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Optimizing oral delivery of next generation probiotics

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ABSTRACT

Background: Probiotic microbes may confer a variety of positive health effects on the host. Until now, development of probiotic products has mainly focused on *Lactobacillus* and *Bifidobacterium* species, which generally tolerate the stresses encountered during processing, storage and delivery well. In recent years, newly-discovered gut microbes have gained attention due to their association with healthy host conditions. However, these microbes, designated next generation probiotics, are often oxygen-sensitive, do not tolerate the established product processing techniques, and need protection during delivery and gastric transit.

Scope and approach: Here, we review the challenges related to development of next generation probiotic products. The applications of current microbial processing and delivery techniques for the oxygen-sensitive next generation probiotics are assessed, and putative process optimizations are discussed.

Key findings and conclusions: Current microbial product processing techniques are not suited for next generation probiotics, thus optimizations or entirely novel processing approaches are needed. Freeze-drying is currently the only method that keeps cells viable during processing and storage, but optimization of the process for individual strains is required, e.g. by adding antioxidants to the drying solution.Oral delivery of live next generation probiotics is poorly investigated. The strains in question are often known to colonize primarily in the colon, and carriers, such as microparticles and microdevices, which have been verified for colon-targeted delivery of drugs, may represent a novel choice as delivery vehicle for next generation probiotics.

1. Introduction

With the discovery of the health-promoting *Lactobacillus* and *Bifidobacterium* species in the beginning of the 20th century (Metchnikoff, 1907)' (Tissier, 1906), the first bricks were laid towards the evolving and recently escalated research field of probiotics, shared by academia and the industry. Today, probiotics are classified as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" by The World Health Organization (World Health Organization, 2001) in which 'health benefits' can broadly be defined as supporting gut homeostasis including immune system stimulation and maintenance (Hill et al., 2014). Investigation of probiotic effects are mainly based on large human-based studies using *Lactobacillus* and *Bifidobacterium* species, but probiotic effects have also been established for other gut microbes including *Bacillus* species, which are widely used in animal production, specific strains of *Escherichia coli*, and for the fungus *Saccharomyces boulardii* (Guarner, Sanders, Eliakim, Fedorak, & Mair, 2017, pp. 1–35). Probiotics are additionally used as supplements in treatment of diarrhea, inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and vaginal infections as well as for modulation of the immune system (Hill et al., 2014).

To investigate putative beneficial properties of a microbe and thereby its potential as a probiotic or therapeutic product, initial screening experiments are typically conducted using active microbial cultures. However, to end up as a probiotic or therapeutic product, the microbe must be able to tolerate the demanding processes needed to create a suitable product that is easy to handle, distribute, store and administrate without significant loss of viability. The process of probiotic fabrication thus includes a number of general factors to consider for optimal survival of non-spore forming microbes from processing to gut delivery (Fig. 1). Several physico-chemical factors including oxygen-exposure, desiccation, osmotic pressure, high temperatures and humidity, affect the viability of the microbes during processing and storage. Furthermore, the harsh gastrointestinal (GI) conditions, characterized by low pH in the stomach and the presence of bile salts in the small intestine, are detrimental to many microbial species (Derrien

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& van Hylckama Vlieg, 2015). Many species of *Lactobacillus* and *Bifidobacterium* are however aero-tolerant or microaerophilic and reasonably tolerant towards many of the environmental changes experienced during processing, storage and GI transit (O'Toole, Marchesi, & Hill, 2017), which, together with well-defined cultivation methods, make them ideal as probiotic products.

The emergence of next generation sequencing techniques has led to the discovery of a number of previously unexplored microorganisms. There is thus a growing interest in indigenous gut microbes such as *Akkermansia muciniphila* and *Faecalibacterium prausnitzii,* which are associated with healthy conditions in the intestines (El Hage, Hernandez-Sanabria, & Van de Wiele, 2017). However, these microbes are challenging to work with due to their extreme sensitivity towards oxygen and often also to gastric conditions encountered after ingestion (Derrien, Vaughan, Plugge, & de Vos, 2004)' (Foditsch et al., 2014). The difficulty in maintaining viability of these sensitive bacteria during common preparation, storage and delivery methods challenges the development of this type of next generation probiotics into commercial products. However, given their health-promoting potential, we here review current advances in oral probiotic delivery strategies with emphasis on processing and delivery of oxygen-sensitive probiotics.

2. Requirements for probiotic activity

2.1. Next-generation probiotics

With newly developed cultivation techniques, fastidious anaerobic microbes from the gut are increasingly being cultured and identified (Lagier et al., 2018). *In vitro* and *in vivo* studies have led to the identification of putative probiotic microbes, such as the before-mentioned *A. muciniphila* and *F. prausnitzii,* which are both associated with a healthy gut microbiota in humans (Dehghanbanadaki et al., 2020)' (Miquel et al., 2013). A feature of some next generation probiotics is the production of short chain fatty acids, of which particularly butyrate is known to support immune homeostasis and human intestinal health (Venegas et al., 2019). Dominant butyrate producers in the human gut include *A. muciniphila*, *F. prausnitzii*, Ruminococcus bromii, as well as Clostridium, *Bacteroides*, *Roseburia*, and *Eubacterium* species (Fattahi, Heidari, & Khosroushahi, 2020). While *Clostridium* species are spore-formers, this is not the case for strains of the other genera. The large group of butyrate producing gut commensals is thus phylogenetically more diverse than the conventional probiotic lactic acid bacteria, and are reported to confer a large variety of probiotic and therapeutic effects (El Hage et al., 2017). *Akkermansia,* which sustains growth on mucin as the sole source of carbon and nitrogen, is generally known for its association with a healthy mucosa and immune modulating properties (Derrien et al., 2011). Additionally, the bacterium has been shown to prevent high fat diet-induced obesity in mice (Everard et al., 2013), improve immunotherapy effects against epithelial tumors in mice transplanted with human microbiota (Routy et al., 2018), and enhance therapeutic drug effects against murine type 2 diabetes (Shin et al., 2014). Members of the *Clostridium* clusters IV, XIVA and XVIII, which all lack virulence factors, are also important immune homeostasis regulators and observations of immune cell induction suggest that they work as therapeutic agents against gut inflammatory disorders (Atarashi et al., 2013). Additionally, *F. prausnitzii* from cluster IV has been reported to ameliorate gut inflammation in colitis patients (Rossi et al., 2016). Evidence from human cell lines and gnotobiotic animal models strongly suggest that the *Bacteroides* genus, which is one of the most common genera found in the human gut microbiota, contains potentially probiotic species with beneficial effects such as amelioration of obesity-related symptoms by *B. uniformis* (Gauffin Cano, Santacruz, Moya, & Sanz, 2012) and *B. acifaciens* (Yang et al., 2017), the preservation of immune homeostasis and prevention of colitis by *B. fragilis* (Erturk-Hasdemir & Kasper, 2018)' (Round & Mazmanian, 2010), cholesterol reduction by *B. dorei* D8 (Gérard et al., 2007), and several important functions related to gut homeostasis by *B. thetaiotaomicron* (Hooper et al., 2001). More recently, the gut commensal *Christensenella minuta,* which is associated with a lean body type in humans, has gained attention for mitigating lifestyle induced obesity and IBD (Goodrich et al., 2014)' (Kropp et al., 2021).

2.2. Probiotic viability in the gut

During the first months of life, the human gut gets colonized by microbes transmitted primarily from the maternal gut (Ferretti et al., 2018) and breast milk (Duranti et al., 2017). After three or four years, the infant gut microbiota develops into a stable, diverse and adult-like

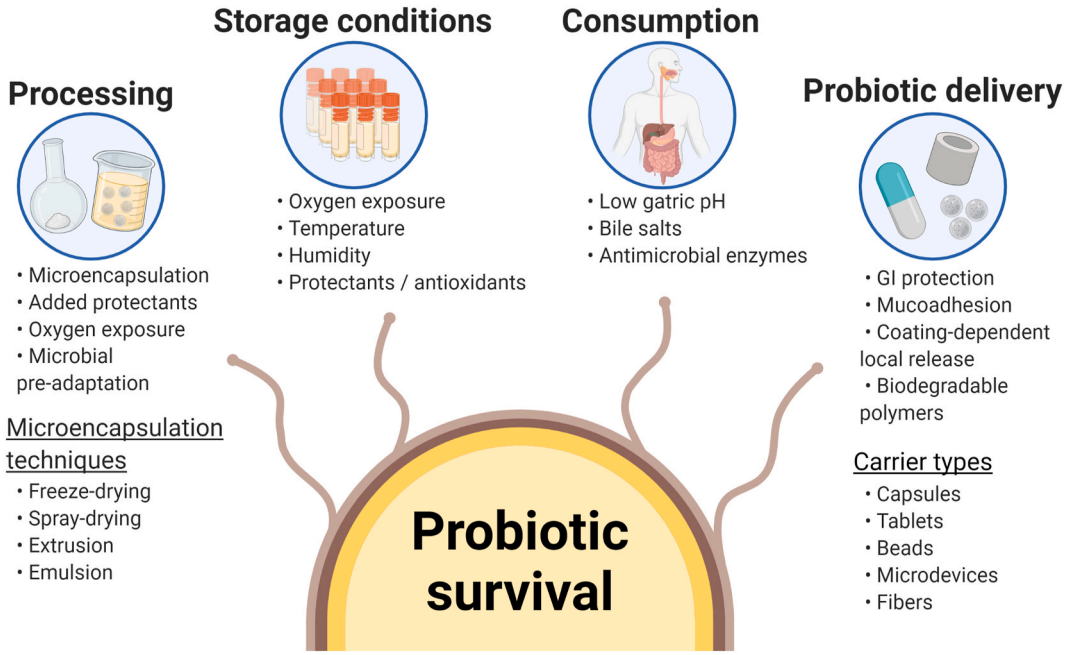

Fig. 1. Overview of factors related to probiotic survival from processing to gut delivery (Created with BioRender.com).

microbial community (Laursen, Bahl, & Licht, 2021). Some colonizers only reside in the gut temporarily during this time period, where many physico-chemichal conditions in the gut are gradually changing (Pannaraj et al., 2017).

The developed, healthy adult microbiota confers a protection mechanism called the 'colonization barrier' (Lawley & Walker, 2013). This phenomenon relies on microbial competition and is useful for preventing pathogenic attacks but also excludes other new-coming microbes such as probiotics. It has thus been described that a new species can only colonize a niche in the gut, if it is able to utilize a limiting nutrient more effectively than other habitants, or if it has another advantageous capacity, such as the ability to adhere to the intestinal wall' (Freter, Brickner, Fekete, Vickerman, & Carey, 1983). Persistent establishment of a probiotic in the gut is, therefore, dependent on **either** the existence of a **free** nutritional and/or spatial niche allowing its proliferation, **or** the ability of the probiotic strain to **outcompete** another strain within this niche. In line with this, it has been shown that existing probiotic products typically do not alter the surrounding indigenous microbiome, and pass through the intestine with minimal proliferation (Laursen et al., 2017)' (Kristensen et al., 2016). However, it should be emphasized that permanent establishment in the gut is not necessarily required for a strain to exert a beneficial effect e.g. by interaction with epithelial receptors or production of bioactive metabolites.

It has been suggested that a probiotic concentration of at least 10^6 CFU/ml in the small intestine and 10^8 in the colon is needed to exert clinical effects (Minelli & Benini, 2008). However, if ingested microbes are able to readily outcompete others in a given niche, the ingested dose of live microbes might be of less importance. Importantly, even though the current definition of probiotics refer to live organisms, also dead microbes may have a probiotic effect. Viable or pasteurized *A. muciniphila* have thus been reported to have similar probiotic effects (Plovier et al., 2017). Initially, it was seen that live, but not autoclaved, *A. muciniphila* counteracted development of high fat diet-induced obesity and dysfunction of the gut barrier in mice (Everard et al., 2013). However, subsequently it was reported that pasteurized *A. muciniphila* had similar effects as the live strain (Plovier et al., 2017). The effects of pasteurization-killed *A. muciniphila* were ascribed to an outer membrane protein, which was stable during pasteurization at 70 °C but not during autoclavation. The intact protein was thus shown to improve the gut barrier integrity and partly counteract the harmful effects of diet-induced obesity (Plovier et al., 2017). Similarly, a heat-killed *L. brevis* strain has been shown to enhance the gut barrier and ameliorate colitis in mice resulting in improved animal survival (Ueno et al., 2011). These examples demonstrate a potential novel approach to create non-viable 'probiotic-derived' products that are easy to produce and have a long shelf life.

Other probiotic effects rely on active microbes and their production of metabolites in the gut, such as alleviation of gut inflammation by SCFAs (Venegas et al., 2019). Gut inflammation may be triggered by a dysbiotic gut community, for example caused by antibiotic treatment that reduces microbial diversity and abundance (Yoon & Yoon, 2018). It has been proposed that development of a stable and diverse microbial gut community is partly dependent on metabolites from lactic acid bacteria and bifidobacteria that are utilized by other commensals, such as the SCFA producers, during infant gut maturation (Laursen et al., 2021). To restore the gut microbiota after dysbiosis, administration of common probiotics, i.e. lactobacilli and bifidobacteria, may provide the same metabolic benefits to restore a diverse microbial community. Although new findings may suggest that this is not always a feasible practice (Suez et al., 2018), probiotics are thus typically recommended to help restore a healthy gut microbiota after antibiotic treatment, even though the probiotic strains themselves are not abundant commensals (Mantegazza et al., 2018). Therefore, delivery of missing gut commensals such as *F. prautsnitzii* to a dysbiotic gut may serve as a more direct approach to restore a healthy community.

2.3. Processing of traditional probiotics

The initial preparation of a traditional non-sporeforming probiotic microbial product after cultivation may include encapsulation of the microbial cells, which is done to protect the microbes during GI transit as well as to improve handling and shelf life, measured as viability during storage, of the product. Commercial probiotic products require a long shelf life as extended storage is often needed. Lowering the intracellular moisture content below 4% and the water activity (a_w) to around 0.1 reduces cellular processes and preserves the cells in an inactive state (Broeckx, Vandenheuvel, Claes, Lebeer, & Kiekens, 2016) while also reducing oxidative damage, and thereby increasing storage stability (Abe, Miyauchi, Uchijima, Yaeshima, & Iwatsuki, 2009). During spray-drying, which is carried out by use of an atomizer or spray nozzle, a bacterial cell suspension is dispersed into air-suspended droplets, from which water is evaporated by heated gas at an outlet temperature up to 60–80 °C, thereby creating a probiotic-containing powder (Broeckx et al., 2016).

For heat-sensitive microbes, freeze-drying, also known as lyophilization, which was applied in 1911 as the first effective encapsulation technique for microbes (Hammer, 1911), is a good alternative. However, this method is more time-consuming and expensive compared to spray-drying' (Broeckx et al., 2016). The principle of freeze-drying is to apply vacuum to a frozen cell suspension followed by a temperature decrease down to somewhere between −20 °C and −80 °C, which allows water molecules to transit directly from solid state to gas by sublimation. The product is a dried cake, which is usually crushed into a powder (Broeckx et al., 2016). By freezing and drying lactic acid bacteria in a vacuum, a drastic improvement in bacterial survival was obtained, compared to cultures dried from the liquid state.

The removal of intracellular water during spray- or freeze-drying may cause a variety of stresses to the bacterial cell including osmotic stress, oxygen damage, and mechanical stress on the membrane. Additionally, ice crystal formation during freeze-drying represents a source of stress (Marcial-Coba, Knøchel, & Nielsen, 2019).The method has thus been optimized in various ways to counteract these stresses. Typically, protecting compounds are added to the drying solution. During the drying process, the cells thus become encapsulated in a powder that includes protectants added to the drying solution. This is referred to as **microencapsulation** (Martín, Lara-Villoslada, Ruiz, & Morales, 2015). Protectants that inhibits the formation of harmful ice crystals during freezing are referred to as cryoprotectants (Juárez Tomás, Ocaña, & Nader-Macías, 2004). Common cryoprotectants include glycerol, sugars and skim milk (Juárez Tomás et al., 2004). Prevention of intracellular crystal formation is also possible using an optimized cooling rate during freezing, which has been tested to be 5 °C/min for *L. casei* (Dimitrellou, Kandylis, & Kourkoutas, 2016).

Some of the most widely used microencapsulating protectants include skim milk powder and the disaccharides trehalose and sucrose, the latter stabilizing polar residues of proteins and membranes by replacing the removed water molecules (Aschenbrenner, Kulozik, & Foerst, 2012). Sugar alcohols and amino acids are also frequently added to drying solutions during desiccation of lactic acid bacteria (Morgan, Herman, White, & Vesey, 2006)' (Marcial-Coba, Knøchel, & Nielsen, 2019). The majority of research into preservation of probiotics focuses on *Lactobacillus* and *Bifidobacterium* species, which typically tolerates different drying techniques including spray-drying to some extend (Huang et al., 2017). However, the high temperatures and presence of oxygen during spray-drying make the technique unfeasible for most other gut microbes, which often do not survive these conditions. The thick peptidoglycan layer covering the membrane of gram-positive bacteria is known to confer protection against physical stresses, which may explain why the gram-positive *Bifidobacterium* species and *Lactobacillus* species tolerate spray-drying better than gram-negative bacteria such as *E. coli* (Pispan, Hewitt, & Stapley, 2013). Novel protectants are still being tested for spray-drying of common probiotic species, such as

chia and flax seed mucilage (Bustamante et al., 2020). Most protectants are food-grade, and are subjected to the appropriate labeling implications for processing aids.

Microencapsulation techniques that do not rely on a drying step include extrusion or emulsification, in which a cell suspension is added to a hardening and stabilizing solution, thereby creating tiny microbe-containing gel or lipid beads (Fig. 2) (Martín et al., 2015)' (Broeckx et al., 2016). Such gels and lipids, referred to as microparticles, may be used as probiotic carriers and are suited for probiotics added to food products such as yoghurt, cheese, and ice cream. However, the microencapsulation techniques are rather slow, which makes large-scale applications challenging (Kailasapathy, 2002). The stressful conditions of cell desiccation is avoided during these types of microencapsulation, and the beads serve as probiotic carriers during delivery (Chen et al., 2012). However, only a few studies have investigated extrusion- or emulsion-based processing of oxygen-sensitive microbes, as described later. Currently, processing of oxygen-sensitive microbes has mainly been tested and optimized by freeze-drying (Bircher, Geirnaert, Hammes, Lacroix, & Schwab, 2018).

2.4. Freeze-drying of next generation probiotics

During desiccation, cell membranes and molecules may be exposed to high levels of oxygen leading to oxygen-induced phospholipid, DNA, and protein damage, which is particularly harmful for oxygen-sensitive bacteria (França, Panek, & Eleutherio, 2007). Addition of antioxidants to the drying solution may protect the bacteria against oxygen damage and enhance viability during processing and storage. By including the antioxidants cysteine and riboflavin to the freeze-drying solution, it was possible to freeze-dry and store the oxygen-sensitive *F. prausnitzii*, which normally survives less than 2 min of air exposure (Duncan, Hold, Harmsen, Stewart, & Flint, 2002), at ambient air for 24 h with around 70% survival (Khan, Van Dijl, & Harmsen, 2014). The same antioxidants were demonstrated also to protect the oxygen-sensitive potential next generation probiotics *B. thetaiotaomicron*, *Roseburia intestinalis*, *Anaerostipes caccae*, *Eubacterium hallii*, *Blautia obeum,* and *F. prausnitzii* (Bircher et al., 2018). Viability after freeze drying with these additives was maintained for all species during anaerobic storage at 4 °C for three months (Bircher et al., 2018). Even though viability was significantly increased by adding protectants during freeze-drying, a loss of more than 1-log was observed for all species except *F. prausnitzii* and *A. caccae*. The large differences in survival among the tested species might be caused by different levels of oxygen-sensitivity, where the most sensitive species may require complete anaerobiosis (Bircher et al., 2018). Also inclusion of the antioxidants ascorbic acid, uric acid and glutathione to the drying solution has been seen to increase survival of *A. muciniphila* during freeze-drying and storage for 30 days (Bellali, Bou Khalil, Fontanini, Raoult, & Lagier, 2020). The survival percentages of specific anaerobic microbes after being freeze-dried with the optimized protocols varies considerably (Table 1).

During bacterial cultivation, small organic solutes may be added to the culture medium to stimulate intracellular accumulation of stress compounds, such as trehalose or glycine betaine, which counteracts osmosis and may protect the cells during subsequent desiccation (Gaucher, Kponouglo, et al., 2019). The bacterial process of adapting to the osmotic conditions of a new environment by changing gene

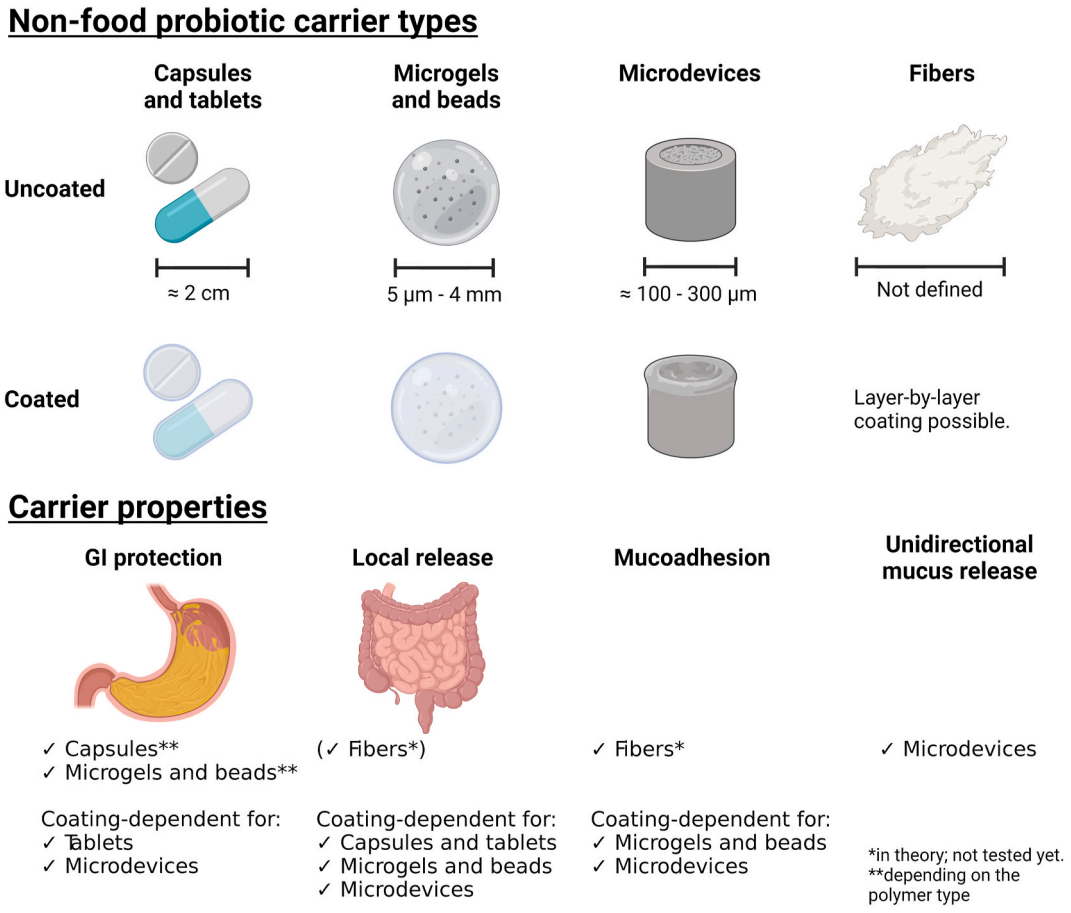

Fig. 2. Potential delivery strategies for next generation probiotics. Capsules, tablets, microdevices, microgels and –beads may be coated to locally release the probiotic whereas probiotic-containing fibers may be sandwiched with other materials to provide similar effects. Sizes: Gel/bead (Rokka & Rantamäki, 2010), microdevices (Mazzoni & Nielsen, 2020). Figure created with BioRender.com.

Table 1
Freeze-drying of probiotic microbes.

Genus	Species	Described	Challenges during freeze drying	Reported optimization	Survival	Refe-rences
Lactobacillus	*spp.*	1907	Commercialized/ standardized			Metchnikoff (1907)
Bifidobacterium	*spp.*	1906	Commercialized/ standardized			Tissier (1906)
Streptococcus	*thermophilus*	Early 1900's	Commercialized/ standardized	Addition of sodium caseinate, skim milk, sucrose, lactose and mono sodium glutamate glutathione to freeze-drying solution.	96%	Sharma et al. (2014)
Escherichia	*coli*	1885	Low survival during freeze-drying. Commercialized (Nissle 1917)	Addition of sucrose, trehalose, skimmed milk, and antioxidants ascorbic acid, uric acid and glutathione to freeze-drying solution.	Increased from 2.67% to 91.67% survival. Decreased to 83.33% after 30 days at 4 °C.	(Hacker & Blum-Oehler, 2007)·(Bellali et al., 2020)
Propioni-bacterium	*freudenreichii*	1928	Low survival during desiccation	Heat and acid treatments as wells as osmoadaptation during cultivation.	Control survival: 43%. With osmo-adaptation: 74.4%. With osmo-adaptation and heat stress: 90%. With osmo-adaptation and acid stress: 96.7%.	(Niel, 1928)·(Gaucher, Kponouglo, et al., 2019)
Anaerostipes	Caccae	2002	Extremely oxygen sensitive	Addition of sucrose and inulin to freeze-drying solution.	From 0.4% to 71% survival. Decreased to 49% after 3 months at 4 °C.	(Schwiertz et al., 2002)·(Bircher et al., 2018)
Bacteroides	thetaiota-omicron	1912 (Reclassified 1919)	Extremely oxygen sensitive	Addition of sucrose and inulin to freeze-drying solution.	From 1% to 4% survival. Decreased to 2% after 3 months at 4 °C.	(Distaso, 1912)·(Castellani & Charlmers, 1919)·(Bircher et al., 2018)
Eubacterium	hallii	1974	Extremely oxygen sensitive	Addition of sucrose and inulin to freeze-drying solution.	From 0.04% to 6% survival. Decreased to 3% after 3 months at 4 °C.	(Holdeman & Moore, 1974)·(Bircher et al., 2018)
Blautia	obeum	1976 (Reclassified 2008)	Extremely oxygen sensitive	Addition of sucrose and inulin to freeze-drying solution.	From 0.02% to 5% survival. Decreased to 0.4% after 3 months at 4 °C.	(Moore, Johnson, & Holdeman, 1976)·(Liu, Finegold, Song, & Lawson, 2008)·(Bircher et al., 2018)
Roseburia	intestinalis	2002	Extremely oxygen sensitive	Addition of sucrose and inulin to freeze-drying solution.	From 0.2% to 8% survival. Decreased to 5% after 3 months at 4 °C.	(Duncan, Hold, Barcenilla, Stewart, & Flint, 2002)·(Bircher et al., 2018)
Akkermansia	*muciniphila*	2004	Oxygen sensitive	1. Addition of sucrose, trehalose, skimmed milk, and antioxidants ascorbic acid, uric acid and glutathione(Bellali et al., 2020). 2. Gel beads created by extrusion followed by freeze-drying and embedment in chocolate(Marcial-Coba, Saaby, Knøchel, & Nielsen, 2019).	1. From 0.36% to 85% survival. Decreased to 80% after 30 days at 4 °C. 2. Anaerobic storage for 2 months resulted in a viability decrease of 71% at 4 °C and 83% at 15 °C.	(Derrien et al., 2004)·(Bellali et al., 2020)·(Marcial-Coba, Saaby, et al., 2019)
Faecalibacterium	*prausnitzii*	2002	Oxygen sensitive	1. Addition of inulin and antioxidants riboflavin and cysteine to freeze-drying solution. 2. Addition of sucrose and inulin to freeze-drying solution(Bircher et al., 2018).	1. 2. From 1% to 32% survival. Stayed at 33% after 3 months at 4 °C.	(Duncan, Hold, Harmsen, et al., 2002)·(Khan et al., 2014)·(Bircher et al., 2018)·(Raise et al., 2020)

expression, designated osmoadaptation, has been known for several decades. However, the protective effects of osmoadaptation during probiotic desiccation are still largely unknown. Recent reports however suggests that growing *Propionibacterium freudenreichii* under hyper-osmotic conditions in combination with heat, acid or oxidative stress results in modulation of the fatty acid composition in the cell membrane and accumulation of specific intracellular compounds significantly increasing survival during both spray- and freeze-drying (Gaucher, Kponouglo, et al., 2019). However, the protective effects of osmoadaptation in *P. freudenreichii* are strain-dependent (Gaucher, Bonnassie, et al., 2019). Similarly, heat or cold shock treatmenta have been reported to boost freeze-drying tolerance of lactic acid bacteria (Reddy, Awasthi, Madhu, & Prapulla, 2009). Osmoadaptation and other stress treatments thus potentially represent a novel set of ways to optimize processing of next generation probiotics.

3. Strategies for improved probiotic delivery

3.1. Capsules and tablets

Commercial probiotic supplements are usually sold as powders in capsules and sachets or compressed into tablets (Fig. 2), which provide easy administration, long storage stability/shelf life, and high consumer acceptance (Villena, Lara-Villoslada, Martínez, & Hernández, 2015). Probiotic powder from sachets are usually dissolved in a liquid prior to consumption where capsules, which are typically made of gelatin or hydroxypropyl methylcellulose (HPMC), and tablets are dissolved in the low pH of the stomach (Chiwele, Jones, & Podczeck, 2000). All of these delivery methods provide low protection of the bacterial cell in the gastric environment. Such methods are well suited for *Lactobacillus* species that typically tolerate the gastric environment and mainly colonize the small intestine. However, as the next-generation probiotics

typically do not tolerate these environments well, they need to be protected during stomach and small intestinal transit in order to reach the colonic environment alive. Colon-targeted coatings, designed to protect the content until the carrier reaches the colon, may be applied to capsules and tablets. Such coatings are degraded depending on a specific trigger, such as changes in pH or colon-specific enzymatic activity, thereby releasing the coated content into the colon (Lee et al., 2020). By coating capsules with a combined pH- and enzyme-triggered coating, a consistent release of probiotics into a simulated colon environment has been observed *in vitro* (Dodoo, Wang, Basit, Stapleton, & Gaisford, 2017). Specific and reliable release strategies are however challenged by the intra- and inter-individual intestinal variability in pH and transit time occurring *in vivo*. This is particularly an issue for murine models used in initial/pre-clinical tests, since only minor pH changes occur throughout the mouse intestinal tract (McConnell, Basit, & Murdan, 2008). Other specific release-triggers are used in the context of receptor-mediated and magnetically driven carriers, which have been applied to specifically target colorectal cancer cells (Lee et al., 2020), but these techniques have not been tested for probiotic delivery yet. Other probiotic delivery systems has recently gained more attention including polymeric carriers such as microparticles, microdevices and fibers (Fig. 2).

3.2. Polymeric carriers

A review from 2020 summarizes the development of polymeric carriers for delivery of active or freeze-dried probiotic microbes (Asgari, Pourjavadi, Licht, Boisen, & Ajalloueian, 2020). None of the 41 studies included in the review have assessed the delivery of next generation probiotics in this type of carriers, revealing a current gap in this field of research.

3.2.1. Microparticles

As described above, probiotic cells may be encapsulated in microparticles, which do not require desiccation and may serve as probiotic carriers. The most common carrier material is the biodegradable polysaccharide alginate, which can be mixed or coated with other compounds, such as poly-L-lysine, to enhance stability and probiotic protection (Asgari et al., 2020)' (Chen et al., 2012)'(Cui, Goh, Kim, Choi, & Lee, 2000). As next generation probiotics need protection through the gastric and small intestinal environment, colon-targeting materials and coatings for microparticles are ideal. A variety of colon-targeted microparticle coatings have been developed for drug delivery, mainly enzyme-sensitive polysaccharides such as chitosan, pectin, inulin, and guar gum (Kotla et al., 2019), but probiotic research using such coatings is limited and mainly consists of *in vitro* experiments based on simulated gastric and intestinal conditions (Asgari et al., 2020). The muco-adhesive properties of these polysaccharides may favor initial bacterial colonization due to prolonged contact of the carrier with the mucus-layer during probiotic release, as observed by increased uptake of orally administered drugs (Bruschi, de Souza Ferreira, & Bassi da Silva, 2020). However, this remains to be experimentally verified. *Lactobacillus* and *Bifidobacterium* species are usually chosen during investigation of probiotic microparticle delivery systems, while only a few studies applied oxygen-sensitive microbes (Marcial-Coba et al., 2018)' (Raise et al., 2020). A water-oil-water double-emulsion encapsulation was successfully used to protect the highly oxygen- and acid-sensitive *A. muciniphila* against gastric and small intestine conditions, but viability severely decreased during storage for 3 day at 4 °C (van der Ark et al., 2017). In general, double-emulsions have poor stability and are prone to coalescence, which challenges their practical application. Another approach is to combine freeze-drying with extrusion- or emulsion-based processing. A significant increase in survival of *A. muciniphila* was observed during simulated gastric and ileal conditions when the bacteria were embedded in xanthan/gellan gum beads followed by freeze-drying, as compared to non-encapsulated cells (Marcial-Coba et al., 2018). However, aerobic storage for 30 days resulted in more than a 1-log viability decrease at 4 °C and a total loss of viable cells at 25 °C (Marcial-Coba et al., 2018). Another study compared survival of *F. prausnitzii* which was either freeze-dried and embedded in lipid Gelucire® beads or embedded in amidated low-methoxyl pectin gel beads followed by freeze-drying (Raise et al., 2020). The freeze-dried gel beads prolonged shelf life but did not protect the acid- and bile-sensitive bacterium against simulated GI conditions, whereas the lipid beads did not prolong shelf life but improved survival under GI conditions (Raise et al., 2020).

A three-step processing technique using microparticles as carriers, a desiccation step to increase storage stability, and a coating to enhance GI protection was tested by Cui et al. (Cui et al., 2000), who embedded *Bifidobacterium bifidum* in alginate microgels followed by poly-L-lysine coating and freeze-drying. Bacterial survival of simulated gastric conditions and during storage at 4 °C was significantly increased compared to control cultures, but viability still decreased about 2-log after 2 h at pH 1.5 and almost 3-log after 16-weeks of storage. For oxygen-sensitive microbes, the viability decrease during storage is thus likely to be higher. A delivery study in healthy human volunteers, dosed twice a day for 14 days with the *B. bifidum* in the same carrier or as unprotected control cultures (Cui, Cao, & Lee, 2007) demonstrated a significantly higher amount of Bifidobacteria in the group receiving the microgel-protected bacteria.

In contrast to what is known for *Lactobacillus* and *Bifidobacterium* species, the limited research investigating extrusion- and emulsion-based processing of next-generation probiotics indicate that these methods alone are not suitable for oxygen sensitive microbes due to low viability during storage. Desiccation is likely to be a crucial step to preserve next generation microbes during storage, and a freeze-drying step may thus be necessary in addition to extrusion or emulsion processing. This could provide a carrier that confers sufficient storage stability and protection during delivery, depending on the coating. However, the process of drying and embedding microbes in microparticles followed by adding a protective coating has not been tested for next generation probiotics.

3.2.2. Microdevices

Microdevices are mainly developed for therapeutic drug delivery to the gut, but the devices may also be applicable as probiotic carriers. The most common types include micropatches, microwells, and microcontainers ranging in size from 100 to 300 μm typically with a square or spherical shape (Fig. 2) (Mazzoni & Nielsen, 2020). They are often made of non-degradable materials, such as silicon or the epoxy-based photoresist SU-8 (Mazzoni & Nielsen, 2020), but these materials are not approved for consumption, leaving them suitable only for proof-of-concept studies. Biodegradable polymers, such as poly-L-lactic acid and poly-ε-caprolactone (Abid et al., 2019), are however also being applied as materials for microdevices and will likely be more suitable as probiotic carriers.

Microdevices are typically loaded with powders, but hydrogels and water- or oil-based liquids can also be loaded, e.g. by inkjet printing (Marizza, Keller, & Boisen, 2013), which are potentially suitable for desiccation-sensitive next-generation microbes. However, the microdevice-loading techniques are quite novel and currently not well-tested for loading of microbes. After loading of the desired content into a microdevice, a coating step usually follows to create a lid and seal off the device.

Specific coatings may confer desired features to the delivery system, such as release at a specific area in the GIT, as described above, or adhesion to the intestinal mucus. Coatings such as Eudragit®-polymers are degraded above specific pH values and commonly used for intestinal release of pharmaceuticals (Mazzoni & Nielsen, 2020). Also enzyme-triggered coatings have been developed based on crosslinking between chitosan and genipin (Kamguyan et al., 2021). Testing of a probiotic-loaded microcontainer delivery device with an

enzyme-triggered coating, suggests that the bacterium is only released in the cecum and colon of rats. Microcontainers may also provide mucoadhesive properties as well as unidirectional release (Christfort et al., 2020), which transiently retain the device and thereby may increase the release of the loaded material into the mucus (Park, Kwon, & Park, 2011). An important issue to consider in the context of probiotic delivery is whether the processing needed for coating (e.g. high temperatures or humidity) will permit bacterial survival.

The spatially heterogeneous gut ecosystem creates different microenvironments where commensals are specialized in colonizing specific sites, such as the mucus layer or colonic crypts (Pereira & Berry, 2017). Thus, introducing a probiotic microbe directly into a preferred gut niche may help to achieve optimal colonization. Such targeted delivery could potentially enhance the initial adhesion and proliferation ability of microbes that adhere to mucin structures and/or feed on nutrients present specifically in the mucus, however, this remains to be demonstrated. The prolonged lag-phase observed for desiccated microbes, including several next generation probiotics (Bircher et al., 2018), may on the other hand mean that the cells get displaced before onset of proliferation, and thereby reduce the effect of local release in the mucus.

3.2.3. *Polymeric fibers*

Like microparticles and -devices, polymeric fibers have mainly been investigated as carriers for oral drug delivery (Feng, Wei, et al., 2020). Fibers are typically produced by electrospinning, a process that uses an electric field to distort and create long, thin polymeric structures from a solution pressed out of a syringe. Desiccated or active microbes may be added to the solution, which often consists of polyvinyl alcohol alone or in combination with other materials, such as alginate, thus creating microbe-embedded fibers with increased storage viability and gastric protection of the microbes (Feng, Huang, et al., 2020). One report suggests that embedding of microbes in a 'sandwich' of two different fiber materials may be a feasible solution for probiotic delivery (Ajalloueian et al., 2022). As electrospun fibers can potentially be made at temperatures permitting bacterial survival (Feng, Wei, et al., 2020) and under anaerobic conditions, they represent a promising carrier for delivery of next generation probiotics, provided that they can be designed to protect the microbes from oxygen exposure after embedding.

4. Concluding remarks

Probiotic bacteria may confer a variety of benefits to the host. Particularly the so-called next-generation of probiotics may release great potential for preventive and therapeutic effects against a variety of conditions (O'Toole et al., 2017)' (El Hage et al., 2017). Most investigations of delivery strategies are carried out with 'classical' probiotics such as *Lactobacillus* and *Bifidobacterium* species. While these strains are very well studied, and therefore appropriate for use in proof-of-concept investigations of novel carriers, they were typically originally selected as probiotics because they are known to be easy to process and store, and robust towards the intestinal environment. Many of the novel delivery strategies therefore have their real potential as carriers of non-spore forming next generation probiotics, which are sensitive to oxygen, pH and bile, and thus more likely to benefit from delivery/release specifically in the colon, but also more prone to be deactivated by the processing steps required to load them into a given carrier. The future challenge is thus to further modify and optimize the novel delivery strategies in a way that allow survival during processing and storage of encapsulated or embedded next generation probiotic strains, and additionally confer protection of these sensitive bacteria in the GI tract until arrival at the target site.

However, microencapsulation methods involving extrusion or emulsion are difficult to apply in large-scale productions and currently result in a significant viability loss of next generation probiotics during storage. As high temperatures and oxygen is unavoidable during spray-drying, this technique is also not suited for next generation probiotics, hence there is a need to focus on more feasible desiccation methods such as freeze-drying. To optimize this process, the protecting effects of antioxidants, osmoadaptation and stress treatment during freeze drying of next generation probiotics should be investigated.

Declarations of interest

The authors have no conflicting interests to declare.

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