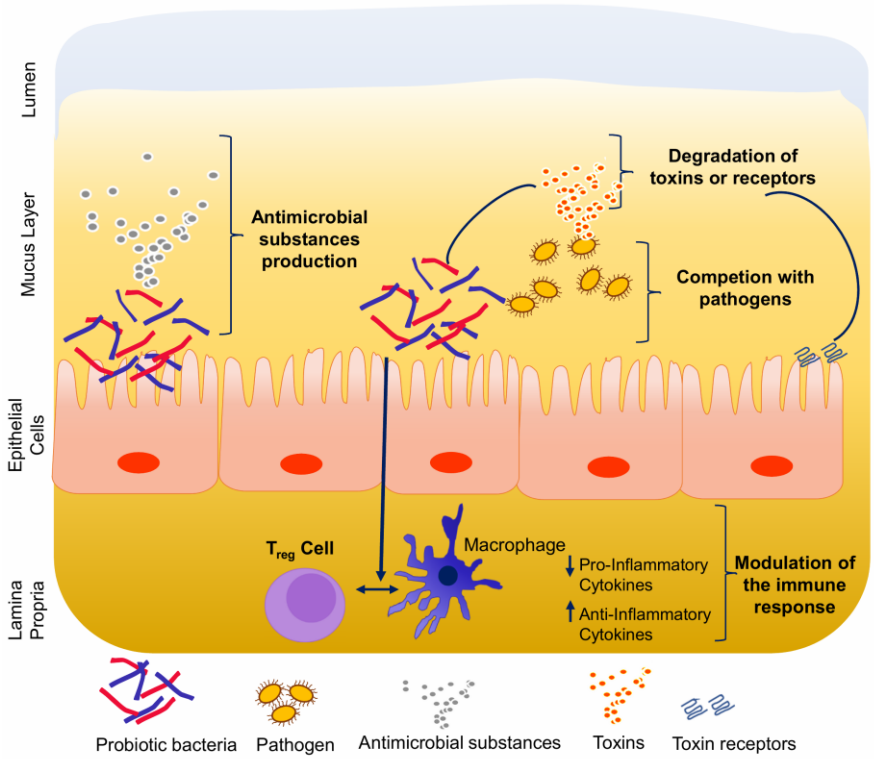

Fig. 2- Mechanisms of action of probiotics

Currently, the microbial strains must meet certain requirements to be considered as a potential probiotic. According to the guidelines suggested by the FAO/WHO, every potential probiotic strain must be correctly identified, followed by several *in vitro* assays with the aim to investigate their functional properties (FAO and WHO 2001). Resistance to gastrointestinal conditions, adherence to mucus and/or intestinal epithelial cells, and antimicrobial activity against potential pathogens are the main properties that potential probiotic must possess. There are numerous commercial probiotics in the market, but still, there is a demand for novel probiotic strains with better properties than existing ones. Thus, additional probiotic characteristics should be considered including, cholesterol reduction ability, antioxidant and anti-cancer effects (Shokryazdan et al. 2017). To note that, probiotic characteristics are not related with the genus or species of a microorganism, but with certain strains of a particular species (Jacobsen et al. 1999). After taxonomic identification and functionality properties research, potential probiotics must be characterized in terms of safety parameters and technological usefulness (FAO and WHO 2001). The safety parame-

ters are related to the origin of strain, the absence of association with pathogens and the antibiotic resistance profile. Meanwhile, technological robustness of probiotic strains is related to their ability to survive and maintain their biological properties throughout the storage and distribution processes (Markowiak and Slizewska 2017).

1.3.2 Conventional and potential next-generation probiotics

Conventionally, probiotics have been isolated from such biological sources as the gut or derived from fermented foods (such as yogurts, fermented milk and cheeses). Importantly, they have been classified as Generally regarded as safe (GRAS) at the strain level by the Food and Drug Administration (FDA) or included in Qualified Presumption of Safety (QPS) at the species level by the European Food Safety Authority (EFSA) (Martín and Langella 2019). As described in Table 2, the probiotics available in the market contain microorganisms mostly belonging to genera *Lactobacillu*s and *Bifidobacterium*. Nevertheless, there are also some members of *Bacillus* and *Streptococcus* for bacteria and yeast strains belonging to the genus *Saccharomyces* (Gomes et al. 2017). These classical probiotic strains have a long history of use and they are well-characterized regarding to safety point of view (Martín and Langella 2019). However, in some cases strains display limited effects on the human gut microbiota evoking the need for a better selection of microbial strains and improvement in the development of their delivery systems (Neef and Sanz 2013).

Table 2- Probiotic microorganisms available in the market. Adapted from Gomes et al 2017

***Lactobacillus* genus**	***Bifidobacterium* genus**	**Other microorganisms**
L. acidophilus	*B. adolescentis*	*Bacillus coagulans*
L. buchneri	*B. animalis*	*Bacillus subtilis*
L. brevis	*B. animalis* subsp. *lactis*	*Streptococcus thermophilus*
L. casei	*B. catenulatum/ pseudocatenulatum*	*Saccharomyces cerevisiae* variant *boulardii*
L. crispatus	*B. bifidum*	
L. delbrueckii subsp. *bulgaricus*	*B. breve*	
L. delbrueckii subsp. *lactis*	*B. lactis*	
L. fermentum	*B. longum*	
L. helveticus		

L. paracasei
L. paracasei subsp. *paracasei*
L. pentosus
L. plantarum
L. reuteri
L. rhamnosus
L. salivarius
L. salivarius subsp. *salivarius*

In the last years, several bacterial species involving genera other than *Lactobacillus* and *Bifidobacterium* with promising outcomes in the treatment and prevention of diverse metabolic and inflammatory diseases have been proposed as potential next-generation probiotics, NGPs for short (Almeida et al. 2019). Non-conventional candidate strains include *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Eubacterium hallii*, *Bacteroides fragilis, Bacteroides uniformis* and members of the Clostridia clusters IV, XIVa, and XVIII (El Hage et al. 2017; Almeida et al. 2019). These novel candidates have been mainly identified based on comparative analysis of microbiota compositions between both healthy and unhealthy subjects (Qin et al. 2012; Lopez-Siles et al. 2015; Martín and Langella 2019). In contrast to classical probiotics, the next generation probiotics are not yet available in the common market. The introduction of these NGPs, also termed as live biotherapeutics products (O'Toole et al. 2017), in the nutraceutical/pharmaceutical market requires a full assessment of safety parameters and an in-depth characterization of their beneficial effects on the host (Brodmann et al. 2017; El Hage et al. 2017). Moreover, the development of delivery vehicles for these novel probiotics are urgently needed due to their stringent survival conditions. These delivery systems should simultaneously confer greater microbial viability, high efficacy of probiotic action and should be safe for human use (Almeida et al. 2019). Thus, NGP introduction and implementation in the market demands a close interaction between research/clinical institutions, pharmaceutical/food industries and regulatory agencies.

1.3.3 Alternatives strategies to promote health benefits: knowing other -biotics

The current interest in actively improving the host health via modulation of the intestinal microbiota is progressively becoming more prominent. Traditionally, this has been an action endeavored by the use of probiotics. However, data suggests that pre-, syn- and postbiotics also exhibit as correspondingly important sources to positively impact human wellness, by improving gut microbiome composition and stimulating optimal immune system function (Miniello et al. 2015).

Prebiotics is a term that encompasses a wide range of substrates that are selectively utilized by friendly host microorganisms, conferring health benefits (Gibson et al. 2017). Indeed, prebiotics selectively stimulates beneficial microorganisms present in the intestinal tract, affecting their fermentation activity and subsequently influencing the short-chain fatty acids (SCFA) level, which leads positive health effects (Van-Den-Abbeele et al. 2013; Sivieri et al. 2014). Commonly, most prebiotics used in human nutrition include fructooligosaccharides, galactooligosaccharides, inulin, xylooligosaccharides, lactitol, lactosucrose, lactulose, soy oligosaccharides and transgalactooligosaccharides (Markowiak and Slizewska 2017). However, several non-carbohydrate structures such as polyphenols, long chain polyunsaturated fatty acids, minerals or vitamins, has also been classified as prebiotic (Steinert et al. 2016). In fact, prebiotics can be naturally present in certain foods since some dietary fibers are prebiotics. But, they may also be added to foods with a purpose to enhance their nutritional and health value (Markowiak and Slizewska 2017). For the classification of a food ingredient as prebiotic, it is required the fulfillment of five criteria, namely, resistance to digestive processes, selective fermentation by potentially beneficial bacteria in the colon, beneficial effect on the health of the host, selective stimulation of probiotics and stability to food processing treatments (Wang 2009). In a sense, pro- and prebiotics are therefore interdependent, in which prebiotics encourage probiotics intestinal population to flourish. The recognition of this close relationship led to the development of synbiotic products. Synbiotics are “mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, thus improving host welfare” (Martín and Langella 2019). Due to the high number of possible combinations involving prebiotics and probiotics, the synbiotics products seems to be a compelling approach for modulation of human gut microbiota (Markowiak and Slizewska 2017). Thus, the health benefits

claimed by synbiotics consumption by humans include increased abundance of lactobacilli and bifidobacteria (Van-Zanten et al. 2014), improvement of clinical course of cirrhotic patients (Fukui 2015), attenuation of inflammatory markers in patients with nonalcoholic fatty liver disease (Eslamparast et al. 2014) and improvement of symptoms associated to atopic dermatitis in children (Ibáñez et al. 2018).
Importantly, recent evidences suggest that bacterial viability is not a mandatory requirement to attain the health-promoting effects since not all mechanisms, nor clinical benefits, are directly associated to the viability of probiotic microorganisms (Choi et al. 2006; Cicenia et al. 2014). Such understanding has been cementing postbiotics as an emerging field of research, revolutionizing probiotic science. Postbiotics, also known as either metabiotics, biogenics, metabolites or cell-free supernatants are soluble factors (products or metabolic byproducts) secreted by live bacteria or released after bacterial lysis (Cicenia et al. 2014; Aguilar-Toalá et al. 2018). Therefore, postbiotics can comprehend enzymes, peptides, teichoic acids, peptidoglycan-derived muropeptides, polysaccharides, cell surface proteins, vitamins, plasmalogens, SCFAs, and organic acids and other complex biomolecules, and they are known to be important in regulating intestinal biological activity (Aguilar-Toalá et al. 2018). These compounds have drawn attention due to their clear chemical structure, dose and safety parameters, long shelf life and the content of various signaling molecules which may have anti-inflammatory, immunomodulatory, anti-obesogenic, antihypertensive, hypocholesterolemic, anti-proliferative and antioxidant effects (Shenderov 2013; Aguilar-Toalá et al. 2018).

1.4 Encapsulation of probiotics

Encapsulation has arisen as a strategy to mitigate some of the limitations to the use of probiotics in food and therapeutic applications. Encapsulation of bioactive components has been used for a long time in many applications in the food and pharmaceutical industries: preventing biodegradation and undesirable chemical reactions, masking flavours, colours and odours, improving solubility, providing sustained and controlled release, etc. (Nedovic et al. 2011; Santiago and Castro 2016). Probiotic encapsulation has been mainly used to protect the cells against an adverse environment rather than a controlled release. The term encapsulation is related to the entrapment of several substances (active ingredients) within another material (encapsulant). Depending on the method and materials used we may obtain

microcarriers, with sizes ranging from 1 to 1000 μm, or nanocarriers with sizes ranging from 1 to 1000 nm (Quintanilla-Carcavajal et al. 2010; Suganya and Anuradha 2017). However, this size threshold is controversial and is not unanimous; for instance, the European Food Safety Authority (EFSA Scientific Committee 2011) refers to nanoparticles as engineered nanomaterials that have at least one dimension in the range of 1–100 nm. As probiotic cells typically have sizes ranging from 1 to 5 μm it is not possible to encapsulate them in nanoparticles. Particle size is also important, particularly for food applications, as it may negatively affect the sensory properties of the food-product (Champagne and Fustier 2007; Heidebach et al. 2012). For instance, Hansen et al. (2002) reported a particle-size below 100 μm to avoid a "gritty" sensation when consumed. However, particles with larger diameters, and thus with higher volume-to-surface-ratio, may have an increased probiotic protective effect (Anal and Singh 2007). A proper balance between sensory and protective properties must be taken into consideration before application of probiotic microparticles in food products. Another difficulty of probiotic encapsulation is the fact that they must be kept alive as their health effects are dependent on their viability after consumption. This means that materials and methodologies used for probiotic microparticles production should be carefully evaluated.
In the following sections, a brief and updated overview of the main materials and techniques used for probiotics microencapsulation is presented. In Table 3, a selection of recently published works (over the last four years) dealing with probiotic encapsulation is presented.

Table 3 - A selection of studies dealing with probiotic encapsulation published in the last four years (2016-2019)

Probiotic strain	**Encapsulant material**	**Encapsulation technique**	**Particle size (μm)**	**Main achievements**	**Reference**
Bacillus coagulans BC	Alginate, chitosan	Layer by layer (LbL)	Not given	Improved survivability *in vitro* simulated gastrointestinal conditions	Anselmo et al. (2016)
L. plantarum NCDC 012, L. casei NCDC 297 and *L. brevis*	β-glucan	Emulsification	Not given	Improved survivability *in vitro* simulated gastrointestinal conditions and under thermal treatments	Shah et al. (2016)
B. bifidum BB01	Xanthan, chitosan	Extrusion	Not given	Improved survivability *in vitro* simulated gastrointestinal conditions. Increased storage stability in yogurt (21 days storage at 4	Chen et al. (2017b)

				°C and 25 °C)	
L. plantarum ATCC 13643	Carboxymethyl cellulose, k-carrageenan	Extrusion	Not given	Improved survivability *in vitro* simulated gastrointestinal conditions	Dafe et al. (2017)
L. acidophilus ATCC 4356	Chitosan, phytic acid	Extrusion (electrostatic)	1300-1500	Improved survivability during storage and exposure to acid	Kim et al. (2017)
L. casei DSM 20011, *L. reuteri* DSM 20016 and *L. delbrueckii* subsp. *bulgaricus* DSM 2008)	Alginate	Extrusion (vibration nozzle)	600-800	Improved survivability under exposure to low acid conditions	Olivares et al. (2017)
L. paracasei A13 and *L. salivarius* subsp. *salivarius* CET 4063	Alginate	Emulsion with high-pressure homogenization	< 100	Improved survivability *in vitro* simulated gastrointestinal conditions. Production of fermented milk with improved functionality and with enhanced sensory properties.	Patrignani et al. (2017)
L. acidophilus TISTR 1338	Alginate, egg albumin, stearic acid and cassava starch	Electrospraying and fluidized bed coating.	450	Improved survivability under moist heat treatment (70 ± 0.5 °C, 100% relative humidity, 30 min.)	Pitigraisorn et al. (2017)
B. animalis subsp. *lactis* BB12	full-fat goat's milk, inulin or oligofructose	Spray drying	Not given	Improved survivability *in vitro* simulated gastrointestinal conditions and under thermal treatments	Verruck et al. (2017)
L. plantarum ATCC 8014	Alginate, Chitosan	Electrospraying	300-400	Improved survivability during storage and exposure to acid	Zaeim et al. (2017)
L. plantarum CECT 220 and *L. casei* CECT 475	Soybean protein concentrate, maltodextrin and oligofructose-	Coacervation followed by spray drying	11	Improved survivability *in vitro* simulated gastrointestinal conditions and storage (25°C, >100 days)	Gonzalez-Ferrero et al. (2018)

	enriched inulin				
L. acidophilus La-5.	Gum Arabic, inulin, hi-maize, and trehalose	Spray drying.	6.7 -19.3	Improved survivability *in vitro* simulated gastrointestinal conditions and thermal resistance. Increased storage stability (120 days, 25°C)	Nunes et al. (2018)
L. acidophilus LA5	Alginate, gelatin, FOS	Extrusion (atomization)	Not given	Improved survivability *in vitro* simulated gastrointestinal conditions and during the storage in yogurt	Silva et al. (2018a)
L. *acidophilus* LA3 and *B. animalis* subsp. *lactis* BLC1	vegetable fat, gum Arabic, gelatin	Spray chilling and electrostatic interaction	79 - 84	Improved survivability *in vitro* simulated gastrointestinal conditions as well as under different stress conditions (low pH, high level of sucrose and NaCl)	Silva et al. (2018b)
L. casei 39392	Whey protein	Electrospraying and transglutaminase crosslinking	3.1	Improved survivability *in vitro* simulated gastrointestinal conditions and storage (20°C, 120 days)	Alehosseini et al. (2019)
B. longum DD98	Alginate, chitosan	Emulsification	190	Improved survivability *in vitro* simulated gastrointestinal conditions, under thermal treatments and storage (25°C, 180 days)	Ji et al. (2019)
B. longum	Soy protein isolate and carrageenan	Complex coacervation	Not given	Improved viability during storage (4 °C), pasteurization (85 °C for 5, 10 and 30 min) and *in vitro* dynamic gastric and intestinal digestion.	Mao et al. (2019)
L. paracasei BGP1 and *L. rhamnosus* 64	Vegetable fat, gum Arabic, gelatin	Complex coacervation	80	Improved stability in the presence of salt and in simulated gastrointestinal conditions. Encapsulated microorganisms maintained their viability and functionality during storage (120 days)	Matos-Jr et al. (2019)
L. plantarum	Gelatin, gum Arabic	Double emulsification and complex coacerva-	66 -106	Improved survivability *in vitro* simulated gastrointestinal conditions and storage (8 °C and − 18 °C, 45 days).	Paula et al. (2019)

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L. acidophilus	Alginate, rice bran, inulin, Hi maize	Extrusion (atomization)	80 -118	Improved survivability *in vitro* simulated gastrointestinal conditions. Alginate, rice bran and Hi- maize microparticles maintained viable probiotics for 120 days. At −18 °C, only inulin remained stable for 120 days. At 7 °C, rice bran and inulin preserved viable probiotics over 120 days of storage	Poletto et al. (2019)
S. boulardii CGMCC 10381and *E. faecium* CGMCC 2516	Alginate	Emulsification	300-500	Improved survivability *in vitro* simulated gastrointestinal conditions and under thermal treatments	Qi et al. (2019)
B. bifidum	Alginate, zein	Extrusion	1210-1720	Improved survivability *in vitro* simulated gastrointestinal conditions and storage (4°C)	Riaz et al. (2019)

1.4.1 Encapsulating materials

The choice of encapsulation materials is of extreme importance to ensure the wanted protection as well as production efficiency and compatibility with the desired application (Chen et al. 2017a). For food applications polysaccharides, proteins and lipids have been the obvious choices and thus the most used encapsulation materials.

1.4.1.1 Polysaccharides

Several polysaccharides such as alginate, pectin, chitosan, gellan gum, k-carrageenan, starch, xanthan gum, etc. have been used for probiotic encapsulation (Călinoiu et al. 2019; Kavitake et al. 2018; Kwiecien and Kwiecien 2018; Martín et al. 2015).
Alginate is probably the most extensively used biopolymer for encapsulation. It is an anionic, linear heteropolysaccharide composed of D-mannuronic and L-guluronic acids. The composition and the sequence of L-guluronic acid and D-mannuronic vary widely, depending on the source, and thus so its functional properties. In the presence of divalent cations al-

ginate forms a gel with an "egg-box" structure (Martín et al. 2015). Beads can be formed by dripping a mixture of a sodium alginate solution with a cell suspension into a solution containing cations (usually Ca^{2+} in the form of $CaCl_2$). Alginate microparticles can be obtained by an external or internal gelation process. In the first case, the microparticles are produced by the formation of a water-in-oil emulsion, usually stabilized by surfactants, such as Tween 80. The alginate is then gelled by the addition of calcium containing solution to the emulsion. In the internal gelation process, alginate is first mixed with an insoluble calcium salt (most often calcium carbonate) before the formation of a water-in-oil emulsion. The addition of an organic acid (most often acetic acid) to the emulsion releases calcium ions (and carbonic acid) which will initiate alginate gelation. Although probiotic bacteria can be well encapsulated into the alginate particles with high viability, the structure of the cross-linking polymers formed by divalent cations are turned out to be porous, which cause the easy entry and exit of H^+ and other detrimental substances leading to the damage of cells (Liu et al. 2019). Other disadvantages are related to the scaling-up of the process that is difficult. These drawbacks can be overcome by combining alginate with other polymers or by coating capsules with other compounds or using different additives for structural modification of the alginate (Kavitake et al. 2018). Silva et al. (2018a) produced microbeads of alginate-gelatin and alginate-gelatin-fructooligosaccharides by external gelation, to improve the viability of *L. acidophilus* LA5 when exposed to the gastrointestinal tract and during storage when added to yogurt. Riaz et al. (2019) used zein as coating material of alginate microbeads to improve survival during gastric transit and storage of *B. bifidum.* Other researchers used chitosan as a coating material. Ji et al. (2019) used chitosan-coated alginate microcapsules obtained by emulsification and internal gelation method to extend the viability of *B. longum* DD98. Anselmo et al. (2016) produced chitosan and alginate microparticles using a LbL encapsulation process (see also section 1.4.2.2.3) to increase *Bacillus coagulans* resistance against acidic and bile salt insults.

Chitosan is also an extensively used biopolymer for probiotic encapsulation used mainly in combination with other polysaccharides (for instance alginate as described above). It is an aminopolysaccharide derived from chitins, composed of β-(1,4) linked D-glucosamine and N-acetyl-D-glucosamine. The main advantages of chitosan coating are a unique cationic character, high biocompatibility, non-toxicity, and biodegradability (Călinoiu et al. 2019). However, it has antimicrobial properties and therefore it's not suitable to be used as sole encapsulant material for creating probiotic delivery systems (Kwiecien and Kwiecien 2018).

Other polysaccharides such as inulin, fructooligosaccharides and other dietary fibers have also been increasingly used not only for their prebiotic properties but also for their protecting capabilities (Table 3).

1.4.1.2 Proteins

Several food proteins have been used alone or combined with other compounds to encapsulate probiotics. Proteins usually have good emulsifying properties and aqueous solutions of most proteins have a relatively low viscosity, even at high concentrations. This facilitates the formation of microparticles with dense gel network that provides a substantial buffering capacity, thereby supporting the idea of a protective barrier between the active ingredients and the surrounding environment (Heidebach et al. 2012). Gelatin, milk proteins and vegetable proteins (soy, pea, cereal proteins) are the most commonly used to encapsulate probiotics.

Gelatin is a protein derived by partial hydrolysis of collagen of animal origin. It has a very special structure and versatile functional propertie and forms a solution of high viscosity in water, which sets to a gel during cooling (Gbassi and Vandamme 2012). Due to its amphoteric nature, it is an excellent candidate for cooperation with anionic polysaccharides such as gellan gum (Burgain et al. 2011). Nawong et al. (2016) developed and characterized novel food-grade gelatin–maltodextrin microparticles crosslinked with transglutaminase that protect the encapsulated *Lactobacillus* spp. under simulated gastrointestinal conditions. Paula et al. (2019) used gelatin and gum Arabic for the encapsulation of *L. plantarum* by a dual process combining double emulsification followed by complex coacervation. The formed microparticles maintained the viability of *L. plantarum* cells during storage for 45 days at 8 °C and −18 °C and high survivability in simulated gastrointestinal conditions.

Milk proteins (caseins and whey proteins) are remarkable encapsulation materials because of their biocompatibility and their gel-forming ability like gelatin under suitable conditions (Kavitake et al. 2018). Due to their structural and physico-chemical characteristics, they are suitable as natural vehicles in probiotics encapsulation (Livbney 2010; Abd El-Salam and El-Shibiny 2015). Microencapsulation of *L. paracasei* and *B. lactis* in casein hydrogels obtained by transglutaminase or rennet crosslinking, increased the encapsulation efficiency, the viability of probiotics in simulated gastric condition as well as during freeze-drying and storage (Heidebach et al. 2012). Alehosseini et al. (2019) used electrospraying technique (see also section 1.4.2.2.2) for the encapsulation of *L. casei* in transglutaminase cross-linked whey protein concentrate/whey protein isolate matrix. Spheri-

cal water-resistant capsules with an average diameter of 3.09 μm were obtained with high encapsulation efficiency and high viability of *L. casei* under the simulated gastrointestinal conditions.

Vegetable proteins are becoming increasingly important to the food industry as a replacement for animal-derived proteins. The application of all-plant-based matrices for encapsulation of organisms could expand probiotic use in food products and markets that restrict the use of animal-derived proteins as an encapsulating material due to low cost, renewability, functionality, and religious, moral, or dietary preferences (Wang et al. 2015). Gonzalez-Ferrero et al. (2018) reported the capacity of soybean protein concentrate for encapsulating probiotics in biodegradable microparticles prepared by coacervation and dried by spray-drying. The resulting microparticles, of about 11 μm, showed a spherical matrix in which bacteria were uniformly distributed. The encapsulation of probiotics increased significantly their stability during storage under controlled conditions (25 °C/60% RH) and enhanced significantly *in vitro* gut resistance. Mao et al. (2019) investigated the roles of soy protein isolate and carrageenan coacervates in microencapsulating *B. longum*. The coacervates were effective in improving the viability of probiotics during storage (4 °C), *in vitro* gastrointestinal digestion, and pasteurization (85 °C). Pulse proteins represent an attractive alternative to soy due to their non-genetically modified status and low risk of allergenicity. Wang et al. (2015) investigated the use of legume proteins from pea, faba bean, and lentil combined with small amounts of alginate for the microencapsulation of *B. adolescentis* ATCC 15703 using an emulsion-based technique. Except for the lentil protein formulation, all microparticles were approximately 20 μm in diameter. Pea protein microparticles provided the greatest protective effect for *B. adolescentis* cells in simulated gastric juice. Varankovich et al. (2015) studied the suitability of pea protein isolate mixed with sodium alginate, iota-carrageenan or gellan gum, as protective materials for acid-sensitive *B. adolescentis* 15703 under simulated stomach conditions. Overall the increase in survivability of the probiotics was similar to all types of capsules. Following a temporal rat feeding study with the test bacterium encapsulated in pea protein isolate -alginate, *B. adolescentis*-specific PCR and qPCR analyses confirmed the presence of DNA from this species in rat feces, but only during the period of capsule intake.

1.4.1.3 Lipids

Lipidic materials have been proposed to be used as potential encapsulation matrices because diffusion of acids, water and oxygen across lipid particle membranes is limited (Lahtinen et al. 2007; Pedroso et al. 2012). However, compared to other encapsulant materials, lipids have been much less explored for probiotic encapsulation. Lipid-based encapsulation usually involves the dispersion of the probiotics in molten fat and subsequent cooling. The melting temperature of the chosen fat material is of crucial importance as it can negatively influence probiotic survivability during the encapsulation process. Pedroso et al. (2012) used an interesterified fat with palm and palm kernel, which has a melting point of 47.5 ℃, to encapsulate *B. lactis* BI-01 and *L. acidophilus* LAC-04 using spray-chilling and found that there was no loss in cell viability in both probiotic bacteria. Similar results were also found by Pedroso et al. (2013) using cocoa butter (melting point of 36.5 ℃) to encapsulate the same probiotic microorganisms. Okuro et al. (2013) co-encapsulated *L. acidophilus* LAC-04 with prebiotics (inulin or polydextrose) in solid lipid microparticles using spray chilling technology (see also section 1.4.2.2.1) and an interesterified fully hydrogenated palm and palm-kernel oil (melting point of 43.34 ℃) as encapsulant matrix. More recently, Amakiri et al. (2015) also developed lipid-based symbiotic particles containing *B. longum* LMG 13197 using glyceryl dipalmitostearate (melting point 57 °C) and inulin. These authors used a double emulsion W/O/W method followed by freeze-drying. This method does not require the melting of the fat material but involves the use of organic solvents (dichloromethane) which can limit the use of these microparticles in food applications.

1.4.2 Microencapsulation methods

As mentioned above, probiotics present two sets of problems when considering encapsulation: their size (typically ranging from 1 to 5 μm diameter), which excludes nanotechnologies, and the fact that they must be kept alive. Moreover, the size of microcarriers may also be another limitation depending on the desired application. Thus, the selection of the encapsulation technology for probiotics needs to consider these and other (cost and operating efficacy, for instance) aspects. The most commonly reported techniques for probiotics encapsulation (conventional techniques) in the scientific literature are extrusion, emulsification and spray-drying. However, other techniques such as electrospinning, spray-chilling, LbL and complex

coacervation, have been increasingly used for probiotic encapsulation (emerging techniques).

1.4.2.1 Conventional encapsulation techniques

1.4.2.1.1 Extrusion

The basic extrusion technique involves the dripping of a hydrocolloid solution (most often alginate) containing probiotic bacteria through a syringe needle into a hardening solution (most often $CaCl_2$). Owing to its simplicity, low cost, gentle conditions and high cell viability, the extrusion method is one of the most popular methods that is widely used to encapsulate probiotics. The main disadvantages of this technology are the process duration, the difficulty of scale-up (Burgain et al. 2011) and the impossibility in producing capsules smaller than 500 μm by a conventional dropwise method (Krasaekoopt et al. 2003). To obviate these drawbacks variations of the basic technique were developed using spray systems, such as vibrating nozzles, air-atomizing nozzles, electrostatic and spinning-disk atomization (Chavarri et al. 2012; Ramos et al. 2018). *Lactobacillus acidophilus* ATCC 4356 encapsulated in a chitosan/phytic acid matrix obtained by ionic gelation with electrostatic extrusion showed improved survival rate under refrigerated storage and simulated gastric conditions (Kim et al. 2017). Similar results were also found by Poletto et al. (2019) using alginate microparticles added with prebiotics (inulin, rice bran or Hi-maize) by extrusion/external gelation using an air-atomization nozzle. Alginate microcapsules obtained by extrusion, with or without double coating, revealed to be suitable to protect *L. paracasei* L26 incorporated in low pH juice fruits since viable cells were approximately 9 log cfu/g after 50 days of storage at 5°C (Rodrigues et al. 2012).
Nowadays, the extrusion method presents a vast diversity of industrial equipment able to be adapted to create particles from different mixtures of polymers and crosslinkers. Furthermore, these equipments are also able to obtain particles' sizes that are not attainable with conventional procedures at a laboratorial scale (Ramos et al. 2018).

1.4.2.1.2 Emulsion

In this method, a small volume of an aqueous hydrocolloid probiotic mixture (discontinuous phase) is emulsified into a larger volume of vegetable

oil (continuous phase). Once a water-in-oil emulsion has been formed, the dispersed hydrocolloid-cell mixture must be insolubilized to form small particles within the oil phase. As discussed in section 1.4.1.1, for alginate hydrogels, this solubilization can be achieved by external or internal gelation. The diameter of microparticles is dependent on the concentration and viscosity of the hydrocolloid solution and its agitation speed (Kavitake et al. 2018). In contrast to extrusion, the emulsion-based technique is easier to scale-up with high bacterial survival rate and producing capsules with a smaller diameter (ranging between 25 and 2000 μm) which are the main added advantages. In turn, its main disadvantage is large size range and shape of microparticles, difficult separation from the different phases (risk of damaging the particles) and higher cost for performance owing to the usage of vegetable oil (Kavitake et al. 2018; Ramos et al. 2018). Microparticles of *B. lactis* BB12 formed by emulsification/internal gelation using alginate as an encapsulating agent provided effective protection under simulated gastrointestinal conditions and 120 days of frozen storage (Holkem et al. 2017).

1.4.2.1.3 Spray-drying

Spray-drying is a well-established process in the food industry to convert liquids into dry powders. This technology has been utilized on probiotic cells with the purpose of not just simply drying, but also as an encapsulation procedure. It consists in spraying the liquid feed in fine droplets (10 to 150 μm) that are directed into a flow of hot and dry air (usually 150°C to 250°C) (Huang et al. 2017). The increase in the air-liquid interface area subsequent to spraying dramatically increases the drying kinetics, and it is commonly admitted that drying occurs within a few seconds (Huang et al. 2017). The main disadvantage of this technology is due to the high temperature, osmotic stress, dehydration and oxygen exposure conditions applied during the process that can result in the damage of probiotic cells. However, proper adjustment and control of the processing conditions such as the inlet and the outlet temperatures or the addition of thermal protectants (such as trehalose or prebiotics) can achieve viable encapsulated probiotics with a desired particle size distribution (Burgain et al. 2011). Microparticles containing gum Arabic mixed with inulin, hi-maize, or trehalose were produced through spray drying to encapsulate *L. acidophilus* La-5 by Nunes et al. (2018). The formulations containing trehalose and hi-maize were the encapsulating matrices with higher protective capacity relative to, respectively, thermal and simulated gastrointestinal conditions.

Whey protein microencapsulation via spray-drying, with or without L-cysteine-HCl of *L. acidophilus* Ki, *L. paracasei* L26 and *B. animalis* BB12 were performed and stored up to 6 months at 5 ºC and 22 ºC under different values of relative air humidity and oxygen (Rodrigues et al. 2011); *L. paracasei* L26 was the least susceptible to storage conditions presenting values above 10^6 cfu g^{-1} by 180 d at 22 ºC, irrespective of relative humidity, and the presence/absence of oxygen and L-cysteine.

1.4.2.2 Emerging encapsulation techniques

1.4.2.2.1 Spray-chilling

Spray-chilling, also known as spray cooling and spray congealing, is similar to spray-drying with respect to the production of fine droplets. However, spray-chilling consists of preparing a solution, dispersion or emulsion containing the active ingredient and a molten matrix (usually a lipid), which is then atomized into a chamber where cold air or liquid nitrogen is injected (Okuro et al. 2013). Owing to its low cost, use of low temperature and easy to scale up nature, spray-chilling is considered a suitable technology for the encapsulation of food ingredients (Liu et al. 2019). However, some technological disadvantages still exist, such as low encapsulation efficiency and the possibility of the expulsion of the active ingredient from the matrix during storage.

1.4.2.2.2 Electrospraying

Electrospraying is also known as electro-hydrodynamic atomization is based on the principle of liquid atomization using electrical forces. The liquid flowing out of a capillary nozzle, at high electric potential, is forced by the electric field to be dispersed into fine droplets (Coghetto et al. 2016a). The size of electrospray droplets can range from hundreds of micrometers down to tens of nanometers. The main advantages of this technique are the fact of operating under mild conditions, simplicity and the possibility of large-scale production. Coghetto et al. (2016b) used the electrospraying technique to microencapsulate *L. plantarum* BL011 in sodium alginate or sodium alginate-citric pectin matrixes. The authors demonstrated the efficiency of this technique to increase the cell survival of *L. plantarum* under simulated gastrointestinal conditions and under refrigeration when compared to free cells. Similar results were obtained by Zaeim et al.

(2017) who also used the electrospraying technique to microencapsulate *L. plantarum* ATCC 8014 in Ca-alginate/chitosan hydrogel.

1.4.2.2.3 Layer-by-layer

The LbL technique involves the alternative adsorption of oppositely charged materials on surfaces, thereby providing a system with tunable properties. The thickness, permeability, strength, and morphology of the layers can be tailored with precision (by altering the pH, ionic strength, wall materials), providing an ambiance with the desired properties (Priya et al. 2011). Unlike other methods, LbL is unique because each individual cell in suspension is coated sequentially, affording complete encapsulation (Priya et al. 2011). The survival rate of *L. acidophilus* encapsulated through LbL self-assembly of the polyelectrolytes chitosan and carboxymethyl cellulose was enhanced in simulated gastrointestinal conditions (Priya et al. 2011). The higher resistance of the encapsulated microorganism was attributed to the impermeability of polyelectrolyte nanolayers to pepsin and pancreatic enzymes. Moreover, it also reduced viability losses of the microorganism during freezing and freeze-drying. Anselmo et al. (2016) reported a LbL method for the encapsulation of *Bacillus coagulans* to protecting it from gastrointestinal challenges while facilitating both mucoadhesion and direct growth on intestinal surfaces.

1.4.2.2.4 Coacervation

This technique exploits the phase separation of one or more incompatible polymers from the initial coating polymer solution under specific temperature, pH or composition of the solution (Coghetto et al. 2016a). The incompatible polymer is added to the coating polymer solution and the dispersion is stirred. Changes in the physical parameters result in the separation of incompatible polymer and deposition of dense coacervate phase surrounding the core material (probiotic cells) resulting in the production of microparticles. The main advantages of this technique are the fact of operating under mild conditions (absence of use of high temperatures or organic solvents), simplicity, low cost and the possibility of incorporating a large amount of microorganisms in relation to the encapsulant (Chavarri et al. 2012). However, the scale-up of coacervation is difficult, since it is a batch process that yields coacervate in an aqueous solution.

This implies that an additional drying process (such as spray-drying) may be necessary, which can be harmful to the probiotic cells.

1.5 Nanotechnology applied to probiotic: "How to go smaller"

Nanotechnology has become a central focus in providing medical treatment advancement, through creating medicine with a unique perspective conducted at scales less than 100 nanometers (Caneus 2017). Application of nanotechnology in designing nanoprobiotics has gained tremendous potential worldwide in the field of nutraceuticals and is expected to grow rapidly in the future, in areas such as agriculture and food industry (Pathak and Akhtar 2019). The application of nanotechnology in the Agriculture and Food sectors are relatively recent compared with its use in drug delivery and pharmaceutical sector (Sozer and Kokini 2009). The basic of probiotics nanotechnology application is currently in the development of nano-encapsulated probiotics, aiming to offer an improvement on the taste, texture, and consistency of nanostructured food ingredients (Chau et al. 2007). However, the application of nanotechnology in food requires some precaution, namely its impact on the environment and human health. Currently, there aren't regulations that specifically control or limit the production of nanosized particles, and this is mainly owing to a lack of knowledge about the risks (Sozer and Kokini 2009). Nanoencapsulation is defined as a technology to pack substances in miniature using techniques such as nanocomposite, nanoemulsification, and nanostructuration and provides final product functionality and controlled release of the core (Sekhon 2010; Song et al. 2012). Nanoencapsulation is desirable to develop designer probiotic bacterial preparations- nanoprobiotics- that could be delivered to certain parts of the gastrointestinal tract where they interact with specific receptors (Sekhon 2010). The designed nanoprobiotics may work as *de novo* vaccines by modifying the immune response, being effective in supplementing various therapies such as irritable bowel syndrome and gastrointestinal infections. Nanosized materials have a distinctive potential to enhance the bioavailability or functionality of nutrients as well as ingredients. As previously mentioned, probiotic size ranges from 1 to 5 μm of diameter, making its nanoencapsulation quite a challenge. However, various nanocarriers are available (Figure 3) and these have been effectively researched to formulate the incorporation of probiotic bacteria (see Table 4).

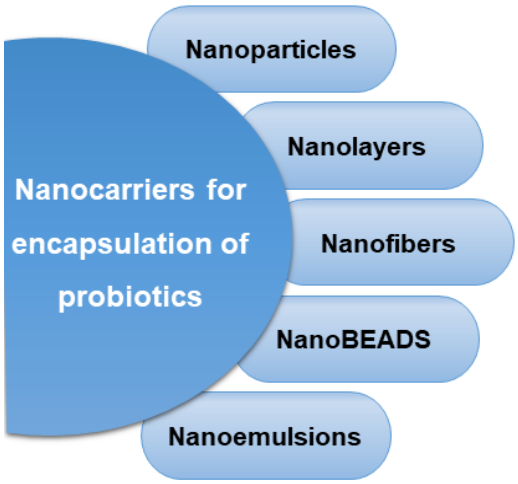

Fig. 3- Different nanocarriers for encapsulation of probiotics.

Table 4 highlights some reports on nanoencapsulation demonstrating the importance of these technologies in probiotics delivery.

Table 2- Delivery of encapsulated probiotics via nanocarriers.

Nanocarriers	**Delivered Probiotics or other micro-organism**	**Main achievements**	**Reference**
Nanoparticles	*Salmonellae* spp.	Inhibition of tumor growth by efficient oral delivery of vascular endothelial growth factor receptor 2 (VEGFR2) with nanoparticle-coated bacteria vectors due to angiogenesis suppression in the tumor vasculature and tumor necrosis.	(Hu et al. 2015)
	Salmonella typhimurium	*Salmonella* surface with the sucrose-linked gold nanoparticles and bacteria biotinylated to link the streptavidin-conjugated fluorophores; Biotin concentration was increased on the membrane surface, leading to an improvement in the tumor destruction.	(Kazmierczak et al. 2015)
	L. plantarum	Selenium nanoparticles to be employed as an immunomodulating agent; Mice treated with selenium nanoparticles- enriched *L. plantarum* induced a reduction of the tumor and increased the mice survival rate com-	(Yazdi et al. 2012)

		pared with the mice treated with free bacteria.	
	L. plantarum	*L. plantarum* PTCC1058 was used for the intracellular synthesis of tellurium nanoparticles; The results demonstrated the potential of *L. plantarum* with deposited tellurium nanoparticles and bacteria devoid of nanoparticles in the reduction of serum cholesterol in mice fed and gavage by a single dose of cholesterol. Bacteria with deposited nanoparticles were more effective than bacteria without nanoparticles in decreasing triglyceride levels.	(Mirjani et al. 2015)
Nanolayers	*Allochromatium vinosum*	Development of LbL nano self-assembled coated bacteria by polyelectrolyte combinations showed that surface charge did neither affect sulfide uptake nor the contact formation between the cells and solid sulfur	(Franz et al. 2010)
	L. acidophilus	The survival of encapsulated bacteria in the gastrointestinal tract was higher than nonencapsulated bacteria; The polyelectrolyte coating also served to reduce viability losses during freezing and freeze-drying.	(Priya et al. 2011)
Nanofibers	*L. acidophilus*	Electrospinning was a capable way for the stable solid formulation of *L. acidophilus*, increasing long-term stability.	(Nagy et al. 2014)
	L. plantarum	The feasibility of encapsulating bacteriocins and lactic acid bacteria into spun nanofibers was demonstrated.	(Heunis et al. 2010)
	L. acidophilus	Bacteria were incorporated into the spinning solution to produce a nanofiber-encapsulated probiotic; Exhibited good bacteria survivability (78.6-90%9 under electrospinning conditions and retained viability at refrigeration temperature during the 21 days storage.	(Fung et al. 2011)
	B. animalis BB12	Prepared nanofiber by electrospinning for encapsulating bifidobacterial strain, in order to improve viability and stability; Exhibited an enhancement of viability up to 40	(López-Rubio et al. 2009)

	days at room temperature and for 130 days under refrigerated conditions in comparison to non-encapsulated bacteria.	
Bacillus spp	Clinical isolate strain was incorporated into nanofibers to fight a periodontal pathogen bacterium; Nanofibers increased the viability of probiotic and storage time. Probiotic was released from nanofibers and the antimicrobial activity against periodontal pathogen bacteria was confirmed.	(Zupančič et al. 2018)

Encapsulation of probiotics into microcapsules or microgels can be used to enhance the viability and stability of probiotics in food and the gastrointestinal tract (as discussed above). However, the porosity is a problem, and so small ions and molecules in the surrounding solutions, such as bile salts or digestive enzymes could easily diffuse into microcapsules, which may lead to degradation of the encapsulated probiotics (Zhang et al. 2017). One strategy to overcome this problem is the addition of inorganic nanoparticles. Yao et al 2018 encapsulated *Pediococcus pentasaceus* Li05 in alginate-gelatin microgels in the absence and presence of magnesium oxide nanoparticles. They demonstrated that probiotic encapsulated in MgO-loaded microgels were more stable than free bacterial cells or those encapsulated in microgels alone. The addition of MgO nanoparticles improves the viability of the probiotics, which can be attributed to its antiacid effects. Nanoparticles may lead to enhanced probiotic viability by filling pores inside the microgels, which may have inhibited the ability of oxygen and hydrogen ions to access the probiotics, because the MgO nanoparticles are able to neutralize the hydrogen ions in the gastric fluids, reducing acid-induced degradation of the probiotics. These nanoparticles can act as a matrix fortifier to fill in the pores generated during freezing drying. In the intestinal fluids, the presence of the MgO nanoparticles also improves the viability of the probiotics, which may again have been because these filled in the pores in the hydrogel matrix inside the microgels, leading to decrease of bile salts diffusion into the microgels and damage of the probiotics (Yao et al. 2018).

Probiotic bacteria are being employed in synthesizing or more specifically biosynthesizing several nanoparticles such as metallic as well as non-metallic nanoparticles (Akhtar and Pathak 2017). Metallic nanoparticles exhibit wide applications in several fields, such as tissue/optical engineering, drugs or cosmetics, biosensors or nanodevices (Akhtar and Pathak 2017). The invention of Domínguez et al (2015) relates to probiotic bacteria, *Lactobacillus casei* and bifidobacteria, with metallic ions and/or metal

nanoparticles, to (i) use probiotics for the prophylaxis/treatment of mineral deficiency diseases; (ii) use the bacteria as contrast agent for imaging of the digestive tract and, (iii) use bacteria for the treatment of cancer. The authors selected a lactic acid bacterium and a bacterium of the genus *Bifidobacterium* comprising at least one metal nanoparticle bound to its surface. Metallic nanoparticles comprised of elements like iron, manganese, cobalt, nickel, calcium, zinc, magnesium, potassium, copper, chromium, selenium, silicon, iodine and combinations thereof (Domínguez Vera et al. 2015).

Besides the nanoparticles strategies that consist of the bioengineered bacteria, there are other strategies that developed nanoparticles aiming to potentiate the prebiotic effect. Ha et al (2016) developed whey protein isolate (WPI) /inulin nano complexes for the delivery of resveratrol, to study how WPI and inulin concentration levels affected the physicochemical properties of nano complexes, and to investigate the potential prebiotic effects of nano complexes. WPI/inulin nano complexes were prepared by using the modified ionic gelation method with $CaCl_2$. They demonstrated that nano complexes formed of inulin exhibited the potential prebiotic effect on *L. acidophilus* ATCC 43121 and the concentration of WPI and inulin were key factors that affected the physicochemical properties of WPI/inulin nano complexes and had a potential prebiotic effect (Ha et al. 2016).

Biodegradable nanoparticles with lyophilized probiotic extract (filtered and lyophilized cell-free supernatant) were studied by Saadatzadeh et al (2012). These nanoparticles were prepared using chitosan/PLGA by double emulsion solvent evaporation technique. Colitis was induced to male Wistar rats and oral gavage of nanoparticles was performed in water for 10 days. The authors observed that free probiotic extract from *L. casei* ATCC 39392, had positive effects in reduction of disease. However, the treatment using probiotic extract-loaded nanoparticles was more efficient in mitigating the experimental colitis in comparison with the highest dose of the free probiotic extract (Saadatzadeh et al. 2012).

Feher (2012) patented a new method for preparing probiotic nanoparticles from natural sources. The invention consists in the preparation of nanoparticles containing probiotic extracts for medical, nutritional, or cosmetic application, for different administration routes: systemic or topical application, enteral or parenteral, oral or intranasal administration. The applications of these probiotic nanoparticles are for the treatment of infective diseases, traumas, age-related diseases, autoimmune diseases, inherited diseases or conatal diseases, as well as functional diseases or disorders. The use of nanoparticles derived from killed probiotics is a novel approach to rebuild the symbiosis of host and probiotics, in the opposite way which suggests that the restoring the mucosal surfaces should be by live probiot-

ics uses. The invention described methods for *in vitro* preparation of nanoparticles from probiotics mimicking as much as possible the *in vivo* physiological processes (Feher 2012).

Many encapsulation techniques have been devised to protect the bacteria from adverse environmental and processing conditions and also in *in vivo* conditions. Commonly, it is reported the immobilization of nanocarriers on the surface of bacteria. However, there are other nanoapproaches to improve the viability of probiotics and their application (Priya et al. 2011). As mentioned above, LbL employed to form the assembly of layers on the surfaces of solids is a novel approach in the development of nanolayers or thin films to be used in drug, nutraceutical, as well as gene delivery and in biosensing (Pathak and Akhtar 2019). This technique, which has all ready been described in section (1.4.2.2.3), can be exploited for the formation of nanocages on living microorganisms too (Priya et al. 2011).

Lyophilization and spray-drying have been the most studied technologies. However, the loss of bacteria viability through damaging of bacteria membranes or other cellular structures, and the time-consuming effect associated are the most important disadvantages for powdered probiotics formulations. Wagner et al. (2015) suggested the of tablets with the formulation of dried bacteria. Tablets can provide easy administration, long-term stability and optimize the adhesion and colonization of bacteria to the epithelial mucosa. However, the high compression force that is needed to form the tablets can cause a significant loss of viability due to mechanical damage of the bacteria (Wagner et al., 2015). To overcome these limitations, the electrospinning is emerging as an attractive alternative method that enables drying of probiotics by the production of nanofibers from electrostatically driven jets of polymer solutions (Zupančič et al. 2018). Nanofibers can be used in wound dressings, as drug delivery systems, and as three-dimensional scaffolds for bone and tissue regeneration. Electrospinning has been introduced as a new method for incorporation of microbial cells into nanofibers, such as *L. acidophilus* or *Bifidobacterium* spp. (López-Rubio et al. 2009; Heunis et al. 2010; Fung et al. 2011; Nagy et al. 2014). Although electrospinning has been shown to be a promising process for probiotic incorporation, the effects of the process, solutions and environment parameters on probiotic viability are still poorly understood. Therefore, Skrlec et al (2019) developed nanofibers loaded with the probiotic *L. plantarum* ATCC 8014. They investigated a method to incorporate bacteria into monolithic poly(ethylene oxide) (PEO) and composite PEO/lyoprotectant (sucrose) nanofibers. PEO was chosen as it is a biocompatible, mucoadhesive and water-soluble polymer that appears not to interfere with the bioactivity of the probiotic. The particular focus on the study was to initially determine the effects of several parameters such as

those related to the environment (temperature and humidity), voltage, solution parameters including the presence of lyoprotectant in the polymer solution as well as bacteria concentration (Škrlec et al. 2019). The authors showed that the most critical parameter for high bacteria viability after the electrospinning was the concentration of probiotic *L. plantarum* in the polymer solution. The relative humidity and voltage during fiber production did not have any vital impact on *L. plantarum* viability. It was observed an improvement in the bacteria viability and also higher survival during storage, when a high concentration of lyoprotectants, namely trehalose, was added. Nanofibers were able to release almost all the *L. plantarum* over 30 min, which is appropriate for local administration. This approach demonstrates the development of a promising local nanodelivery system based on the use of probiotic-loaded nanofibers that can provide high loading and long shelf-life (Škrlec et al. 2019).

There are other forms to encapsulate probiotic bacteria, such as nanoBEADS and nanoemulsion. However, these strategies are poorly explored for nanoencapsulation of probiotics. NanoBEADS means nanosized bacteria-enable autonomous delivery systems, a new concept of nanoparticles linked to the probiotic surface. Traore et al (2014) developed nanoscale NanoBEADS comprised of flagellated *Escherichia coli* bacterium loaded with an assembly of spherical polystyrene nanoparticles. Flagellated bacteria possess specific self-propulsion features, capable of moving through the highly viscous fluid and porous semisolid environments effectively (Traore et al. 2014; Pathak and Akhtar 2019)

Nanoemulsion is formulated using coarse emulsions by reducing the emulsion droplet dimension with high energy techniques such as high-pressure homogenization, microfluidization, and high-power ultrasound. Nanoemulsion is mostly used to load drugs and to provide effective drug delivery but also can be used to initiate the release and absorption of loaded bioactive and food agents. Nanoemulsions were designed to enhance the bioavailability of lipophilic bioactive agents in fruits and vegetables (Pathak and Akhtar 2019).

1.6 Conclusions

Nanotechnology, based on particle size, is a continuously growing field of interest in the realm of health promotion and disease prevention involving a multifaceted approach covering science, engineering and technology.

Current knowledge of the human gut microbiota and its role in gut health homeostasis (microbiota dysbiosis and chronic inflammatory diseases onset), have prompted the use of probiotics, prebiotics, symbiotics, and the derived postbiotics, as efficient health promoting strategies to maintain or recover the normal mucosal immunity and intestinal ecosystem balance. Within this preventive therapy concept the use of commensal bacteria as probiotics is currently being explored paving the way to a new type of probiotics commonly called Next-Generation Probiotics where *Akkermansia muciniphila* or *Faecalibacterium prausnitzii*, among others, are promising candidates. To ensure successful delivery of probiotic benefits to the consumer several criteria are needed including high yields and stability and solutions must be able to meet with requirements that ensure high performance and quality, including recommended dose at time of consumption. An integrated selection of production process, product formulation and strain leads to high-quality probiotics to be included in a wide variety of delivery vectors to meet consumer needs.

Innovative approaches to guarantee optimal probiotic delivery and efficacy within the gastrointestinal tract have looked upon encapsulation strategies to entrap and protect more sensitive probiotic strains from the harsh environmental conditions encountered during production, storage and gastrointestinal tract passage. The emergence of nanomedicine has made nanotechnology a promising tool to foster probiotic strains efficacy in gut health. Despite the constraints associated with probiotic bacteria average size not being at the nano-scale, different nano-carriers based on size, composition, morphology, surface area and charge, provide interesting opportunities for the food sector, including nanoparticles, nanofibers, nanoBEADS and nanoemulsions. This large array of structural arrangements make nanosystems quite versatile and effective.

Nevertheless, dietary applications of these nanosystems is still in its early infancy and while the effectiveness of partnering nanotechnology with current probiotic/prebiotic/symbiotic approaches seems promising and of broad potential, additional studies are required to understand their dietary/preventive/clinical role, potential risks and health promotion capacity.

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