



Optimizing oral delivery of next generation probiotics

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ARTICLE INFO

Keywords:

Next generation probiotics
Microbial processing
Freeze-drying
Preservation/storage
Oral probiotic delivery
Gut microbiota

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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<https://doi.org/10.1016/j.tifs.2021.11.034>

Received 5 July 2021; Received in revised form 24 November 2021; Accepted 30 November 2021

Available online 2 December 2021

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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

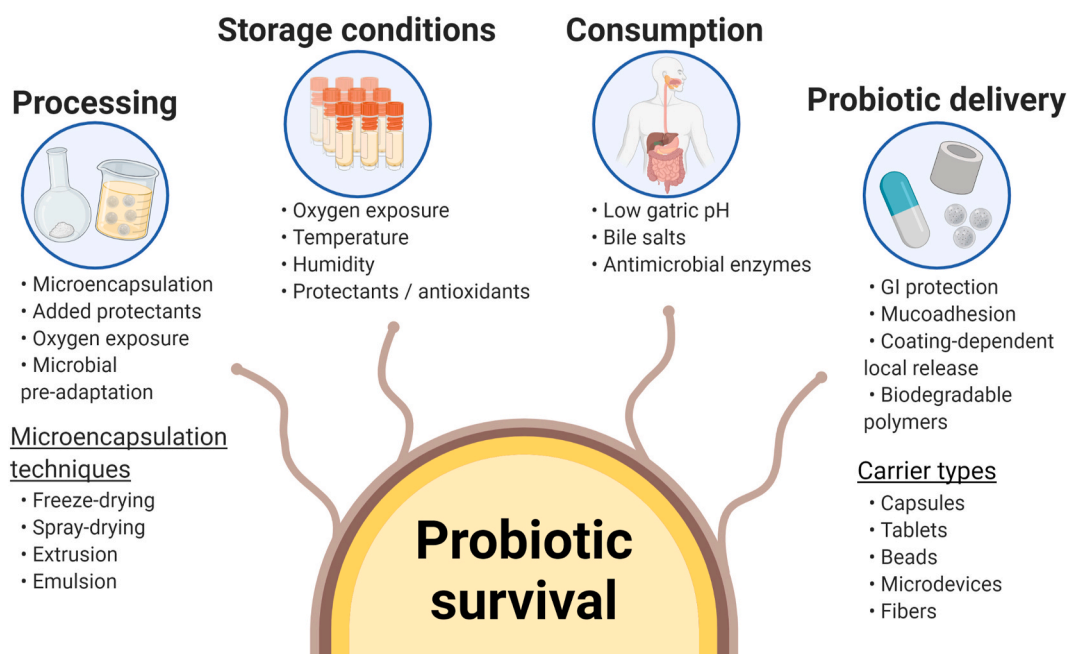


Fig. 1. Overview of factors related to probiotic survival from processing to gut delivery (Created with BioRender.com).

microbial community (Laursen, Bahl, & Licht, 2021). Some colonizers only reside in the gut temporarily during this time period, where many physico-chemical conditions in the gut are gradually changing (Panaraj 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 inhabitants, 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. prausnitzii* 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, Kandyliis, & 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 extent (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, Geirmaert, Hammes, Lacroix, & Schwab, 2018).

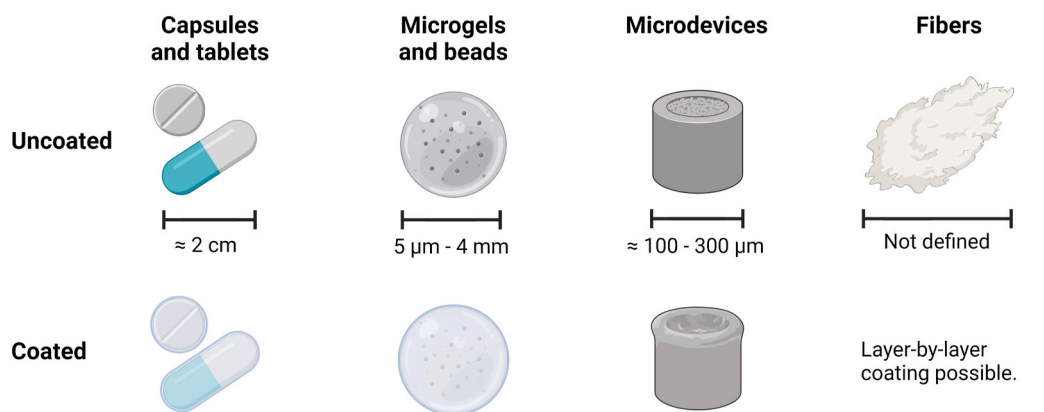
2.4. Freeze-drying of next generation probiotics

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 Diji, & 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

Non-food probiotic carrier types



Carrier properties

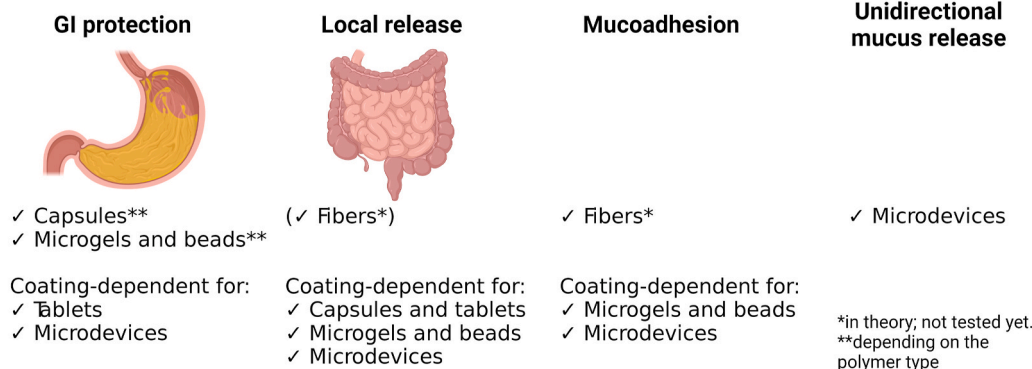


Fig. 2. Potential delivery strategies for next generation probiotics. Capsules, tablets, microdevices, microgels and –beads may be coated to locally release the probiotic whereas probiotic-containing fibers may be sandwiched with other materials to provide similar effects. Sizes: Gel/bead (Rokka & Rantamäki, 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	References
<i>Lactobacillus</i>	<i>spp.</i>	1907	Commercialized/standardized			Metchnikoff (1907)
<i>Bifidobacterium</i>	<i>spp.</i>	1906	Commercialized/standardized			Tissier (1906)
<i>Streptococcus</i>	<i>thermophilus</i>	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)
<i>Escherichia</i>	<i>coli</i>	1885	Low survival during freeze-drying. Commercialized (Nisse 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)
<i>Propionibacterium</i>	<i>freudenreichii</i>	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)
<i>Anaerostipes</i>	<i>Caccae</i>	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)
<i>Bacteroides</i>	<i>thetaitota-omicron</i>	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)
<i>Eubacterium</i>	<i>hallii</i>	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)
<i>Blautia</i>	<i>obeum</i>	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)
<i>Roseburia</i>	<i>intestinalis</i>	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)
<i>Akkermansia</i>	<i>muciniphila</i>	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, Knöchel, & 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)
<i>Faecalibacterium</i>	<i>prausnitzii</i>	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. 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 hyperosmotic 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 treatments 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, & Podczeczek, 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, & Ajallouei, 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 micro-environments 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 (Ajallouei 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.

Funding

This work was funded by the Novo Nordic Foundation through the Interdisciplinary Synergy Grant 'MIMIO - Microstructures, microbiota and oral delivery' (NNF17OC0026910), given to AB and TR.

Acknowledgements

The authors thank Pi Westi Bondegaard, Priscila Guerra, Katja Ann Kristensen and the MIMIO team at IDUN for the great collaboration that led to the insights resulting in this review.

References

- Abe, F., Miyauchi, H., Uchijima, A., Yaeshima, T., & Iwatsuki, K. (2009). Effects of storage temperature and water activity on the survival of bifidobacteria in powder form. *International Journal of Dairy Technology*, 62(2), 234–239.
- Abid, Z., Strindberg, S., Javed, M. M., Mazzoni, C., Vaut, L., Nielsen, L. H., et al. (2019). Biodegradable microcontainers-towards real life applications of microfabricated systems for oral drug delivery. *Lab on a Chip, Royal Society of Chemistry*, (17), 2905–2914.
- Ajallouei, F., Guerra, P. R., Bahl, M. I., Torp, A. M., Hwu, E., Te Licht, T. R., et al. (2022). Multi-layer PLGA-pullulan-PLGA electrospun nanofibers for probiotic delivery *Food Hydrocolloids* (Vol. 123, p. 107112). Elsevier.
- van der Ark, K. C. H., Nugroho, A. D. W., Berton-Carabin, C., Wang, C., Belzer, C., de Vos, W. M., et al. (2017). Encapsulation of the therapeutic microbe *Akkermansia muciniphila* in a double emulsion enhances survival in simulated gastric conditions. *Food Research International*, 102, 372–379. Elsevier.
- Aschenbrenner, M., Kulozik, U., & Foerst, P. (2012). Evaluation of the relevance of the glassy state as stability criterion for freeze-dried bacteria by application of the Arrhenius and WLF model. *Cryobiology, Elsevier Inc*, 65(3), 308–318.
- Asgari, S., Pourjavadi, A., Licht, T. R., Boisen, A., & Ajallouei, F. (2020). Polymeric carriers for enhanced delivery of probiotics. In *Advanced drug delivery reviews* (Vols. 161–162, pp. 1–21). Elsevier B.V..
- Atarashi, K., Tanoue, T., Oshima, K., Suda, W., Nagano, Y., Nishikawa, H., et al. (2013). Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. *Nature*, 500(7461), 232–236.
- Bellali, S., Bou Khalil, J., Fontanini, A., Raoult, D., & Lagier, J. C. (2020). A new protectant medium preserving bacterial viability after freeze drying. *Microbiological Research*, 236, 126454. Elsevier.
- Bircher, L., Geirnaert, A., Hammes, F., Lacroix, C., & Schwab, C. (2018). Effect of cryopreservation and lyophilization on viability and growth of strict anaerobic human gut microbes. *Microbial Biotechnology*, 11(4), 721–733.
- Broeckx, G., Vandenheuvel, D., Claes, I. J. J., Lebeer, S., & Kiekens, F. (2016). Drying techniques of probiotic bacteria as an important step towards the development of novel pharmaceuticals. *International Journal of Pharmaceutics*, 505(–2), 303–318.
- Bruschi, M. L., de Souza Ferreira, S. B., & Bassi da Silva, J. (2020). Mucoadhesive and mucus-penetrating polymers for drug delivery, nanotechnology for oral drug delivery. available at: <https://doi.org/10.1016/b978-0-12-818038-9.00011-9>.
- Bustamante, M., Laurie-Martínez, L., Vergara, D., Campos-Vega, R., Rubilar, M., & Shene, C. (2020). Effect of three polysaccharides (inulin, and mucilage from chia and flax seeds) on the survival of probiotic bacteria encapsulated by spray drying. *Applied Sciences*, 10(13), 1–17.
- Castellani, A., & Charlmers, A. J. (1919). *Manual of tropical medicin* (3rd ed.). New York: William Wood & Co.
- Chen, S., Zhao, Q., Ferguson, L. R., Shu, Q., Weir, I., & Garg, S. (2012). Development of a novel probiotic delivery system based on microencapsulation with protectants. *Applied Microbiology and Biotechnology*, 93(4), 1447–1457.
- Chiwele, I., Jones, B. E., & Podczek, F. (2000). The shell dissolution of various empty hard capsules. *Chemical and Pharmaceutical Bulletin*, 48(7), 951–956.
- Christfort, J. F., Guillot, A. J., Melero, A., Thamdrup, L. H. E., Garrigues, T. M., Boisen, A., et al. (2020). Cubic microcontainers improve in situ colonic mucoadhesion and absorption of amoxicillin in rats. *Pharmaceutics*, 12(4), 1–16.
- Cui, J. H., Cao, Q. R., & Lee, B. J. (2007). Enhanced delivery of bifidobacteria and fecal changes after multiple oral administrations of bifidobacteria-loaded alginate poly-L-lysine microparticles in human volunteers. *Drug Delivery*, 14(5), 265–271.
- Cui, J. H., Goh, J. S., Kim, P. H., Choi, S. H., & Lee, B. J. (2000). Survival and stability of bifidobacteria loaded in alginate poly-L-lysine microparticles. *International Journal of Pharmaceutics*, 210(1–2), 51–59.

- Dehghanbanadaki, H., Aazami, H., Keshavarz Azizi Raftar, S., Ashrafi, F., Ejtahed, H. S., Hashemi, E., et al. (2020). Global scientific output trend for Akkermansia muciniphila research: A bibliometric and scientometric analysis. *BMC Medical Informatics and Decision Making*, *20*(1), 1–12.
- Derrien, M., Van Baarlen, P., Hooiveld, G., Norin, E., Müller, M., & de Vos, W. M. (2011). Modulation of mucosal immune response, tolerance, and proliferation in mice colonized by the mucin-degrader Akkermansia muciniphila. *Frontiers in Microbiology*, *2*, 1–14.
- Derrien, M., & van Hylckama Vlieg, J. E. T. (2015). Fate, activity, and impact of ingested bacteria within the human gut microbiota. *Trends in Microbiology*. Elsevier Ltd, 1 June.
- Derrien, M., Vaughan, E. E., Plugge, C. M., & de Vos, W. M. (2004). Akkermansia muciniphila gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. *International Journal of Systematic and Evolutionary Microbiology*. *International Journal of Systematic and Evolutionary Microbiology*, *54*(5), 1469–1476.
- Dimitrellou, D., Kandyli, P., & Kourkoutas, Y. (2016). Effect of cooling rate, freeze-drying, and storage on survival of free and immobilized Lactobacillus casei ATCC 393. *Lebensmittel-Wissenschaft und -Technologie- Food Science and Technology*, *69*, 468–473. Elsevier Ltd.
- Distaso, A. (1912). Contribution a l'étude sur l'intoxication intestinale. *Zentralbl Bakteriol Parasitenkd Infektionskr Hyg Abt I*, *62*, 433–468.
- Dodoo, C. C., Wang, J., Basit, A. W., Stapleton, P., & Gaisford, S. (2017). Targeted delivery of probiotics to enhance gastrointestinal stability and intestinal colonisation. *International Journal of Pharmaceutics*. Elsevier B.V., *530*(1–2), 224–229.
- Duncan, S. H., Hold, G. L., Barcenilla, A., Stewart, C. S., & Flint, H. J. (2002). Roseburia intestinalis sp. nov., a novel saccharolytic, butyrate-producing bacterium from human faeces. *International Journal of Systematic and Evolutionary Microbiology*, *52* (5), 1615–1620.
- Duncan, S. H., Hold, G. L., Harmsen, H. J. M., Stewart, C. S., & Flint, H. J. (2002). Growth requirements and fermentation products of Fusobacterium prausnitzii, and a proposal to reclassify it as Faecalibacterium prausnitzii gen. nov., comb. nov. *International Journal of Systematic and Evolutionary Microbiology*, *52*(6), 2141–2146.
- Duranti, S., Lugli, G. A., Mancabelli, L., Armanini, F., Turroni, F., James, K., et al. (2017). Maternal inheritance of bifidobacterial communities and bifidophages in infants through vertical transmission. *Microbiome*. BioMed Central Ltd, *5*(1), 282.
- El Hage, R., Hernandez-Sanabria, E., & Van de Wiele, T. (2017). Emerging trends in 'smart probiotics': Functional consideration for the development of novel health and industrial applications. *Frontiers in Microbiology*, *8*, 1–11.
- Erturk-Hasdemir, D., & Kasper, D. L. (2018). Finding a needle in a haystack: Bacteroides fragilis polysaccharide a as the archetypical symbiosis factor. *Annals of the New York Academy of Sciences*, *1417*(1), 116–129.
- Everard, A., Belzer, C., Geurts, L., Ouwerkerk, J. P., Druart, C., Bindels, L. B., et al. (2013). Cross-talk between Akkermansia muciniphila and intestinal epithelium controls diet-induced obesity. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(22), 9066–9071.
- Fattahi, Y., Heidari, H. R., & Khosroushahi, A. Y. (2020). Review of short-chain fatty acids effects on the immune system and cancer. *Food Bioscience*, *38*, 100793. Elsevier Ltd.
- Feng, K., Huang, R., meng, Wu, R., qing, W., shan, Y., et al. (2020). A novel route for double-layered encapsulation of probiotics with improved viability under adverse conditions. *Food Chemistry*, Elsevier, *310*, 125977.
- Feng, K., Wei, Y., shan, H., gen, T., Linhardt, R. J., Zong, M. hua, et al. (2020). Colon-targeted delivery systems for nutraceuticals: A review of current vehicles, evaluation methods and future prospects. *Trends in Food Science & Technology*, *102*, 203–222. Elsevier.
- Ferretti, P., Pasolli, E., Tett, A., Asnicar, F., Gorfer, V., Fedi, S., et al. (2018). Mother-to-Infant microbial transmission from different body sites shapes the developing infant gut microbiome. *Cell Host & Microbe*, *24*(1), 133–145. e5.
- Foditsch, C., Santos, T. M. A., Teixeira, A. G. V., Pereira, R. V. V., Dias, J. M., Gaeta, N., et al. (2014). Isolation and characterization of faecalibacterium prausnitzii from calves and piglets. *PLoS One*, *9*(12), 1–9.
- França, M. B., Panek, A. D., & Eleutherio, E. C. A. (2007). Oxidative stress and its effects during dehydration. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *146*(4), 621–631.
- Freter, R., Brickner, H., Fekete, J., Vickerman, M. M., & Carey, K. E. (1983). Survival and implantation of Escherichia coli in the intestinal tract. *Infection and Immunity*, *39*(2), 686–703.
- Gaucher, F., Bonnassie, S., Rabah, H., Leverrier, P., Pottier, S., Jardin, J., et al. (2019). Benefits and drawbacks of osmotic adjustment in Propionibacterium freudenreichii. *Journal of Proteomics*, Elsevier, *204*, 103400.
- Gaucher, F., Kponoulo, K., Rabah, H., Bonnassie, S., Ossemond, J., Pottier, S., et al. (2019). Propionibacterium freudenreichii CIRM-BIA 129 osmoadaptation coupled to acid-adaptation increases its viability during freeze-drying. *Frontiers in Microbiology*, *10*, 1–15.
- Gauffin Cano, P., Santacruz, A., Moya, Á., & Sanz, Y. (2012). Bacteroides uniformis CECT 7771 ameliorates metabolic and immunological dysfunction in mice with high-fat-diet induced obesity. *PLoS One*, *7*(7). <https://doi.org/10.1371/journal.pone.0041079>
- Gérard, P., Lepercq, P., Leclerc, M., Gavini, F., Raibaud, P., & Juste, C. (2007). Bacteroides sp. strain D8, the first cholesterol-reducing bacterium isolated from human feces. *Applied and Environmental Microbiology*, *73*(18), 5742–5749.
- Goodrich, J. K., Waters, J. L., Poole, A. C., Sutter, J. L., Koren, O., Blekhan, R., et al. (2014). Human genetics shape the gut microbiome. *Cell*, Elsevier Inc, *159*(4), 789–799.
- Guarner, F., Sanders, M. E., Eliakim, R., Fedorak, R., & Mair, A. Le (2017). *World gastroenterology organisation global guidelines: Probiotics and prebiotics february 2017*.
- Hacker, J., & Blum-Oehler, G. (2007). In appreciation of theodor escherich. *Nature Reviews Microbiology*, *5*(12), 902.
- Hammer, B. W. (1911). A note on the vacuum desiccation of bacteria. *The Journal of Medical Research*, *24*(1), 527–530.
- Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology*, *11*(8), 506–514.
- Holdeman, L. V., & Moore, W. E. C. (1974). New genus, Coprococcus, twelve new species, and emended descriptions of four previously described species of bacteria from human feces. *International Journal of Systematic and Evolutionary Microbiology*, *24*(2), 260–277.
- Hooper, L. V., Wong, M. H., Thelin, A., Hansson, L., Falk, P. G., & Gordon, J. I. (2001). Molecular analysis of commensal host-microbial relationships in the intestine. *Science*, *291*(5505), 881–884.
- Huang, S., Vignolles, M. L., Chen, X. D., Le Loir, Y., Jan, G., Schuck, P., et al. (2017). Spray drying of probiotics and other food-grade bacteria: A review. *Trends in Food Science & Technology*, *63*, 1–17.
- Juárez Tomás, M. S., Ocaña, V. S., & Nader-Macias, M. E. (2004). Viability of vaginal probiotic lactobacilli during refrigerated and frozen storage. *Anaerobe*, *10*(1), 1–5.
- Kailasapathy, K. (2002). Microencapsulation of probiotic bacteria: Technology and potential applications. *Current Issues in Intestinal Microbiology*, *3*(1), 39–48.
- Kamguyan, K., Torp, A., Christfort, J., Guerra, P., Licht, T., Hagner Nielsen, L., et al. (2021). Colon-specific delivery of bioactive agents using genipin-crosslinked chitosan coated microcontainers. *ACS Applied Materials & Interfaces*, *4*(1), 752–762.
- Khan, M. T., Van Dijk, J. M., & Harmsen, H. J. M. (2014). Antioxidants keep the potentially probiotic but highly oxygen-sensitive human gut bacterium Faecalibacterium prausnitzii alive at ambient air. *PLoS One*, *9*(5). <https://doi.org/10.1371/journal.pone.0096097>
- Kotla, N. G., Rana, S., Sivaraman, G., Sunnapu, O., Vemula, P. K., Pandit, A., et al. (2019). Bioresponsive drug delivery systems in intestinal inflammation: State-of-the-art and future perspectives. *Advanced Drug Delivery Reviews* (Vol. 146, pp. 248–266). Elsevier B.V..
- Kristensen, N. B., Bryrup, T., Allin, K. H., Nielsen, T., Hansen, T. H., & Pedersen, O. (2016). Alterations in fecal microbiota composition by probiotic supplementation in healthy adults: A systematic review of randomized controlled trials. *Genome Medicine*, BioMed Central, *8*(1), 52.
- Kropp, C., Le Corf, K., Relizani, K., Tambosco, K., Martinez, C., Chain, F., et al. (2021). The keystone commensal bacterium Christensenella minuta DSM 22607 displays anti-inflammatory properties both in vitro and in vivo. *Scientific Reports*. Nature Publishing Group UK, *11*(1), 1–12.
- Lagier, J.-C., Dubourg, G., Million, M., Cadoret, F., Bilen, M., Fenollar, F., et al. (2018). Culturing the human microbiota and culturomics. *Nature Reviews Microbiology*, *16*, 540–550.
- Laursen, M. F., Bahl, M. I., & Licht, T. R. (2021). Settlers of our inner surface – factors shaping the gut microbiota from birth to toddlerhood. *FEMS Microbiology Reviews*, Oxford University Press (OUP). <https://doi.org/10.1093/femsre/ruab001>. available at:
- Laursen, M. F., Laursen, R. P., Larnkjær, A., Michaelsen, K. F., Bahl, M. I., & Licht, T. R. (2017). Administration of two probiotic strains during early childhood does not affect the endogenous gut microbiota composition despite probiotic proliferation. *BMC Microbiology*, *17*(1), 175.
- Lawley, T. D., & Walker, A. W. (2013). Intestinal colonization resistance. *Immunology*, *138*(1), 1–11.
- Lee, S. H., Bajracharya, R., Min, J. Y., Han, J. W., Park, B. J., & Han, H. K. (2020). Strategic approaches for colon targeted drug delivery: An overview of recent advancements. *Pharmaceutics*, *12*(1). <https://doi.org/10.3390/pharmaceutics12010068>
- Liu, C., Finegold, S. M., Song, Y., & Lawson, P. A. (2008). Reclassification of Clostridium coccoides, Ruminococcus hansenii, Ruminococcus hydrogenotrophicus, Ruminococcus loti, Ruminococcus productus and Ruminococcus schinkii as Blautia coccoides gen. nov., comb. nov., Blautia hansenii comb. nov., Blautia hydroge. *International Journal of Systematic and Evolutionary Microbiology*, *58*(8), 1896–1902.
- Mantegazza, C., Molinari, P., D'Auria, E., Sonnino, M., Morelli, L., & Zuccotti, G. V. (2018). Probiotics and antibiotic-associated diarrhea in children: A review and new evidence on lactobacillus rhamnosus gg during and after antibiotic treatment. *Pharmacological Research*, Elsevier Ltd, *128*, 63–72.
- Marcial-Coba, M. S., Cieplak, T., Cahú, T. B., Blennow, A., Knöchel, S., & Nielsen, D. S. (2018). Viability of microencapsulated Akkermansia muciniphila and Lactobacillus plantarum during freeze-drying, storage and in vitro simulated upper gastrointestinal tract passage. *Food and Function*, Royal Society of Chemistry, *9*(11), 5868–5879.
- Marcial-Coba, M. S., Knöchel, S., & Nielsen, D. S. (2019). Low-moisture food matrices as probiotic carriers. *FEMS Microbiology Letters*, *366*(2), 1–11.
- Marcial-Coba, M. S., Saaby, L., Knöchel, S., & Nielsen, D. S. (2019). Dark chocolate as a stable carrier of microencapsulated Akkermansia muciniphila and Lactobacillus casei. *FEMS Microbiology Letters*, *366*(2), 1–6.
- Marizza, P., Keller, S. S., & Boisen, A. (2013). Inkjet printing as a technique for filling of micro-wells with biocompatible polymers. *Microelectronic Engineering*, *111*, 391–395. Elsevier B.V.
- Martin, M. J., Lara-Villoslada, F., Ruiz, M. A., & Morales, M. E. (2015). Microencapsulation of bacteria: A review of different technologies and their impact on the probiotic effects. *Innovative Food Science & Emerging Technologies*, *27*, 15–25.

- Mazzoni, C., & Nielsen, L. H. (2020). *Microdevices to successfully deliver orally administered drugs, nanotechnology for oral drug delivery*. Elsevier Inc. <https://doi.org/10.1016/b978-0-12-818038-9.00012-0>. available at:
- McConnell, E. L., Basit, A. W., & Murdan, S. (2008). Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. *Journal of Pharmacy and Pharmacology*, *60*(1), 63–70.
- Metchnikoff, E. (1907). *The prolongation of life: Optimistic studies*. New York: G P Putnam's Sons, English trans. Mitchell, P. Chalmers.
- Minelli, E. B., & Benini, A. (2008). Relationship between number of bacteria and their probiotic effects. *Microbial Ecology in Health and Disease*, *20*(4), 180–183.
- Miquel, S., Martín, R., Rossi, O., Bermúdez-Humarán, L. G., Chatel, J. M., Sokol, H., et al. (2013). Faecalibacterium prausnitzii and human intestinal health. *Current Opinion in Microbiology*, *16*(3), 255–261.
- Moore, W. E. C., Johnson, J. L., & Holdeman, L. V. (1976). Emendation of bacteroidaceae and butyrivibrio and descriptions of desulfomonas gen. Nov. And ten new species in the genera desulfomonas, butyrivibrio, Eubacterium, Clostridium, and Ruminococcus. *International Journal of Systematic Bacteriology*, *26*(2), 238–252.
- Morgan, C. A., Herman, N., White, P. A., & Vesey, G. (2006). Preservation of microorganisms by drying; A review. *Journal of Microbiological Methods*, *66*(2), 183–193.
- Niel, C. B. van (1928). *The propionic acid bacteria*. Haarlem: Uitgeverszaak and Boissevain and Co.
- O'Toole, P. W., Marchesi, J. R., & Hill, C. (2017). Next-generation probiotics: The spectrum from probiotics to live biotherapeutics. *Nature Microbiology*, *Macmillan Publishers Limited*, *2*, 1–6.
- Pannaraj, P. S., Li, F., Cerini, C., Bender, J. M., Yang, S., Rollie, A., et al. (2017). Association between breast milk bacterial communities and establishment and development of the infant gut microbiome. *JAMA Pediatrics*, *171*(7), 647–654.
- Park, K., Kwon, I. C., & Park, K. (2011). Oral protein delivery: Current status and future prospect. *Reactive and Functional Polymers*, *71*(3), 280–287. Elsevier Ltd.
- Pereira, F. C., & Berry, D. (2017). Microbial nutrient niches in the gut. *Environmental Microbiology*, *19*(4), 1366–1378.
- Pispan, S., Hewitt, C. J., & Stapley, A. G. F. (2013). Comparison of cell survival rates of *E. coli* K12 and *L. acidophilus* undergoing spray drying. *Food and Bioprocess Processing, Institution of Chemical Engineers*, *91*(4), 362–369.
- Plovier, H., Everard, A., Druart, C., Depommier, C., Van Hul, M., Geurts, L., et al. (2017). A purified membrane protein from Akkermansia muciniphila or the pasteurized bacterium improves metabolism in obese and diabetic mice. *Nature Medicine, Nature Publishing Group*, *23*(1), 107–113.
- Raise, A., Dupont, S., Iaconelli, C., Caliri, C., Charriau, A., Gervais, P., et al. (2020). Comparison of two encapsulation processes to protect the commensal gut probiotic bacterium Faecalibacterium prausnitzii from the digestive tract. In *Journal of drug delivery science and technology* (Vol. 56, p. 101608). Elsevier.
- Reddy, K. B. P. K., Awasthi, S. P., Madhu, A. N., & Prapulla, S. G. (2009). Role of cryoprotectants on the viability and functional properties of probiotic lactic acid bacteria during freeze drying. *Food Biotechnology*, *23*(3), 243–265.
- Rokka, S., & Rantamäki, P. (2010). Protecting probiotic bacteria by microencapsulation: Challenges for industrial applications. *European Food Research and Technology*, *231* (1), 1–12.
- Rossi, O., Van Berkel, L. A., Chain, F., Tanweer Khan, M., Taverne, N., Sokol, H., et al. (2016). Faecalibacterium prausnitzii A2-165 has a high capacity to induce IL-10 in human and murine dendritic cells and modulates T cell responses. *Scientific Reports, Nature Publishing Group*, *6*, 1–12.
- Round, J. L., & Mazmanian, S. K. (2010). Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(27), 12204–12209.
- Routy, B., Le Chatelier, E., Derosa, L., Duong, C. P. M., Alou, M. T., Daillère, R., et al. (2018). Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. *Science*, *359*(6371), 91–97.
- Schwartz, A., Hold, G. L., Duncan, S. H., Gruhl, B., Collins, M. D., Lawson, P. A., et al. (2002). Anaerostipes caccae gen. nov., sp. nov., a new saccharolytic, acetate-utilising, butyrate-producing bacterium from human faeces. *Systematic & Applied Microbiology*, *25*(1), 46–51.
- Sharma, R., Sanodiya, B. S., Thakur, G. S., Jaiswal, P., Sharma, A., & Bisen, P. S. (2014). Standardization of lyophilization medium for Streptococcus thermophilus subjected to viability escalation on freeze drying. *Microbiology Research*, *5*(1), 1–3.
- Shin, N. R., Lee, J. C., Lee, H. Y., Kim, M. S., Whon, T. W., Lee, M. S., et al. (2014). An increase in the Akkermansia spp. population induced by metformin treatment improves glucose homeostasis in diet-induced obese mice. *Gut*, *63*(5), 727–735.
- Suez, J., Zmora, N., Zilberman-Schapira, G., Mor, U., Dori-Bachash, M., Bashardes, S., et al. (2018). Post-antibiotic gut mucosal microbiome reconstitution is impaired by probiotics and improved by autologous FMT. *Cell, Elsevier Inc*, *174*(6), 1406–1423.
- Tissier, H. (1906). Traitement des infections intestinales par la méthode de la flore bactérienne de l'intestin. *Crit. Rev. Soc. Biol.*, *60*, 359–361.
- Ueno, N., Fujiya, M., Segawa, S., Nata, T., Moriichi, K., Tanabe, H., et al. (2011). Heat-killed body of lactobacillus brevis SBC8803 ameliorates intestinal injury in a murine model of colitis by enhancing the intestinal barrier function. *Inflammatory Bowel Diseases*, *17*(11), 2235–2250.
- Venegas, D. P., De La Fuente, M. K., Landskron, G., González, M. J., Quera, R., Dijkstra, G., et al. (2019). Short chain fatty acids (SCFAs) mediated gut epithelial and immune regulation and its relevance for inflammatory bowel diseases. *Frontiers in Immunology*, *10*. <https://doi.org/10.3389/fimmu.2019.00277>
- Villena, M. J. M., Lara-Villoslada, F., Martínez, M. A. R., & Hernández, M. E. M. (2015). Development of gastro-resistant tablets for the protection and intestinal delivery of Lactobacillus fermentum CECT 5716. *International Journal of Pharmaceutics*, *487* (1–2), 314–319.
- World Health Organization. (2001). Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria. *Food and Agriculture Organization of the United Nations and World Health Organization Expert Consultation Report*.
- Yang, J. Y., Lee, Y. S., Kim, Y., Lee, S. H., Ryu, S., Fukuda, S., et al. (2017). Gut commensal Bacteroides acidifaciens prevents obesity and improves insulin sensitivity in mice. *Mucosal Immunology*, *10*(1), 104–116.
- Yoon, M. Y., & Yoon, S. S. (2018). Disruption of the gut ecosystem by antibiotics. *Yonsei Medical Journal*, *59*(1), 4–12.