# CHAPTER 24. NEXT-GENERATION PROBIOTICS

Joana Cristina Barbosa [a]*, Daniela Machado [a], Diana Almeida [a], José Carlos Andrade [b], Ana Maria Gomes [a], Ana Cristina Freitas [a†]

[a] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal

[b] CESPU, Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde, Rua Central de Gandra, 1317, 4585-116 Gandra PRD, Portugal

* Corresponding author: jcbarbosa@ucp.pt

## ABSTRACT

In the last years, the scientific community has recognized that specific microbial strains resident in the intestinal ecosystem play a key role in human health, participating in several functions beneficial to the host. Such microorganisms have been termed as next-generation probiotics and they are presently considered as food/nutraceutical supplements and biotherapeutic products. However, most of the next-generation probiotic candidates are nutritionally demanding and highly sensitive to aerobic conditions, which translates into several technological challenges concerning large-scale production and appeals to the development of suitable delivery systems able to promote viability and functionality of such probiotic strains. In this chapter, we will present an overall perspective of next-generation probiotics candidates in terms of their health beneficial effects, the delivery systems developed and employed to protect them, and related regulation framework and risk assessment targeting relevant criteria for commercialization in food and pharmaceutical markets.

### KEYWORDS

Biotherapeutics; Delivery systems; Dysbiosis; Gut microbiota; Novel foods; Probiotics

## INTRODUCTION

The consumption of probiotics, although unintentional, has a story as old as human history, dating from the first-ever produced fermented foods and beverages, upon the implementation of agriculture and farming techniques by our ancestors (Gasbarrini et al., 2016; Khan and Malik, 2019; Ozen and Dinleyici, 2015). A timeline of landmarks in probiotics evolution is presented in Figure 1. What started as a food preservation method, to ensure longer storage periods, was later associated with beneficial effects to consumers, both in terms of nutrition and disease prevention and/or treatment (Ozen and Dinleyici, 2015). The modern history of probiotics starts with the works of Pasteur and Metchnikoff: the first identified the microorganisms responsible for fermentation, while the second proposed the pioneering idea of the relationship between diet, gut microbiota, and the general individual health and aging [Figure 1; (Gasbarrini et al., 2016; Ozen and Dinleyici, 2015)].

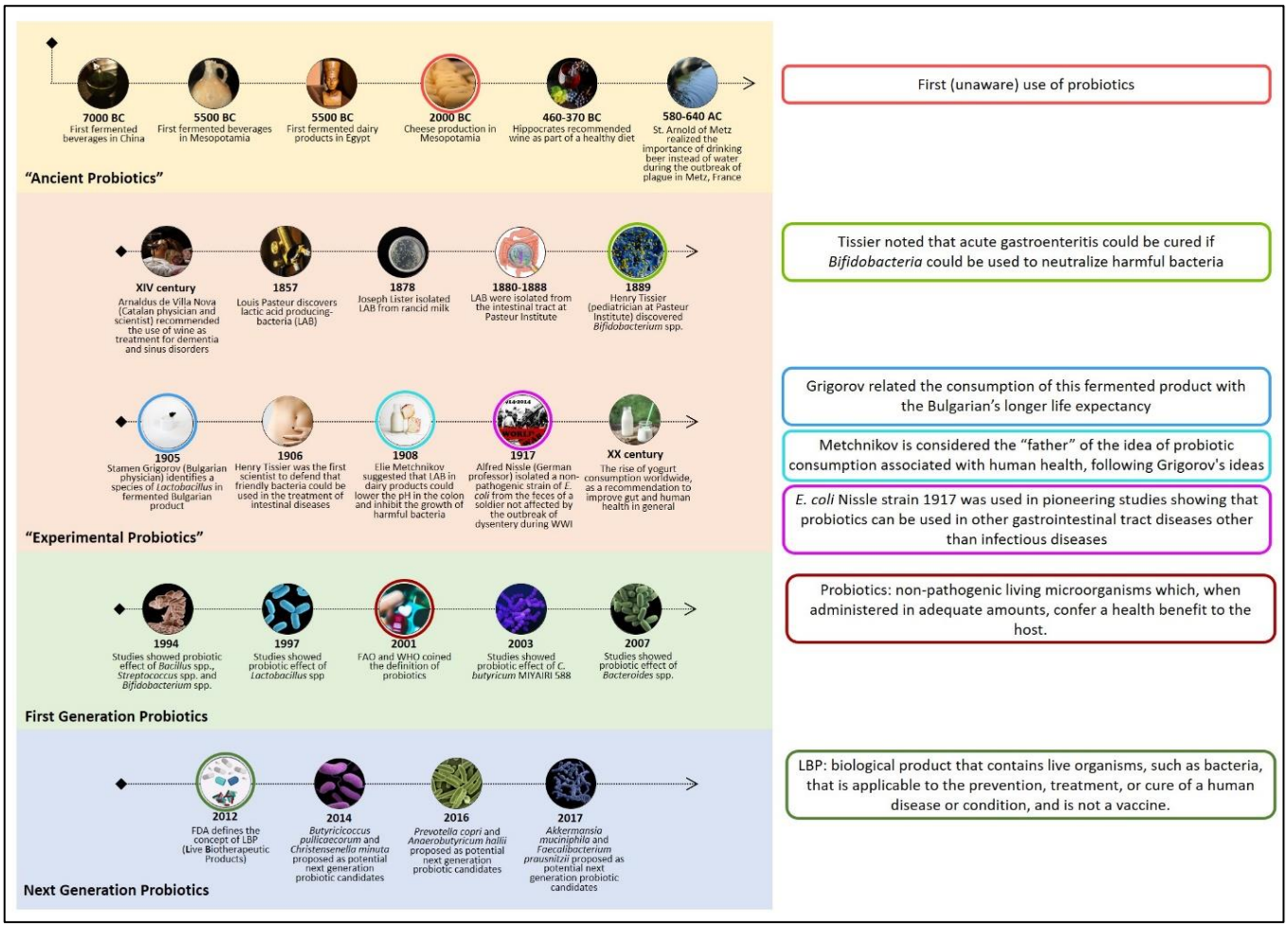


**Figure 1. Probiotics timeline.** This timeline provides a compilation of milestones that punctuate the history and the story of probiotics; it starts with the first, unaware, consumption of "ancient" probiotics, and continues highlighting the main contributors for the understanding of the probiotics concept and evidence collected for the bacterial strains designated as classical probiotics. More recently, several bacterial strains are being proposed as next-generation probiotics, the focus of this chapter.

Since then, the definition of probiotics was established – "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014) – and several bacterial strains have been widely applied in human consumption due to their well-established beneficial effects. These known "classical" probiotics include several strains from the genera *Bacillus*, *Lactobacillus,* and *Bifidobacterium* and are usually associated with fermented foods, mainly dairy products (Dahiya et al., 2019; Gomes et al., 2017; Sornplang and Piyadeatsoontorn, 2016).

According to the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO), the human gastrointestinal tract (GIT) is presented as the recommended source of probiotics for human consumption (FAO/WHO, 2002; Zielińska et al., 2018). Studies demonstrated that strains isolated from human GIT and isolates originated from food show distinct characteristics, for instance, in terms of adhesion capacity and resistance to GIT passage, as well as in the effectiveness in modulating the hosts' microbiota (Monteagudo-Mera et al., 2012). Additionally, commensal strains isolated from human GIT are considered safe for human consumption as probiotics (de Melo Pereira et al., 2018; Zielińska et al., 2018). Gut microbiota play a key role in the regulation of the metabolic and inflammatory profiles of the host, contributing to cellular homeostasis and global health. An imbalance in the composition and proportion of gut microbiota strains – dysbiosis – is often associated with several illnesses, such as diabetes, obesity, and immune system disorders (Almeida et al., 2020; Khan and Malik, 2019).

In this context, various commensal microbial genera and species identified among the gut microbiota, that have never been applied before in the food industry, are being proposed as Next-Generation Probiotics (NGP), and are currently under intensive investigation (Andrade et al., 2020; Saarela, 2019). Among the most promising candidates are strains of the species *Akkermansia muciniphila*, *Faecalibacterium prausnitzii* (former *Fusobacterium prausnitzii*), *Anaerobutyricum hallii* (formerly *Eubacterium hallii*), as well as *Bacteroides* spp., *Roseburia* spp. and *Clostridium butyricum* (Almeida et al., 2020; Dahiya et al., 2019; Khan and Malik, 2019; Saarela, 2019). These commensal bacteria are associated with a healthy status of the gut microbiota when present in adequate amounts, and have demonstrated promising results in terms of promoting health in model studies. Interestingly, this list of new potential probiotic candidates includes not only Gram-positive bacteria – which are the most commonly used by tradition –, but also Gram-negative bacteria, as is the case of *A. muciniphila*, *E. coli,* and *Bacteroides* spp. (Khan and Malik, 2019). Gram-negative bacteria are usually regarded as potentially harmful, due to the presence of lipopolysaccharides (LPS) anchored on their outer membrane (Sperandeo et al., 2017). Bacterial LPS is a pyrogenic substance that has been

associated with several diseases, which include chronic gut inflammation, diabetes, neurological degeneration, and liver damage (Wassenaar and Zimmermann, 2018). However, human microbiota, in particular, gut commensals, include several Gram-negative bacteria (Hugon et al., 2015); in fact, there is increasing evidence that Gram-negative bacteria play an important role in immune response modulation and seem to have a positive impact when applied in both animal and preclinical human models (Cani and de Vos, 2017; Dao et al., 2016; Kandasamy et al., 2017; Ottman et al., 2017).

Thus, there is a considerable number of new probiotic candidates among the human commensals, which are being isolated from the human gut and characterized, envisaging a possible application in human consumption (Figure 2). Several variables have to be considered when investigating a strain as a potential probiotic: starting with the accurate taxonomic identification to the strain level, moving to the characterization of the potential health benefits to the consumer, and ensuring that the strain does not carry harmful traits; at the end, the candidate strain must also fulfill all the requirements of the regulatory authorities as well as be suitable for industrial manipulation (Khan and Malik, 2019; de Melo Pereira et al., 2018). It is worth mentioning that gut commensal bacteria often have specific requirements in terms of nutrients, oxygen, and pH, which makes them sometimes hard to handle under laboratorial conditions, presenting a fastidious behavior or demanding extensive growth optimization (Clark, 2019). Upon the selection steps, an appropriate method for delivering the probiotic has to be developed, to ensure the administration of adequate amounts of the live bacteria to the consumer's gut, preferably incorporated into a food or beverage matrix (Šipailienė and Petraitytė, 2018).

Table 1 provides information on bacterial strains that are currently being regarded as promising candidates for the NGP category. Particularly, next-generation probiotic candidates *A. muciniphila*, *F. prausnitzii,* and *A. hallii* will be described in further detail, as they are under great scrutiny for future applications. Their potential uses and the most promising developments in this field will be described. Finally, delivery strategies currently under development and the recent advances in this area will be addressed, as well as safety and regulatory issues that NGP candidates need to fulfill to reach the market and, ultimately, human consumption.

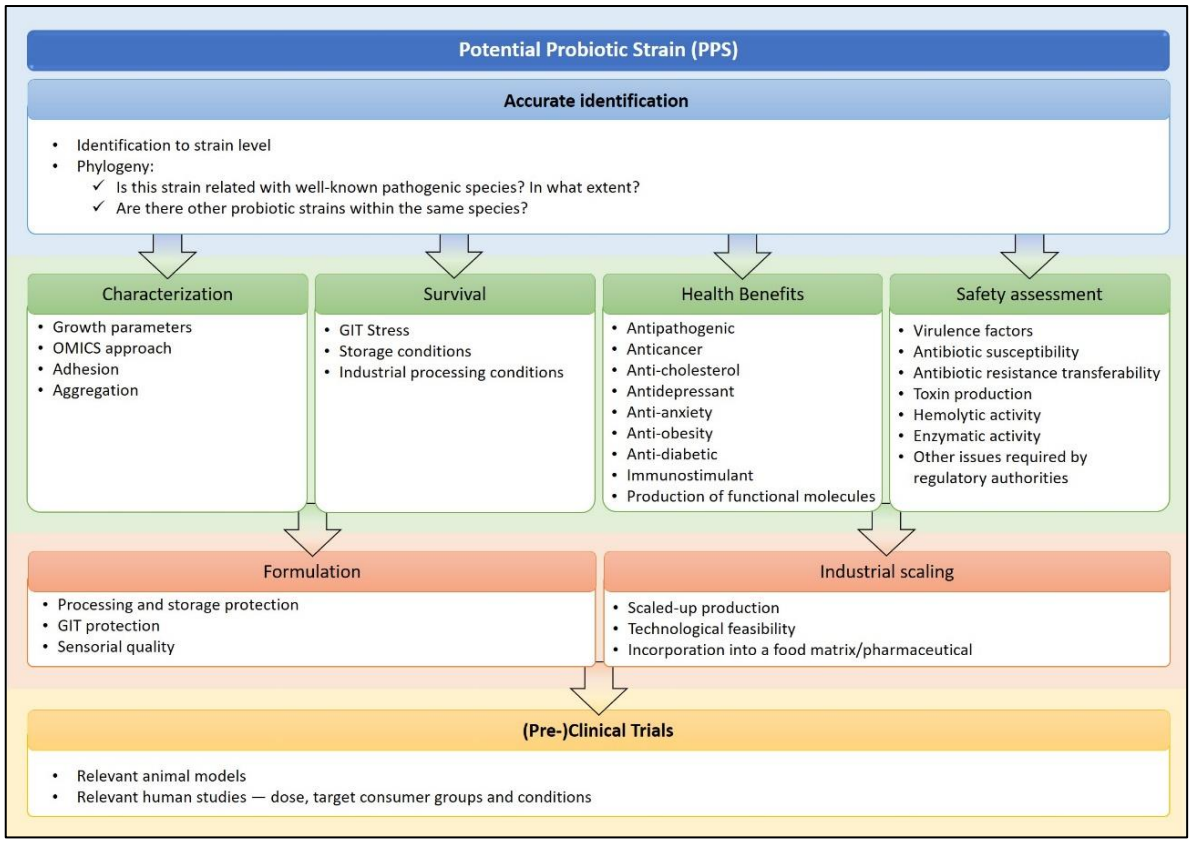


**Figure 2. Probiotics selection pipeline.** Several steps must be followed to ensure the effectiveness, safety, and technological feasibility of a bacterial strain, to meet the requirements of a probiotic. Although in the earlier described probiotics the benefit was proved even before knowing the agent causing such effect, as the interest in discovering and producing probiotics grows, so the need for adequate regulation is a demand, with clearly defined steps that should be fulfilled by all the next-generation probiotic candidates.

## NEXT-GENERATION PROBIOTICS MECHANISMS AND POTENTIAL HEALTH APPLICATIONS

Scientific evidence supporting the importance of gut beneficial microorganisms in many facets of human physiology has been long established through randomized controlled trials and observational studies. The recent opportunity for the cultivation of hitherto unknown human commensals – a major advantage of the "-omics" revolution – has provided new insights into the relationship between disease onset and dysbiotic gut microbiota. However, the effect of gut community members on human health is not solely determined by their presence/abundance in the gut ecosystem, but also by the products resulting from their collective genetic (microbiome) and metabolic activity (microbiota-derived metabolites), and NGP are no exception. These metabolites can derive directly from bacteria or the transformation of dietary or host-derived substrates. The effects of many bacterial metabolites, including short chain fatty acids (SCFA; acetate, propionate and butyrate), folate, indoles, secondary bile acids, trimethylamine-N-oxide (TMAO), serotonin, gamma amino butyric acid (GABA), have been characterized. Under different concentrations, these bacterial metabolites play important roles in modulating host physiology (Agus et al., 2021).

Hereafter, the mechanistic fashion by which some of the emerging NGP positively impact human disease and health will be discussed – from their influence on the immune system to less expected areas such as neurobiology – and how they can putatively modify disease trajectory. It is important to note that, although each NGP possesses its own microbial identity and potential, some of the mechanisms underlying their beneficial effects may have overlapping elements with other NGP, a trend common to classical probiotic strains.

## IMPACT OF NGP-DERIVED METABOLITES ON HOST PHYSIOLOGY

Many of the acknowledged effects attributed to the gut microbiome in human health are the result of microbiota-derived metabolites, including, short-chain fatty acids, antimicrobial compounds, extracellular proteins, among others (Figure 3). Interestingly, the microbial gut environment represents a highly dynamic and intricate bionetwork that does not reflect the impact of an individual species via a specific mechanism, but it is rather a cascade of trophic complexity that leads to community stability.

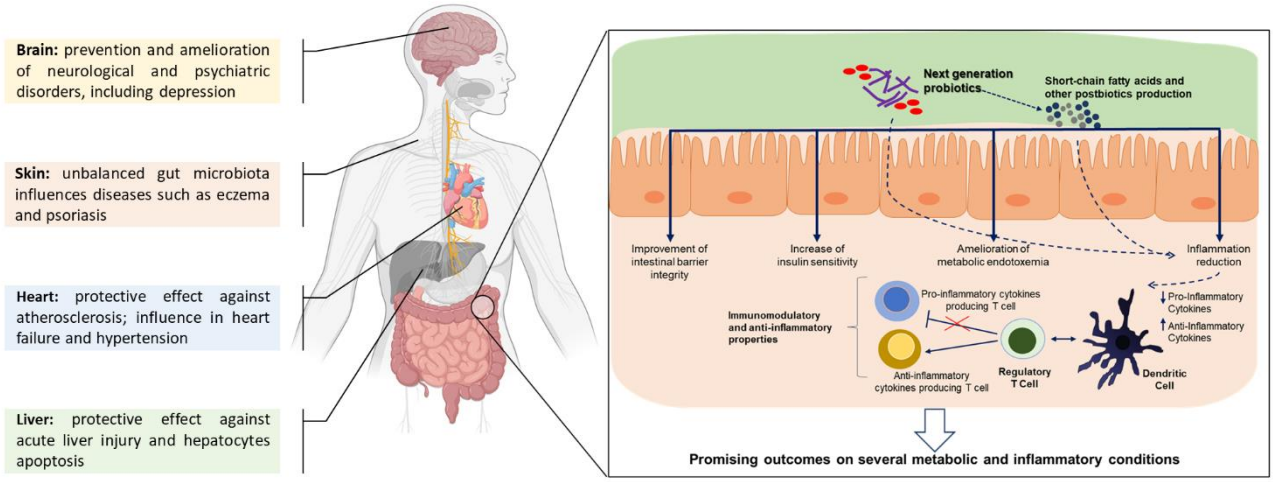


**Figure 3. Probiotics mechanisms of action.** The right panel contains a graphical summary of probiotic physiological interactions in gut epithelium responsible for maintaining host health. The metabolism of prebiotic compounds by probiotic bacteria leads to the production of postbiotic molecules, such as SCFA. Short-chain fatty acids are linked to distinct beneficial effects on the host improving the intestinal barrier integrity which prevents metabolic endotoxemia, a key characteristic of metabolic disorders such as T2D and insulin resistance; it also exerts immunoregulatory properties promoting an anti-inflammatory phenotype. On the left, the main organs that are affected by the activity of NGP, in an extent of their application beyond the gut health. Partially designed with BioRender.

### Short-Chain Fatty Acids

There is no bacterial feature that illustrates better this sophisticated cooperation other than the interaction of gut microorganisms through the production and utilization of SCFA, a

phenomenon termed 'cross-feeding' (Sung et al., 2017). Through the production of these organic acids, most gut commensals exert their beneficial effects on the host while modulating other gut community members (Figure 3). Thus, SCFA can be pointed as a more robust health indicator than bacterial strain abundance (de la Cuesta-Zuluaga et al., 2019). These metabolites result from the saccharolytic fermentation of dietary carbohydrates by anaerobic intestinal microorganisms and display an associated biochemical impact on the host (Sivieri et al., 2014; Van-Den-Abbeele et al., 2013). It is important to highlight that SCFA influence a variety of aspects of host health by mainly promoting metabolic and immunologic homeostasis, and gut barrier integrity (Deleu et al., 2021). In pure culture, the SCFA most commonly produced by the different anaerobic gut bacteria are acetate, butyrate, and propionate, and they all perform crucial yet distinct roles in human health.

- ***Acetate***

Acetate stands as the most predominant SCFA found in feces, and its production is determined by a balance between proteolytic and saccharolytic fermentation (Hernández et al., 2019). Evidence shows that acetate acts on G-protein coupled receptors which secrete gut hormones (e.g., glucagon-like peptide-1 and peptide YY), positively affecting host energy and substrate metabolism. Acetate may also be responsible for increased fatty acid synthesis, energy production via the tricarboxylic acid (TCA) cycle, and metabolic capacity improvement via 5'AMP-activated protein kinase (AMPK) (Hernández et al., 2019). In addition, studies on the biological influence of acetate in colorectal cells have demonstrated that acetate leads to the inhibition of proliferation, induction of apoptosis, promotion of lysosomal membrane permeabilization with release of cathepsin D and alteration of the energetic metabolism through the modulation of monocarboxylate transporters expression (Gomes et al., 2020). These mechanistic effects on human health emphasize the importance of maintaining the abundance of acetate-producing microorganisms in the gut community. *Akkermansia muciniphila* is an NGP that possesses such ability through its mucolytic nature, as it degrades mucin – the main component of the intestinal mucus layer – (Derrien et al., 2017), which also contributes to the protective role of the intestinal mucosal layer (more on that later). *Bacteroides xylanisolvens* is another potential NGP that, through the fermentation of xylan and other sugars, can produce acetate among other SCFA (Chassard et al., 2008). As any acetate-producer, these NGP not only directly impact host health, but also participate in a cross-feeding chain that is essential to maintain the gut milieu (Belzer et al., 2017), as they provide acetate for the production of perhaps the most important SCFA of them all: butyrate.

- ***Butyrate***

In the gastrointestinal environment, butyrate is predominantly produced by *Firmicutes*, a phylum which comprises *Faecalibacterium prausnitzii* (Duncan et al., 2002), *Anaerobutyricum hallii* (Duncan et al., 2004b), and *Butyricicoccus pullicaecorum* (Eeckhaut et al., 2008) species. Butyrate is considered of paramount importance, since i) it represents the main energy source for colonocytes; ii) it demonstrates anticarcinogenic potential via the induction of colonic regulatory T cells; iii) it is associated with the improvement of metabolic syndrome by the activation of intestinal gluconeogenesis through a cAMP-dependent mechanism; and iv) it is linked to the inhibition of inflammatory responses (Brodmann et al., 2017). Evidence shows that, in a dysbiotic gut microbiota community, the abundance of butyrate producers is often reduced when compared to healthy controls (Rivera-Chávez et al., 2016). Thus, due to several beneficial features that aid gastrointestinal health, the consumption of butyrate-producing bacteria is viewed as a strategy to increase butyrate production in the gut, and the NGP mentioned earlier are pointed as strong candidates (Andrade et al., 2020). As mentioned, many acetate-consuming butyrogenic bacteria require the presence of acetate in pure culture for optimal growth, and that is the case of *F. prausnitzii* (Duncan et al., 2004a) and *B. pullicaecorum* (Eeckhaut et al., 2008). *Anaerobutyricum hallii,* on the other hand, depends on lactate formation by primary degraders such as *Bifidobacterium* spp. to produce butyrate in the absence of a glucose source, as it is not able to metabolize complex oligo- and polysaccharides (Schwab et al., 2017; Scott et al., 2014). Since lactate accumulation is associated with several intestinal disorders (Duncan et al., 2004b), this ability also presents *A. hallii* as a relevant member for the balance of the intestinal metabolism. Conversely, *A. hallii* also contributes to the production of another health-promoting SCFA: propionate.

- ***Propionate***

This SCFA plays a role as an energy source for colonic epithelial cells and acts as a hepatic gluconeogenic precursor, reducing adiposity by decreasing the production of hepatic glucose (Martín and Langella, 2019). Additionally, it is thought to interact with G-protein coupled receptors and fatty acid receptors, playing a role in glucose homeostasis and satiety signaling (Reichardt et al., 2014). As previously stated, *A. hallii* contributes to the formation of intestinal propionate through the use of 1,2-propanediol (1,2-PD), another important intermediary metabolite in the cross-feeding network (Engels et al., 2016). In fact, propionate can also be generated via the succinate pathway, and *Prevotella copri* – another emerging NGP – has been shown to contribute to the succinate pool, as it is its main fermentation product (De Vadder et al., 2016).

**Other NGP-related Metabolites**

Next-generation probiotics do not only contribute to the homeostasis of SCFA levels in the gut environment; they can also mechanistically influence host health through the production of other important bioactive compounds that exert specific effects on the host. Next-generation probiotics are also associated with the suppression of inflammation through several distinct mechanisms. *Faecalibacterium prausnitzii* was shown to secrete a small peptide, named microbial anti-inflammatory molecule (MAM), that has demonstrated an inhibitory effect towards the nuclear factor-κB (NF-κB) pathway, which was translated into the improvement of the inflammatory condition in murine models (Quévrain et al., 2016). This molecule was also reported to reduce Th1 and Th17 – proinflammatory cytokines – in colonic tissues of inflammation murine models (Breyner et al., 2017). A biofilm-forming *F. prausnitzii* strain was also reported to produce an anti-inflammatory extracellular polymeric matrix (EPM) which stimulates the increase of anti-inflammatory cytokines production such as interleukin-10 (IL-10) and IL-2 (Rossi et al., 2015). Indeed, *F. prausnitzii* is mainly characterized by its anti-inflammatory activity within the gut and a decreased relative abundance of this strain is negatively correlated with inflammatory conditions such as Crohn's disease (CD) (Sokol et al., 2008). In the same way, *A. muciniphila* was shown to increase the production of 2-oleoylglycerol (2-OG), a bioactive lipid that belongs to the endocannabinoid system which stimulates the secretion of glucagon-like peptides and contributes to the control of intestinal inflammation (Dao et al., 2016). Interestingly, a pili-like surface protein named Amuc_1100 seems to be one of the prominent reasons by which *A. muciniphila* is linked to amelioration of metabolic conditions such as type 2 diabetes (T2D) and obesity. Amuc_1100 showed stability at temperatures used in pasteurization and it was reported to interact with toll-like receptors (TLR), which are a class of innate immune system proteins that trigger an immune regulatory signaling cascade by recognizing conserved molecular patterns (Ottman et al., 2017; Plovier et al., 2016). Likewise, *Bacteroides fragilis* exerts immunomodulatory effects by producing polysaccharide A and outer membrane vesicles, which activate TLR and regulatory T cells (Sun et al., 2019).

Another mechanism of action associated with probiotic benefits displayed by some NGP candidates is the antimicrobial activity against pathogenic bacteria. This can include the production of antimicrobial substances, like hydrogen peroxide, bacteriocins, or organic acids (e.g., SCFA). Thus, potential NGP do not only antagonize pathogens via the production of SCFA but also with the secretion of other bioactive compounds. *Anaerobutyricum hallii* was reported to metabolize glycerol, producing 3-hydroxypropionaldehyde (3-HPA) or reuterin (Fekry et al., 2016), a compound with demonstrated antimicrobial activity against various microorganism

groups (Schaefer et al., 2010). It was also found that *A. muciniphila* can induce Paneth cells - highly specialized secretory epithelial cells located in the crypts of the small intestine – in producing antimicrobial peptides such as the regenerating islet-derived protein 3 gamma (RegIII-γ), which is part of one class of antimicrobials expressed within the intestine (Everard et al., 2013).

The potential role of *Akkermansia* in contributing to the synthesis and/or degradation of tryptophan metabolites has been investigated as well. Tryptophan metabolites like anthranilate, tryptamine, and 4,6-dihydroxyquinoline are well established as ligands for the aryl hydrocarbon receptor (AhR) and modulate inflammation in multiple cell types. Tryptophan metabolism also plays an important role in impeding colorectal cancer development through inhibiting inflammation, repairing the gut barrier structure, and interacting with beneficial microorganisms in the gut. Yang and collaborators studied the effect of diet and loss of AhR in intestinal epithelial cells on the correlation between the fecal microbiome and metabolome using wild type (WT) and intestinal epithelial cell-specific AhR knockout mice maintained on high-fat or low-fat diet (Yang et al., 2020). Changes in the fecal microbial community and the fecal metabolome were determined using 16S rRNA sequencing and untargeted metabolomics, respectively. The study showed that diet has a more pronounced impact on mice fecal microbiota composition than the effect of the loss of AhR. In contrast, metabolomic analysis revealed that the loss of AhR in intestinal epithelial cells had a more pronounced effect on metabolite profile compared to diet. Integration analysis of microbiome and metabolome identified *Akkermansia*, unclassified Clostridiales, and unclassified *Desulfovibrionaceae* as key contributors to the synthesis and/or utilization of tryptophan metabolites. The unclassified Clostridiales was a main contributor to the synthesis of anthranilate, while *A. muciniphila* was predicted to participate in the degradation of tryptamine and the synthesis of 4,6-dihydroxyquinoline. Thus, the contribution of *Akkermansia* to the synthesis and/or degradation of tryptophan metabolites is likely to be associated with the modulation of inflammatory process with beneficial effects on the host. The study also highlights the use of multi-omic analysis to investigate the relationship between the microbiome and metabolome and identifies possible taxa that can be targeted to manipulate the microbiome for colorectal cancer treatment (Yang et al., 2020).

**The Integrity of the Mucosal Layer**

Accumulating evidence from animal studies supports the hypothesis that many diseases stem from the abrogation of intestinal integrity (Chelakkot et al., 2018). Indeed, the condition commonly described as “leaky gut” essentially means the existence of a compromised gut

barrier, which allows the diffusion of possible immunogenic substances, such as toxins and bacterial products, to the bloodstream. This causes the activation of the immune system, mainly creating a systemic low-grade type of inflammatory state (Michielan and D'Incà, 2015). Indeed, some of the metabolites mentioned earlier, namely SCFA, have an instrumental part in maintaining and strengthening the integrity of this layer. When SCFA producers decrease, secretion of these metabolites suffers a toll, and that might translate to possible disease onset. In this context, one of the most prominent NGP seems to be *A. muciniphila*, which was found to protect from low-grade systemic inflammation due to its ability to instigate mucin production – mainly by colonic mucus turnover – which increases enterocyte monolayer integrity, thereby reducing gut permeability (Reunanen et al., 2015). Colonization of the mucus layer by *A. muciniphila* induces the proliferation of other mucus-colonizing bacteria due to the production of oligosaccharides and acetate upon degradation of mucus; this will impair the proliferation of pathogenic bacteria leading to their competitive exclusion within the mucosa (Belzer and De Vos, 2012).

Furthermore, *A. muciniphila* and *F. prausnitzii* were shown to produce extracellular vesicles (EVs), which were reportedly to modulate the intestinal barrier permeability. EVs are released by all domains of life and represent a general, evolutionary conserved mechanism of intercellular communication. EVs contain a variety of bioactive molecules, such as proteins, lipids, nucleic acids, and small-molecule metabolites that play a key role in the biology of bacteria, and the growing appreciation of the functional significance of the human microbiota in health and disease has triggered a marked interest in the functional role of bacterial EVs and communication with the host (Jahromi and Fuhrmann, 2021). Recently, *A. muciniphila* has been reported as a beneficial bacterium that reduces gut barrier disruption and insulin resistance in T2D murine models, and the role of *A. muciniphila*-derived EVs in the regulation of gut permeability was evaluated (Chelakkot et al., 2018). Fecal samples of healthy controls showed more EVs in comparison with those from T2D mice. Moreover, EV administration enhanced tight junction function, reduced body weight gain and improved glucose tolerance in high-fat diet-induced diabetic mice. The direct effect of EVs on human epithelial cells was evaluated and the permeability of lipopolysaccharide-treated Caco-2 cell line was reduced upon treatment with *A. muciniphila*-derived EVs. Interestingly, the expression of occludin was increased by EV treatment (Chelakkot et al., 2018). Similarly, *F. prausnitzii* was also found to stimulate the production of mucin and tight-junction proteins (Carlsson et al., 2013; Rossi et al., 2015). The effect of *F. prausnitzii* and its EVs on mRNA expression levels of tight junction protein genes (*ZO1* and *OCLN*) was evaluated in Caco-2 cells by quantitative real-time PCR (Moosavi et al., 2020). A significant increase in the expression of *ZO1* (zonula occludens-1) and *OCLN* (occluding) genes

was induced by EVs treatment, but not by *F. prausnitzii*. Overall, these studies suggest that EVs derived from NGP may act as a functional moiety for modulation of gut permeability and the resulting regulation of intestinal barrier integrity can improve metabolic functions in mammalian cells.

## GUT-MICROBIOTA AXES

At this point, it is clear the potential effects that the various NGP can exert on human health, and more specifically, the possible implications in a broad spectrum of conditions that go beyond the GIT. Indeed, recent findings reiterate the interconnectivity of the gut microbiota and specific organs/systems (Figure 3). This communication is accomplished mainly via the circulating bioactive microbiota-derived metabolites, forming a multidirectional communication network, in a concept coined as "gut axes". As follows, we will briefly describe some of the known effects of emerging NGP on diseases of the various organ systems.

- **Gut-Cardiovascular Axis**

Undoubtedly, cardiovascular diseases are among the conditions where NGP have been demonstrated to have profound effects. In fact, a key link between gut microbiota, gut permeability, and the vascular system has been reported (Li et al., 2016). Specifically, daily oral gavage with *A. muciniphila* attenuated atherosclerotic lesions in $Apoe^{-/-}$ mice model by ameliorating metabolic endotoxemia-induced inflammation through the restoration of the gut barrier. These findings highlight the protective role of *A. muciniphila* against the development and progression of atherosclerosis, the main contributor to cardiovascular mortality (Hansson et al., 2006; Li et al., 2016). Furthermore, alterations in the relative abundance of intestinal commensal bacterial species commonly classified as NGP were reported in certain cardiovascular disorders. For instance, *F. prausnitzii* abundance was inversely correlated with chronic heart failure, which is known as an end-stage syndrome of many cardiovascular diseases (Cui et al., 2018). The relative abundance of other bacterial species (including, besides the abovementioned, *Anaerobutyricum*, *Prevotella*, *Bacteroides,* or *Parabacteroides*) was analyzed in several independent studies, revealing a highly diverse correlation with diseases such as atherosclerosis, atrial fibrillation, hypertension, or heart failure (for a review, see Jin *et al.*, 2020). Most studies intend to directly relate the abundance with the observed health condition; however, beneficial or detrimental effects might result from the interactions between the whole microbial environment and with the host, making it difficult to correctly assign the role of each individual, without a deeper mechanistic understanding of the microbiome and their

metabolites. Thus, additional information is still required to accurately relate the effectiveness of NGP in preventing or treating cardiovascular diseases. Noteworthily, some specific metabolites produced by NGP strains, including SCFA, were shown to have a modulatory effect on blood pressure (Wang and Zhao, 2018). This is a good indicator that the presence of adequate amounts of SCFA-producing bacteria – or involved in the cross-feeding SCFA metabolism – within the human gut microbiome (*A. muciniphila*, *A. hallii*, *F. prausnitzii*, among others), might, indeed, represent a benefit in patients with this condition.

- **Gut-Brain Axis**

Besides the existence of an enteric nervous system – known as the body's 'second brain' –, evidence demonstrates that the gut microbial community is reported to influence the central nervous system through a bidirectional communication system, known as the 'brain-gut-microbiome axis'. Indeed, the association between gut microbiome alterations and changes in neural development and some mental illnesses has been established (Rogers et al., 2016). Currently, therapeutic manipulation of the microbiome with the ingestion of probiotic bacteria – psychobiotic – has been pointed as a possible strategy to mitigate some of the effects of such conditions, in which *Bifidobacterium* and *Lactobacillus* are predominantly the most investigated genera (Sarkar et al., 2016). Notwithstanding, recent studies have linked reduced levels of anti-inflammatory, butyrate-producing bacteria, to neurological conditions such as depression. In a preclinical study, Hao et al. (2019) found that when exposed to stress, murine models treated with *F. prausnitzii* (ATCC 27766) displayed higher levels of SCFA in the cecum, higher levels of plasma cytokines IL-10, reduced corticosterone and IL-6 levels, and suppressed the upregulation of inflammatory cytokines. This positive correlation with brain health and neurological disease protective effects has prompted the possibility of using this strain as a complementary prophylactic and therapeutic strategy on depression and anxiety symptoms. In another study, it was observed that, when compared with healthy individuals, patients with major depressive disorder (MDD) had lower levels of *Faecalibacterium* and other related members (at the genus level), which are known butyrate-producers (Liu et al., 2020). As mentioned before, butyrate has the ability to strengthen the integrity of the epithelial gut barrier and displays anti-inflammatory effects. The decrease of butyrate-producing microorganisms can lead to the loss of that protective mechanism and thus, the translocation of compounds that activate low-grade systemic inflammation increases, a characteristic trait of a substantial number of depressed individuals (Marrone and Coccurello, 2020). Other potential NGP psychobiotic has been evaluated for the effects on the treatment of MDD. Major depressive disorder patients treated with a *Clostridium butyricum* MIYAIRI 588-based synbiotic, along with antidepressants,

demonstrated a favorable response with a significant improvement in anxiety and depressive scale scores (Miyaoka et al., 2018). Thus, evidence confirms the alterations in the abundance of various NGP within the microbiome profile of patients with psychiatric disorders. Despite the promising scenario, there is no distinguishable microbiome motif that could guide researchers towards a specific set of strains as a method of preventing mental disease or alleviating symptoms. Whether it is by probiotic supplementation or promotion of the growth of these beneficial bacteria via a more customized prebiotic ingestion, this creates the possibility of a holistic psychotherapeutic approach for future interventions, since a "healthy" gastrointestinal environment seems key in symptom improvement.

- **Other relevant "axes"**

As mentioned before, studies are still required to assess the full extent of NGP effects upon consumption. However, correlational studies have shown a broad range of influence that goes far beyond the gut itself. Besides the heart and the brain, the liver seems to be another organ that is positively affected by the presence of *A. muciniphila* within the gut microbiota. Wu and co-workers have shown a protective effect mediated by this species in mice with acute liver injury, through the decrease of pro-inflammatory cytokines and reduced apoptosis of hepatocytes (Wu et al., 2017). The therapeutic potential of *A. muciniphila* in treating metabolic dysfunction-associated fatty liver disease (MAFLD) has been described. Although the mechanism involved is not fully understood, *A. muciniphila* efficiently reversed hepatic steatosis, inflammatory dysfunction, and liver injury in diet-induced obese mice models (Rao et al., 2021). The treatment with *A. muciniphila* resulted in effective increase of mitochondrial oxidation and bile acid metabolism in the gut-liver axis, improvement of oxidative stress-induced cell apoptosis in the gut, leading to the restructuring of the gut microbiota composition. These metabolic enhancements occurred with increased L-aspartate levels in the liver that derived from the gut. Similar beneficial metabolic effects were obtained with the administration of L-aspartate, suggesting that the anti-MAFLD activity of *A. muciniphila* is associated with lipid oxidation and improved gut-liver interactions through modulation of L-aspartate metabolism (Rao et al., 2021). Furthermore, a novel mechanism linking intestinal *A. muciniphila* and the aryl hydrocarbon receptor (AhR) to saccharin/sucralose-induced nonalcoholic fatty liver disease (NAFLD) in mice has been proposed (Shi et al., 2021). Saccharin/sucralose consumption altered the gut microbial community structure, with significant depletion of *A. muciniphila* abundance in the cecal contents of mice, resulting in disruption of intestinal permeability and a high level of serum lipopolysaccharide, which likely contributed to systemic inflammation and caused NAFLD in mice. Saccharin/sucralose also markedly decreased microbiota-derived AhR ligands

and colonic AhR expression, which are closely associated with many metabolic syndromes. Metformin or fructo-oligosaccharide supplementation significantly restored *A. muciniphila* and AhR ligands in sucralose-consuming mice, consequently ameliorating NAFLD (Shi et al., 2021). Consequently, these findings suggest that *A. muciniphila* could be a promising NGP with therapeutic application on liver disorders.

Case-control studies involving patients with skin diseases have shown variable results. For instance, Tan and colleagues demonstrated that *A. muciniphila* was reduced in subjects with psoriasis (Tan et al., 2018). Similarly, *A. muciniphila* and *F. prausnitzii* abundance were reported to be depleted in infants with atopic disease (Candela et al., 2012). In contrast, both species were enriched in children with eczema which can be also a symptom of atopic disease (Zheng et al., 2016). Although these studies focus mainly on the species level, it is important to highlight that certain subspecies can have a distinct behavior in terms of abundance, as shown by Song and co-workers. These researchers reported an enrichment in a clade of *F. prausnitzii* subspecies in patients with atopic dermatitis (Song et al., 2016). This disparity between results highlights the need for further studies to understand the role of NGP in the prevention and treatment of skin diseases through the manipulation of gut microbiota.

**PRODUCTION CONSTRAINTS AND CRAFTY DELIVERY SYSTEMS**

Large scale use of NGP poses several and unique challenges. One of the challenges is related to oxygen, since many of the NGP are obligate anaerobes and thus, must be handled in oxygen-free atmospheres throughout the manufacturing process and storage. Practical and scalable downstream processing solutions are needed to enable cost-effective and efficient manufacture and storage of commercial products.

*Faecalibacterium prausnitzii* was shown to be extremely sensitive to oxygen, as exposure to ambient air for more than 2 min inhibited all subsequent bacterial growth (Duncan et al., 2002). Still, it has been found that it can tolerate low levels of oxygen by adherence to the gut mucosa where oxygen diffuses from epithelial cells, through an extracellular electron shuttle of flavins and thiols to transfer electrons to oxygen (Khan et al., 2012). Based on these findings, the same research group, developed a formulation containing the antioxidants cysteine and riboflavin plus the cryoprotectant inulin, which enabled the maintenance of *F. prausnitzii* DSM 17677 viability in aerobic environment for 24 h (Khan et al., 2014). Improved formulations were obtained by addition of the bulking agents corn starch and wheat bran, facilitating the handling. On the other hand, *A. muciniphila* once thought to be a strict anaerobe was shown recently to be aerotolerant (Machado et al., 2020). When exposed to an aerobic environment in temperatures ranging from 4 to 37$^{o}$C, *A. muciniphila* DSM 22959 exhibited a high oxygen

tolerance up to 72 h, though a higher oxygen tolerance was exhibited at 4◦C. This intrinsic oxygen tolerance of *A. muciniphila* may allow the future implementation of a wider range of handling procedures, which facilitates its large-scale use.

Another challenge is related to the composition of the growth media, which must be suitable for human use and generate high biomass yields. Ideally, particularly for therapeutic proposes, the medium components should be defined and preferably of non-animal origin. As mentioned before, *A. muciniphila* is a mucin-degrading bacterium, since it uses mucin as carbon and nitrogen sources. In order to ensure good growth yields, mucin (present in the mucus layer lining of the intestinal tract of animals) is usually added to some base medium, such as brain heart infusion (BHI) broth (Li et al., 2021). van der Ark and colleagues have devised a defined minimal medium containing L-threonine and N-acetylglucosamine or N-acetylgalactosamine, which were shown to be essential for *A. muciniphila* growth (van der Ark et al., 2017). Nevertheless, a potentially scalable preservation and preparation protocol for the use of viable *A. muciniphila* in therapeutic interventions has already been reported by Ouwerkerk et al. (2017) using a mucin-based medium. Cell growth and all subsequent procedures were performed under strict anaerobic conditions, with several quality assessment and control procedures, to ensure cell viability. *Akkermansia muciniphila* preserved at -80 °C in glycerol-amended medium showed high long-term viability for over 1 year (97.9±4.5%) and could be shipped to other locations on dry-ice without detectable loss of viable cells. De Vos and Seegers (2017) have developed a medium for culturing *A. hallii* to high biomass yields, composed only by food grade components and free of any animal sources. This medium includes sugars (such as a glucose or sucrose), yeast extract, a plant protein hydrolysate (such as soy peptone) and acetate (de Vos and Seegers, 2017).

Besides the challenges faced during the manufacturing process and storage, NGP must be formulated to ensure the release of high numbers of viable cells [$10^7$-$10^9$ CFU per product dose are usually recommended for conventional probiotics (Hill et al., 2014; Hungin et al., 2018)] in the right place, so they can effectively recolonize the gut. After ingestion, bacteria will face a harsh physicochemical and biological environment composed of low pH levels, digestive enzymes, and bile salts which could affect their cell structure (Barer, 2014). Thus, delivery systems that assure that these sensitive anaerobic commensal strains exert efficaciously their beneficial effects must be envisaged. Table 2 summarizes NGP delivery systems that are reported in the scientific literature. A relatively simple approach consists in freeze-drying the bacteria, to promote stabilization, prior to their encapsulation in enteric-coated gelatin capsules. In one study, *B. pullicaecorum* was first anaerobically freeze-dried in horse serum supplemented with trehalose and cysteine and then encapsulated in hydroxypropyl

methylcellulose (HPMC) capsules. The system showed good storage stability, as only 1 log reduction was observed after 7 months at 4°C (Eeckhaut et al., 2014). Later, Boesmans et al. (2018), using a similar encapsulation technique, showed that HPMC capsules containing a freeze-dried culture of *B. pullicaecorum* 25-3$^T$ preserved its viability over an 8-month storage period at 4°C (in heat-sealed aluminum sachets). The capsules were sealed and coated with a pH-resistant coating consisting of the enteric polymer cellulose acetate phthalate and the plasticizer diethyl phthalate and were used in a human intervention trial. These capsules were shown to be safe and well-tolerated by the participant volunteers, without causing disruptive alterations in the composition or metabolic activity of health-associated microbiota (Boesmans et al., 2018).

Allouche et al. (2018) developed probiotic tablets by direct compression of a mixture of three excipients (microcrystalline cellulose, hydroxypropyl methylcellulose phthalate or HPMC) with *F. prausnitzii* (I4573) previously freeze-dried with sucrose. Using this approach, *F. prausnitzii* displayed high stability during 28 days of anaerobic storage (anaerobic atmosphere at 25 °C and 11% of relative humidity and in absence of light). However, these researchers highlighted the need to find alternatives to anaerobic storage as well as the urgency to develop an optimal coating to protect bacteria against gastric acidity. Taking this in consideration, Raise et al. (2020) designed and compared two encapsulation processes that could deliver bacteria in a viable and functional form to the intestine. One process consisted of mixing previously freeze-dried *F. prausnitzii* with a melted hydrophobic matrix (Gelucire® 43/01) to encapsulate them into a solid lipid base after cooling. The resulting lipid mixture was then used to fill gelatin capsules. The other process was the classical encapsulation by the extrusion method, using amidated low-methoxyl pectin and a calcium salt (ionotropic gelation). The resulting beads were subsequently freeze-dried. All the encapsulation processes were performed inside an anaerobic chamber. Both formulations improved the survival of bacteria in the stomach and distal jejunum buffers during a digestive exposure test. However, only lipid inclusion provided sufficient protection suitable for therapeutic application. Nevertheless, Gelucire® poorly stabilized bacteria during storage, unlike the freeze-dried beads. Earlier, van der Ark et al. (2018) succeeded in encapsulating *A. muciniphila* CIP 107961$^T$ in a double water-oil-water emulsion and in protecting these bacteria effectively during their passage in the digestive tract. However, this encapsulation process did not allow stabilization for more than 72 h of anaerobic storage at 4°C. Marcial-Coba et al. (2018) also used the extrusion method to encapsulate *A. muciniphila* DSM22959. They used a xanthan/gellan gum matrix for cell immobilization with a subsequent freeze-drying step, in which various combinations of cryoprotective agents were employed. The use of higher sugar cryoprotectants [sweetener agave syrup 10 % (w/v)] combined with xanthan/gellan gum matrix,

550 in the form of freeze-dried microcapsules, provided the higher encapsulation efficiency (76.2%).
551 Furthermore, this formulation was able to enhance *A. muciniphila* survival during in vitro GIT
552 conditions. Later, the same research group investigated the efficacy of dark chocolate as a
553 carrier for xanthan/gellan encapsulated *A. muciniphila* (Marcial-Coba et al., 2019). Embedding
554 in dark chocolate conferred an efficient protection to encapsulated *A. muciniphila* reflected, not
555 only, in a viability loss < 1 log CFU.$g^{-1}$ and a final concentration ≥ 7 log CFU.$g^{-1}$ after 60 days of
556 storage, but also, in a high survival rate after *in vitro* gastric transit at pH 3, when compared to
557 free cells. Moreover, in a hedonic sensory test, dark chocolate containing microcapsules were
558 not significantly different from two commercially available chocolates. To the best of our
559 knowledge, this is the first and only report regarding the addition of a NGP in a food product.
560 More recently, Chang and colleagues demonstrated the possibility of using spray-drying for
561 encapsulation of *A. muciniphila* (Chang et al., 2020). This technique is particularly interesting to
562 be used in large-scale processing, namely in the food industry; the main challenge being that
563 high temperatures are employed, which can be deleterious for the microbial cells. The authors
564 used succinate-grafted alginate doped with epigallocatechin-3-gallate (EGCG) which resulted in
565 improved storage in aqueous environments and increased survival under simulated GIT
566 conditions (Chang et al., 2020). Recently, laboratory-scale electrospinning has been
567 demonstrated to be a suitable solid formulation technology for NGP. Vass et al. (2020) used this
568 technique to incorporate *C. butyricum* (mixture of cells and spores) into polymer-free fibers of
569 a water-soluble cyclodextrin matrix (Hydroxypropyl- β cyclodextrin). The viability of the
570 electrospun sporulated anaerobic bacteria was preserved after 1 year of aerobic storage at
571 ambient temperature. Moreover, the bacteria-containing fibers were grindable by an oscillating
572 mill, and the milled fibrous powder could be tableted by direct compression after mixing with
573 tableting excipients. None of the processing steps (drying, milling, and tableting) caused
574 significant reduction in bacterial viability. It should be noted that spores are usually highly
575 resistant to stress compared to vegetative cells and, therefore, easier to keep viable for long
576 storage periods. Spores of *C. butyricum* were also encapsulated by Zheng and colleagues using
577 a different approach. These authors used host-guest chemistry to encapsulate bacteria with
578 chemically modified prebiotic dextran (Zheng et al., 2020). The prebiotics-encapsulated
579 probiotic spores were found to specifically enrich in colon cancers after oral administration. In
580 the lesion, dextran was fermented by *C. butyricum*, and thereby produced anti-cancer short-
581 chain fatty acids.
582 Until now the up-scale of NGPs has been hindered mainly by extreme oxygen sensitivity
583 exhibited by several NGP, which poses several challenges at different levels (production,
584 stability, storage, and target delivery). Despite these difficulties, it is worth mentioning that a

commercial product containing NGP is already available to help manage type 2 diabetes (Pendulum Glucose Control – https://pendulumlife.com ). The product designed as a "probiotic medical food" (contains inulin, *A. muciniphila, Clostridium beijerinckii, C. butyricum, Bifidobacterium infantis* and *A. hallii*) was tested in a clinical trial and was shown to be safe and well tolerated and to improve postprandial glucose control (Perraudeau et al., 2020). No details on the manufacture or stabilization procedures of the product were given.

## NEXT-GENERATION PROBIOTICS: REGULATION FRAMEWORK AND RISK ASSESSMENT

Advanced knowledge regarding the pivotal role of gut microbiota in human health has increased the interest in using intestinal commensal microorganisms as probiotics. Such probiotics may be delivered to the consumer as a food product, a dietary supplement or a drug (Andrade et al., 2020). However, to introduce these NGP in the market, product developers should address a key question: what is the intended use? In fact, the regulatory difference between dietary supplements and drugs is very clear: food supplements are intended to maintain or improve a healthy state in a healthy or at-risk population, while drugs are intended to have therapeutic or prophylactic effect in human diseases (Cordaillat-Simmons et al., 2020). In the European Union, the responsible regulatory agency for novel foods is the European Food Safety Authority (EFSA), while drugs are regulated by the European Medicines Agency. On the other hand, in the United States of America, both categories are regulated by the Food and Drug Administration [FDA; (Cordaillat-Simmons et al., 2020; O'Toole et al., 2017)].

To avoid the misleading characterization of the product nature and its regulatory status, FDA created, in 2012, a novel category designated live biotherapeutic product (LBP) to define "a biological product that: 1) contains live organisms, such as bacteria; 2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and 3) is not a vaccine" (FDA, 2016). As of 2019, when European Pharmacopoeia Commission published a general monograph laying down harmonized requirements for LBP for human use, LBP are officially accepted as a novel category of drug products in European markets (European Pharmacopoeia Commission, 2019). Regarding the quality, safety, and effectiveness parameters for commercialization approval, the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use provides guidelines that include important recommendations that aid developers in fulfilling all requirements that LBP products, among other medicinal products, must meet and are expected by the regulatory authorities (Cordaillat-Simmons et al., 2020).

The authorisation to place a novel food on the European market, including those that contain probiotic microorganisms, is oriented by Regulation 2015/2283 (European Commission, 2015).

Following this regulation, EFSA developed scientific and technical guidance for the preparation and presentation of applications for the authorization of novel foods. This guidance states that the applications should provide a description of the novel food, production process, compositional data, specification, proposed uses and use levels, anticipated intake of the novel food, as well as, history of use of the novel food and/or its source, absorption, distribution, metabolism, excretion, nutritional, toxicological and allergenicity data (EFSA Panel on Dietetic Products Nutrition and Allergies et al., 2016). Considering all the data provided in the application, EFSA will evaluate the safety of novel foods under the proposed conditions of use (EFSA Panel on Dietetic Products Nutrition and Allergies et al., 2016).

Within the scope of safety assessment of novel foods, EFSA developed a qualified presumption of safety (QPS) approach, which aims to provide a harmonized generic pre-evaluation procedure for safety risk assessment of microorganisms intentionally added to food/feed (EFSA, 2007; EFSA Panel on Biological Hazards et al., 2021). The QPS list was first published in 2007 and has been regularly revised and updated. The establishment of QPS status is made based on four requirements: 1) "taxonomic identification" must be well defined; 2) the available "body of knowledge" must be sufficient to conclude on human/animal exposure by food/ feed; 3) the lack of pathogenic and virulence properties, including absence of acquired genes encoding resistance to antimicrobials relevant for humans and animals, must be established and substantiated ("safety"); 4) "intended use" must be clearly described (EFSA, 2007; EFSA Panel on Biological Hazards et al., 2021). Most bacteria recommended for the QPS list belong to the *Bifidobacterium* and *Lactobacillus* genera, where their long history of safe use contributed to the acquisition and maintenance of this status (Brodmann et al., 2017; EFSA Panel on Biological Hazards et al., 2020). However, some of the NGP discussed throughout this chapter were only recently characterized and thus present a challenge to both the scientific community and regulatory agencies (Brodmann et al., 2017). In this scope, EFSA recently assessed the possibility of integration of two NGP species in the QPS status list, namely *A. muciniphila* and *C. butyricum*. However, the committee decided not to recommend both strains for the QPS status; regarding *A. muciniphila*, due to safety concerns, and in the case of *C. butyricum*, the fact that some strains contain pathogenicity factors excluded its consideration for further QPS evaluation (EFSA Panel on Biological Hazards et al., 2020).

Another system, similar to the QPS approach, and called Generally Recognized As Safe (GRAS) system, was established by FDA in the USA (Food and Drug Administration, 2019). However, there are dissimilarities between the two approaches; for instance, the GRAS guidelines apply to food additives in general, whereas QPS is focused on microorganisms only; GRAS addresses a specific substance or organism (working at the strain level), in opposition to the QPS system,

655 that evaluates taxonomic units (frequently species level for bacteria and yeasts, or families for
656 viruses); finally, the GRAS status is determined by the FDA and/or external experts, while QPS
657 status is determined by EFSA only (EFSA Panel on Biological Hazards et al., 2017).
658 Despite advances and updates in the regulatory framework of probiotics, the commercialization
659 of NGP in several modalities (as novel food products, dietary supplement, or LBP) is a multi-actor
660 endeavour broadly dependent from the close interaction between research institutions,
661 pharmaceutical industries, and regulatory agencies (Almeida et al., 2020).
662

663 **CONCLUSIONS AND FUTURE PERSPECTIVES**

664 Despite the increasing number of studies regarding probiotics, more investigation is still
665 required to go beyond the identified association between healthy and disease state gut
666 microbiota and the impact that probiotic ingestion might elicit. This is particularly relevant when
667 considering NGP, whose characterization is still in progress and can benefit from the emergence
668 of new and more efficient scientific tools. Next-generation probiotics derived from the human
669 gut commensals are promising candidates to be used as indicators of human health as well as
670 to mitigate some clinical situations, with particular impact on the fight against metabolic and
671 inflammatory dysbiosis-derived diseases. These rapidly growing conditions are reaching
672 epidemic proportions, presenting new challenges to both clinicians and researchers. Due to the
673 continuous investigation regarding the role of the human microbiome in the host's health, novel
674 microorganisms are expected to emerge in the next years as new potential probiotics, increasing
675 the number of putative NGP available. Most of the potential gut-derived NGP have specific
676 nutritional requirements and present a strict anaerobic character, which poses real challenges
677 in what concerns their cultivation, their achieving biomass containing high viable cell numbers
678 and even their viability maintenance for long periods. Thus, tailored strategies must be
679 developed and optimized to produce and stabilize the different strains, without impairing their
680 biological functionality, while providing a delivery form that is safe and efficient for human
681 consumption. In this context, the development and implementation of novel probiotics in the
682 market requires a close interaction between research institutions, pharmaceutical industries,
683 and regulatory agencies.
684

## REFERENCES


Agus A, Clément K, Sokol H. Gut microbiota-derived metabolites as central regulators in metabolic disorders. Gut 2021;70:1174–82. https://doi.org/10.1136/gutjnl-2020-323071.

Allouche R, Dupont S, Charriau A, Gervais P, Beney L, Chambin O. Optimized tableting for extremely oxygen-sensitive probiotics using direct compression. Int J Pharm 2018;538:14–20. https://doi.org/10.1016/j.ijpharm.2018.01.010.

Almeida D, Machado D, Andrade JC, Mendo S, Gomes AM, Freitas AC. Evolving trends in next-generation probiotics: a 5W1H perspective. Crit Rev Food Sci Nutr 2020;60:1783–96. https://doi.org/10.1080/10408398.2019.1599812.

Andrade JC, Almeida D, Domingos M, Seabra CL, Machado D, Freitas AC, et al. Commensal Obligate Anaerobic Bacteria and Health: Production, Storage, and Delivery Strategies. Front Bioeng Biotechnol 2020;8:550. https://doi.org/10.3389/fbioe.2020.00550.

van der Ark KCH, Aalvink S, Suarez-Diez M, Schaap PJ, de Vos WM, Belzer C. Model-driven design of a minimal medium for *Akkermansia muciniphila* confirms mucus adaptation. Microb Biotechnol 2018;11:476–85. https://doi.org/10.1111/1751-7915.13033.

van der Ark KCH, Nugroho ADW, Berton-Carabin C, Wang C, Belzer C, de Vos WM, et al. Encapsulation of the therapeutic microbe *Akkermansia muciniphila* in a double emulsion enhances survival in simulated gastric conditions. Food Res Int 2017;102:372–9. https://doi.org/10.1016/j.foodres.2017.09.004.

Barer MR. Bacterial Growth, Culturability and Viability. Mol. Med. Microbiol. Second Ed., vol. 1–3, Elsevier Ltd; 2014, p. 181–99. https://doi.org/10.1016/B978-0-12-397169-2.00010-X.

Belzer C, Chia LW, Aalvink S, Chamlagain B, Piironen V, Knol J, et al. Microbial metabolic networks at the mucus layer lead to diet-independent butyrate and vitamin B12 production by intestinal symbionts. MBio 2017;8:e00770-17. https://doi.org/10.1128/mBio.00770-17.

Belzer C, De Vos WM. Microbes inside - from diversity to function: The case of *Akkermansia*. ISME J 2012;6:1449–58. https://doi.org/10.1038/ismej.2012.6.

Boesmans L, Valles-Colomer M, Wang J, Eeckhaut V, Falony G, Ducatelle R, et al. Butyrate Producers as Potential Next-Generation Probiotics: Safety Assessment of the Administration of *Butyricicoccus pullicaecorum* to Healthy Volunteers. MSystems 2018;3:94–112. https://doi.org/10.1128/msystems.00094-18.

Breyner NM, Michon C, de Sousa CS, Vilas Boas PB, Chain F, Azevedo VA, et al. Microbial Anti-Inflammatory Molecule (MAM) from *Faecalibacterium prausnitzii* Shows a Protective Effect on DNBS and DSS-Induced Colitis Model in Mice through Inhibition of NF-κB Pathway. Front Microbiol 2017;8:114. https://doi.org/10.3389/fmicb.2017.00114.

Brodmann T, Endo A, Gueimonde M, Vinderola G, Kneifel W, de Vos WM, et al. Safety of Novel Microbes for Human Consumption: Practical Examples of Assessment in the European Union. Front Microbiol 2017;8:1725. https://doi.org/10.3389/fmicb.2017.01725.

Bunesova V, Lacroix C, Schwab C. Mucin Cross-Feeding of Infant Bifidobacteria and *Eubacterium hallii*. Microb Ecol 2018;75:228–38. https://doi.org/10.1007/s00248-017-1037-4.

Candela M, Rampelli S, Turroni S, Severgnini M, Consolandi C, De Bellis G, et al. Unbalance of intestinal microbiota in atopic children. BMC Microbiol 2012;12:95. https://doi.org/10.1186/1471-2180-12-95.

Cani PD, de Vos WM. Next-generation beneficial microbes: The case of *Akkermansia muciniphila*. Front Microbiol 2017;8. https://doi.org/10.3389/fmicb.2017.01765.

Carlsson AH, Yakymenko O, Olivier I, Håkansson F, Postma E, Keita A V, et al. *Faecalibacterium prausnitzii* supernatant improves intestinal barrier function in mice DSS colitis. Scand J Gastroenterol 2013;48:1136–44. https://doi.org/10.3109/00365521.2013.828773.

Chang Y, Yang Y, Xu N, Mu H, Zhang H, Duan J. Improved viability of *Akkermansia muciniphila* by encapsulation in spray dried succinate-grafted alginate doped with epigallocatechin-3-gallate. Int J Biol Macromol 2020;159:373–82. https://doi.org/10.1016/j.ijbiomac.2020.05.055.

Chassard C, Delmas E, Lawson PA, Bernalier-Donadille A. *Bacteroides xylanisolvens* sp . nov ., a xylan- degrading bacterium isolated from human faeces. Int J Syst Evol Microbiol 2008;58:1008–13. https://doi.org/10.1099/ijs.0.65504-0.

Chelakkot C, Ghim J, Ryu SH. Mechanisms regulating intestinal barrier integrity and its pathological implications. Exp Mol Med 2018;50:103. https://doi.org/10.1038/s12276-018-0126-x.

Clark H. Culturing anaerobes. Nat Res 2019 2019;163:6257.

Cordaillat-Simmons M, Rouanet A, Pot B. Live biotherapeutic products: the importance of a defined regulatory framework. Exp Mol Med 2020;52:1397–406. https://doi.org/10.1038/s12276-020-0437-6.

Cui X, Ye L, Li J, Jin L, Wang W, Li S, et al. Metagenomic and metabolomic analyses unveil dysbiosis of gut microbiota in chronic heart failure patients. Sci Rep 2018;8. https://doi.org/10.1038/s41598-017-18756-2.

Dahiya DK, Renuka, Dangi AK, Shandilya UK, Puniya AK, Shukla P. New-Generation Probiotics: Perspectives and Applications. Microbiome Metabolome Diagnosis, Ther. other Strateg. Appl., Elsevier Inc.; 2019, p. 417–24. https://doi.org/10.1016/B978-0-12-815249-2.00044-0.

Dao MC, Everard A, Aron-Wisnewsky J, Sokolovska N, Prifti E, Verger EO, et al. *Akkermansia muciniphila* and improved metabolic health during a dietary intervention in obesity: Relationship with gut microbiome richness and ecology. Gut 2016;65:426–36.

https://doi.org/10.1136/gutjnl-2014-308778.
De Vadder F, Kovatcheva-Datchary P, Zitoun C, Duchampt A, Bäckhed F, Mithieux G. Microbiota-Produced Succinate Improves Glucose Homeostasis via Intestinal Gluconeogenesis. Cell Metab 2016;24:151–7. https://doi.org/10.1016/J.CMET.2016.06.013.
Deleu S, Machiels K, Raes J, Verbeke K, Vermeire S. Short chain fatty acids and its producing organisms: An overlooked therapy for IBD? EBioMedicine 2021;66:103293. https://doi.org/10.1016/j.ebiom.2021.103293.
Derrien M, Belzer C, de Vos WM. *Akkermansia muciniphila* and its role in regulating host functions. Microb Pathog 2017;106:171–81. https://doi.org/10.1016/j.micpath.2016.02.005.
Derrien M, Collado MC, Ben-Amor K, Salminen S, De Vos WM. The mucin degrader *Akkermansia muciniphila* is an abundant resident of the human intestinal tract. Appl Environ Microbiol 2008;74:1646–8. https://doi.org/10.1128/AEM.01226-07.
Derrien M, Vaughan EE, Plugge CM, de Vos WM. *Akkermansia municiphila* gen. nov., sp. nov., a human intestinal mucin-degrading bacterium. Int J Syst Evol Microbiol 2004;54:1469–76. https://doi.org/10.1099/ijs.0.02873-0.
Duncan SH, Hold GL, Harmsen HJM, Stewart CS, Flint HJ, Hold GL, et al. Growth requirements and fermentation products of *Fusobacterium prausnitzii*, and a proposal to reclassify it as *Faecalibacterium prausnitzii* gen. nov., comb. nov. Int J Syst Evol Microbiol 2002;52:2141–6. https://doi.org/10.1099/00207713-52-6-2141.
Duncan SH, Holtrop G, Lobley GE, Calder AG, Stewart CS, Flint HJ. Contribution of acetate to butyrate formation by human faecal bacteria. Br J Nutr 2004a;91:915–23. https://doi.org/10.1079/bjn20041150.
Duncan SH, Louis P, Flint HJ. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl Environ Microbiol 2004b;70:5810–7. https://doi.org/10.1128/AEM.70.10.5810-5817.2004.
Eeckhaut V, Ducatelle R, Sas B, Vermeire S, Van Immerseel F. Progress towards butyrateproducing pharmabiotics: *Butyricicoccus pullicaecorum* capsule and efficacy in TNBS models in comparison with therapeutics. Gut 2014;63:367. https://doi.org/10.1136/gutjnl-2013-305293.
Eeckhaut V, Van Immerseel F, Teirlynck E, Pasmans F, Fievez V, Snauwaert C, et al. *Butyricicoccus pullicaecorum* gen. nov., sp. nov., an anaerobic, butyrate-producing bacterium isolated from the caecal content of a broiler chicken. Int J Syst Evol Microbiol 2008;58:2799–802. https://doi.org/10.1099/ijs.0.65730-0.
EFSA. Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA - Opinion of the Scientific Committee. EFSA J

2007;5:587. https://doi.org/10.2903/j.efsa.2007.587.
EFSA Panel on Biological Hazards, Koutsoumani K, Allende A, Alvarez-Ordónez A, Bolton D, Bover-Cid S, et al. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 12: suitability of taxonomic units notified to EFSA until March 2020. EFSA J 2020;18:6174. https://doi.org/10.2903/j.efsa.2020.6174.
EFSA Panel on Biological Hazards, Koutsoumanis K, Allende A, Alvarez-Ordónez A, Bolton D, Bover-Cid S, et al. Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 13: suitability of taxonomic units notified to EFSA until September 2020. EFSA J 2021;19:6377. https://doi.org/10.2903/j.efsa.2021.6377.
EFSA Panel on Biological Hazards, Ricci A, Allende A, Bolton D, Chemaly M, Davies R, et al. Scientific Opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA. EFSA J 2017;15:4664. https://doi.org/10.2903/j.efsa.2017.4664.
EFSA Panel on Dietetic Products Nutrition and Allergies, Turck D, Bresson J-L, Burlingame B, Dean T, Fairweather-Tait S, et al. Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. EFSA J 2016;14:4594. https://doi.org/10.2903/j.efsa.2016.4594.
Engels C, Ruscheweyh HJ, Beerenwinkel N, Lacroix C, Schwab C. The common gut microbe *Eubacterium hallii* also contributes to intestinal propionate formation. Front Microbiol 2016;7. https://doi.org/10.3389/fmicb.2016.00713.
European Commission. REGULATION (EU) 2015/2283 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliam. 2015.
European Pharmacopoeia Commission. 3053E General monograph on Live biotherapeutic products. 2019.
Everard A, Belzer C, Geurts L, Ouwerkerk JP, Druart C, Bindels LB, et al. Cross-talk between *Akkermansia muciniphila* and intestinal epithelium controls diet-induced obesity. Proc Natl Acad Sci 2013;110:9066–71. https://doi.org/10.1073/pnas.1219451110.
FAO/WHO. Probiotics in food Health and nutritional properties and guidelines for evaluation. FAO Food Nutr Pap 2002;85:1–56.
FDA. Early clinical trials with live biotherapeutic products: chemistry, manufacturing, and control information. Guid Ind 2016:1–20.
Fekry MI, Engels C, Zhang J, Schwab C, Lacroix C, Sturla SJ, et al. The strict anaerobic gut microbe *Eubacterium hallii* transforms the carcinogenic dietary heterocyclic amine 2-amino-1-

methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Environ Microbiol Rep 2016;8:201–9. https://doi.org/10.1111/1758-2229.12369.
Food and Drug Administration. Generally Recognized as Safe (GRAS) 2019.
Gasbarrini G, Bonvicini F, Gramenzi A. Probiotics History. J Clin Gastroenterol 2016;50:S116–9. https://doi.org/10.1097/MCG.0000000000000697.
Geirnaert A, Steyaert A, Eeckhaut V, Debruyne B, Arends JBA, Van Immerseel F, et al. *Butyricicoccus pullicaecorum*, a butyrate producer with probiotic potential, is intrinsically tolerant to stomach and small intestine conditions. Anaerobe 2014;30:70–4. https://doi.org/10.1016/j.anaerobe.2014.08.010.
Gomes AM, Andrade JC, Freitas AC. The Use of Probiotics in the Food Industry. In: Rodriguez JML, García FJC, editors. Strateg. Obtaining Heal. Foods, Nova Science Publishers, Inc.; 2017.
Guo P, Zhang K, Ma X, He P. *Clostridium* species as probiotics: potentials and challenges. J Anim Sci Biotechnol 2020;11. https://doi.org/10.1186/s40104-019-0402-1.
Hansson GK, Robertson A-KL, Söderberg-Nauclér C. Inflammation and Atherosclerosis. Annu Rev Pathol Mech Dis 2006;1:297–329. https://doi.org/10.1146/annurev.pathol.1.110304.100100.
Hao Z, Wang W, Guo R, Liu H. *Faecalibacterium prausnitzii* (ATCC 27766) has preventive and therapeutic effects on chronic unpredictable mild stress-induced depression-like and anxiety-like behavior in rats. Psychoneuroendocrinology 2019;104:132–42. https://doi.org/10.1016/j.psyneuen.2019.02.025.
Hayashi H, Shibata K, Sakamoto M, Tomita S, Benno Y. *Prevotella copri* sp. nov. and *Prevotella stercorea* sp. nov., isolated from human faeces. Int J Syst Evol Microbiol 2007;57:941–6. https://doi.org/10.1099/ijs.0.64778-0.
Hernández MAG, Canfora EE, Jocken JWE, Blaak EE. The short-chain fatty acid acetate in body weight control and insulin sensitivity. Nutrients 2019;11. https://doi.org/10.3390/nu11081943.
Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, et al. Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat Rev Gastroenterol Hepatol 2014;11:506–14. https://doi.org/10.1038/nrgastro.2014.66.
Hillman ET, Kozik AJ, Hooker CA, Burnett JL, Heo Y, Kiesel VA, et al. Comparative genomics of the genus *Roseburia* reveals divergent biosynthetic pathways that may influence colonic competition among species. Microb Genomics 2020;6:7–24. https://doi.org/10.1099/mgen.0.000399.
Holdeman L V., Moore WEC. New genus, *Coprococcus*, twelve new species, and emended descriptions of four previously described species of bacteria from human feces. Int J Syst

Bacteriol 1974;24:260–77. https://doi.org/10.1099/00207713-24-2-260.
Hugon P, Dufour JC, Colson P, Fournier PE, Sallah K, Raoult D. A comprehensive repertoire of prokaryotic species identified in human beings. Lancet Infect Dis 2015;15:1211–9. https://doi.org/10.1016/S1473-3099(15)00293-5.
Hungin APS, Mitchell CR, Whorwell P, Mulligan C, Cole O, Agréus L, et al. Systematic review: probiotics in the management of lower gastrointestinal symptoms – an updated evidence-based international consensus. Aliment Pharmacol Ther 2018;47:1054–70. https://doi.org/10.1111/apt.14539.
Isa K, Oka K, Beauchamp N, Sato M, Wada K, Ohtani K, et al. Safety assessment of the *Clostridium butyricum* MIYAIRI 588® probiotic strain including evaluation of antimicrobial sensitivity and presence of *Clostridium* toxin genes in vitro and teratogenicity in vivo. Hum Exp Toxicol 2016;35:818–32. https://doi.org/10.1177/0960327115607372.
Jahromi LP, Fuhrmann G. Bacterial extracellular vesicles: Understanding biology promotes applications as nanopharmaceuticals. Adv Drug Deliv Rev 2021;173:125–40. https://doi.org/10.1016/j.addr.2021.03.012.
Jin L, Shi X, Yang J, Zhao Y, Xue L, Xu L, et al. Gut microbes in cardiovascular diseases and their potential therapeutic applications. Protein Cell 2020:1–14. https://doi.org/10.1007/s13238-020-00785-9.
Kandasamy S, Vlasova AN, Fischer DD, Chattha KS, Shao L, Kumar A, et al. Unraveling the differences between Gram-positive and Gram-negative probiotics in modulating protective immunity to enteric infections. Front Immunol 2017;8:334. https://doi.org/10.3389/fimmu.2017.00334.
Khan MT, Van Dijl JM, Harmsen HJM. Antioxidants keep the potentially probiotic but highly oxygen-sensitive human gut bacterium *Faecalibacterium prausnitzii* alive at ambient air. PLoS One 2014;9:e96097–e96097. https://doi.org/10.1371/journal.pone.0096097.
Khan MT, Duncan SH, Stams AJM, Van Dijl JM, Flint HJ, Harmsen HJM. The gut anaerobe *Faecalibacterium prausnitzii* uses an extracellular electron shuttle to grow at oxic-anoxic interphases. ISME J 2012;6:1578–85. https://doi.org/10.1038/ismej.2012.5.
Khan ST, Malik A. Next-Generation Probiotics Their Molecular Taxonomy and Health Benefits. Heal. Saf. Asp. Food Process. Technol., Springer Nature Switzerland; 2019, p. 1–672. https://doi.org/10.1007/978-3-030-24903-8.
de la Cuesta-Zuluaga J, Mueller NT, Álvarez-Quintero R, Velásquez-Mejía EP, Sierra JA, Corrales-Agudelo V, et al. Higher fecal short-chain fatty acid levels are associated with gut microbiome dysbiosis, obesity, hypertension and cardiometabolic disease risk factors. Nutrients 2019;11. https://doi.org/10.3390/nu11010051.

Ley RE. Gut microbiota in 2015: *Prevotella* in the gut: Choose carefully. Nat Rev Gastroenterol Hepatol 2016;13:69. https://doi.org/10.1038/nrgastro.2016.4.

Li J, Lin S, Vanhoutte PM, Woo CW, Xu A. *Akkermansia muciniphila* Protects Against Atherosclerosis by Preventing Metabolic Endotoxemia-Induced Inflammation in Apoe −/− Mice. Circulation 2016;133:2434–46. https://doi.org/10.1161/CIRCULATIONAHA.115.019645.

Li Z, Hu G, Zhu L, Sun Z, Jiang Y, Gao M jie, et al. Study of growth, metabolism, and morphology of *Akkermansia muciniphila* with an in vitro advanced bionic intestinal reactor. BMC Microbiol 2021;21:1–12. https://doi.org/10.1186/s12866-021-02111-7.

Liu RT, Rowan-Nash AD, Sheehan AE, Walsh RFL, Sanzari CM, Korry BJ, et al. Reductions in anti-inflammatory gut bacteria are associated with depression in a sample of young adults. Brain Behav Immun 2020;88:308–24. https://doi.org/10.1016/j.bbi.2020.03.026.

Machado D, Almeida D, Seabra CL, Andrade JC, Gomes AM, Freitas AC. Uncovering *Akkermansia muciniphila* resilience or susceptibility to different temperatures, atmospheres and gastrointestinal conditions. Anaerobe 2020;61:2–5. https://doi.org/10.1016/j.anaerobe.2019.102135.

Marcial-Coba MS, Cieplak T, Cahú TB, Blennow A, Knøchel S, Nielsen DS. Viability of microencapsulated Akkermansia muciniphila and Lactobacillus plantarum during freeze-drying, storage and in vitro simulated upper gastrointestinal tract passage. Food Funct 2018;9:5868–79. https://doi.org/10.1039/C8FO01331D.

Marcial-Coba MS, Saaby L, Knøchel S, Nielsen DS. Dark chocolate as a stable carrier of microencapsulated Akkermansia muciniphila and Lactobacillus casei. FEMS Microbiol Lett 2019;366. https://doi.org/10.1093/femsle/fny290.

Marrone MC, Coccurello R. Dietary fatty acids and microbiota-brain communication in neuropsychiatric diseases. Biomolecules 2020;10. https://doi.org/10.3390/biom10010012.

Martín R, Langella P. Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. Front Microbiol 2019;10:1047. https://doi.org/10.3389/fmicb.2019.01047.

Martín R, Miquel S, Benevides L, Bridonneau C, Robert V, Hudault S, et al. Functional characterization of novel *Faecalibacterium prausnitzii* strains isolated from healthy volunteers: A step forward in the use of *F. prausnitzii* as a next-generation probiotic. Front Microbiol 2017;8:1226. https://doi.org/10.3389/fmicb.2017.01226.

de Melo Pereira GV, de Oliveira Coelho B, Magalhães Júnior AI, Thomaz-Soccol V, Soccol CR. How to select a probiotic? A review and update of methods and criteria. Biotechnol Adv 2018;36:2060–76. https://doi.org/10.1016/j.biotechadv.2018.09.003.

Michielan A, D'Incà R. Intestinal Permeability in Inflammatory Bowel Disease: Pathogenesis,

930 Clinical Evaluation, and Therapy of Leaky Gut. Mediators Inflamm 2015;2015:1–10.
931 https://doi.org/10.1155/2015/628157.
932 Miyaoka T, Kanayama M, Wake R, Hashioka S, Hayashida M, Nagahama M, et al. *Clostridium*
933 *butyricum* MIYAIRI 588 as Adjunctive Therapy for Treatment-Resistant Major Depressive
934 Disorder: A Prospective Open-Label Trial. Clin Neuropharmacol 2018;41:151–5.
935 https://doi.org/10.1097/WNF.0000000000000299.
936 Monteagudo-Mera A, Rodríguez-Aparicio L, Rúa J, Martínez-Blanco H, Navasa N, García-
937 Armesto MR, et al. In vitro evaluation of physiological probiotic properties of different lactic
938 acid bacteria strains of dairy and human origin. J Funct Foods 2012;4:531–41.
939 https://doi.org/10.1016/j.jff.2012.02.014.
940 Moosavi SM, Akhavan Sepahi A, Mousavi SF, Vaziri F, Siadat SD. The effect of *Faecalibacterium*
941 *prausnitzii* and its extracellular vesicles on the permeability of intestinal epithelial cells and
942 expression of PPARs and ANGPTL4 in the Caco-2 cell culture model. J Diabetes Metab Disord
943 2020;19:1061–9. https://doi.org/10.1007/s40200-020-00605-1.
944 O'Toole PW, Marchesi JR, Hill C. Next-generation probiotics: the spectrum from probiotics to
945 live biotherapeutics. Nat Microbiol 2017;2:17057.
946 https://doi.org/10.1038/nmicrobiol.2017.57.
947 Ottman N, Reunanen J, Meijerink M, Pietila TE, Kainulainen V, Klievink J, et al. Pili-like proteins
948 of *Akkermansia muciniphila* modulate host immune responses and gut barrier function. PLoS
949 One 2017;12:1–18. https://doi.org/10.1371/journal.pone.0173004.
950 Ouwerkerk JP, Aalvink S, Belzer C, De Vos WM. Preparation and preservation of viable
951 *Akkermansia muciniphila* cells for therapeutic interventions. Benef Microbes 2017;8:163–9.
952 https://doi.org/10.3920/BM2016.0096.
953 Ozen M, Dinleyici EC. The history of probiotics: The untold story. Benef Microbes 2015;6:159–
954 65. https://doi.org/10.3920/BM2014.0103.
955 Perraudeau F, McMurdie P, Bullard J, Cheng A, Cutcliffe C, Deo A, et al. Improvements to
956 postprandial glucose control in subjects with type 2 diabetes: a multicenter, double blind,
957 randomized placebo-controlled trial of a novel probiotic formulation. BMJ Open Diabetes Res
958 Care 2020;8:e001319. https://doi.org/10.1136/bmjdrc-2020-001319.
959 Plovier H, Everard A, Druart C, Depommier C, Van Hul M, Geurts L, et al. A purified membrane
960 protein from *Akkermansia muciniphila* or the pasteurized bacterium improves metabolism in
961 obese and diabetic mice. Nat Med 2016;23:107–13. https://doi.org/10.1038/nm.4236.
962 Quévrain E, Maubert MA, Michon C, Chain F, Marquant R, Tailhades J, et al. Identification of an
963 anti-inflammatory protein from *Faecalibacterium prausnitzii*, a commensal bacterium deficient
964 in Crohn's disease. Gut 2016;65:415–25. https://doi.org/10.1136/GUTJNL-2014-307649.

965 Raise A, Dupont S, Iaconelli C, Caliri C, Charriau A, Gervais P, et al. Comparison of two
966 encapsulation processes to protect the commensal gut probiotic bacterium *Faecalibacterium*
967 *prausnitzii* from the digestive tract. J Drug Deliv Sci Technol 2020;56:101608.
968 https://doi.org/10.1016/j.jddst.2020.101608.
969 Rao Y, Kuang Z, Li Chan, Guo S, Xu Y, Zhao D, et al. Gut *Akkermansia muciniphila* ameliorates
970 metabolic dysfunction-associated fatty liver disease by regulating the metabolism of L-
971 aspartate via gut-liver axis. Gut Microbes 2021;13:1–19.
972 https://doi.org/10.1080/19490976.2021.1927633.
973 Reichardt N, Duncan SH, Young P, Belenguer A, McWilliam Leitch C, Scott KP, et al.
974 Phylogenetic distribution of three pathways for propionate production within the human gut
975 microbiota 2014;8:1323–35. https://doi.org/10.1038/ismej.2014.14.
976 Reunanen J, Kainulainen V, Huuskonen L, Ottman N, Belzer C, Huhtinen H, et al. *Akkermansia*
977 *muciniphila* adheres to enterocytes and strengthens the integrity of the epithelial cell layer.
978 Appl Environ Microbiol 2015;81:3655–62. https://doi.org/10.1128/AEM.04050-14.
979 Rivera-Chávez F, Zhang LF, Faber F, Lopez CA, Byndloss MX, Olsan EE, et al. Depletion of
980 Butyrate-Producing Clostridia from the Gut Microbiota Drives an Aerobic Luminal Expansion of
981 Salmonella. Cell Host Microbe 2016;19:443–54. https://doi.org/10.1016/j.chom.2016.03.004.
982 Rogers GB, Keating DJ, Young RL, Wong M-L, Licinio J, Wesselingh S. From gut dysbiosis to
983 altered brain function and mental illness: mechanisms and pathways. Mol Psychiatry
984 2016;21:738–48. https://doi.org/10.1038/mp.2016.50.
985 Rossi O, Khan MT, Schwarzer M, Hudcovic T, Srutkova D, Duncan SH, et al. *Faecalibacterium*
986 *prausnitzii* Strain HTF-F and Its Extracellular Polymeric Matrix Attenuate Clinical Parameters in
987 DSS-Induced Colitis. PLoS One 2015;10:e0123013.
988 https://doi.org/10.1371/journal.pone.0123013.
989 Saarela MH. Safety aspects of next generation probiotics. Curr Opin Food Sci 2019;30:8–13.
990 https://doi.org/10.1016/j.cofs.2018.09.001.
991 Sarkar A, Lehto SM, Harty S, Dinan TG, Cryan JF, Burnet PWJ. Psychobiotics and the
992 Manipulation of Bacteria–Gut–Brain Signals. Trends Neurosci 2016;39:763–81.
993 https://doi.org/10.1016/j.tins.2016.09.002.
994 Schaefer L, Auchtung TA, Hermans KE, Whitehead D, Borhan B, Britton RA. The antimicrobial
995 compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with
996 thiol groups. Microbiology 2010;156:1589–99. https://doi.org/10.1099/mic.0.035642-0.
997 Schwab C, Ruscheweyh H-J, Bunesova V, Pham VT, Beerenwinkel N, Lacroix C. Trophic
998 Interactions of Infant *Bifidobacteria* and *Eubacterium hallii* during L-Fucose and Fucosyllactose
999 Degradation. Front Microbiol 2017;8:95. https://doi.org/10.3389/fmicb.2017.00095.

1000 Scott KP, Martin JC, Duncan SH, Flint HJ. Prebiotic stimulation of human colonic butyrate-
1001 producing bacteria and *Bifidobacteria*, in vitro. FEMS Microbiol Ecol 2014;87:30–40.
1002 https://doi.org/10.1111/1574-6941.12186.
1003 Shetty SA, Zuffa S, Bui TPN, Aalvink S, Smidt H, De Vos WM. Reclassification of *Eubacterium*
1004 *hallii* as *Anaerobutyricum hallii* gen. nov., comb. nov., and description of *Anaerobutyricum*
1005 *soehngenii* sp. nov., a butyrate and propionate-producing bacterium from infant faeces. Int J
1006 Syst Evol Microbiol 2018;68:3741–6. https://doi.org/10.1099/ijsem.0.003041.
1007 Shi Z, Lei H, Chen G, Yuan P, Cao Z, Ser H-L, et al. Impaired Intestinal *Akkermansia muciniphila*
1008 and Aryl Hydrocarbon Receptor Ligands Contribute to Nonalcoholic Fatty Liver Disease in Mice.
1009 MSystems 2021;6. https://doi.org/10.1128/msystems.00985-20.
1010 Šipailienė A, Petraitytė S. Encapsulation of Probiotics: Proper Selection of the Probiotic Strain
1011 and the Influence of Encapsulation Technology and Materials on the Viability of Encapsulated
1012 Microorganisms. Probiotics Antimicrob Proteins 2018;10:1–10.
1013 https://doi.org/10.1007/s12602-017-9347-x.
1014 Sivieri K, Morales MLV, Saad SMI, Adorno MAT, Sakamoto IK, Rossi EA. Prebiotic effect of
1015 fructooligosaccharide in the simulator of the human intestinal microbial ecosystem (SHIME
1016 model). J Med Food 2014;17:894–901. https://doi.org/10.1089/jmf.2013.0092.
1017 Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermúdez-Humarán LG, Gratadoux J-J, et al.
1018 *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut
1019 microbiota analysis of Crohn disease patients. Proc Natl Acad Sci 2008;105:16731–6.
1020 https://doi.org/10.1073/PNAS.0804812105.
1021 Song H, Yoo Y, Hwang J, Na YC, Kim HS. *Faecalibacterium prausnitzii* subspecies-level dysbiosis
1022 in the human gut microbiome underlying atopic dermatitis. J Allergy Clin Immunol
1023 2016;137:852–60. https://doi.org/10.1016/j.jaci.2015.08.021.
1024 Sornplang P, Piyadeatsoontorn S. Probiotic isolates from unconventional sources: a review. J
1025 Anim Sci Technol 2016;58. https://doi.org/10.1186/s40781-016-0108-2.
1026 Sperandeo P, Martorana AM, Polissi A. Lipopolysaccharide biogenesis and transport at the
1027 outer membrane of Gram-negative bacteria. Biochim Biophys Acta - Mol Cell Biol Lipids
1028 2017;1862:1451–60. https://doi.org/10.1016/j.bbalip.2016.10.006.
1029 Sun F, Zhang Q, Zhao J, Zhang H, Zhai Q, Chen W. A potential species of next-generation
1030 probiotics? The dark and light sides of *Bacteroides fragilis* in health. Food Res Int
1031 2019;126:108590. https://doi.org/10.1016/j.foodres.2019.108590.
1032 Sung J, Kim S, Cabatbat JJT, Jang S, Jin YS, Jung GY, et al. Global metabolic interaction network
1033 of the human gut microbiota for context-specific community-scale analysis. Nat Commun
1034 2017;8:1–12. https://doi.org/10.1038/ncomms15393.

1035 Tamanai-Shacoori Z, Smida I, Bousarghin L, Loreal O, Meuric V, Fong SB, et al. *Roseburia* spp.: A
1036 marker of health? Future Microbiol 2017;12:157–70. https://doi.org/10.2217/fmb-2016-0130.
1037 Tan H, Zhai Q, Chen W. Investigations of Bacteroides spp. towards next-generation probiotics.
1038 Food Res Int 2019;116:637–44. https://doi.org/10.1016/j.foodres.2018.08.088.
1039 Tan L, Zhao S, Zhu W, Wu L, Li J, Shen M, et al. The *Akkermansia muciniphila* is a gut microbiota
1040 signature in psoriasis. Exp Dermatol 2018;27:144–9. https://doi.org/10.1111/exd.13463.
1041 Van-Den-Abbeele P, Venema K, Van-De-Wiele T, Verstraete W, Possemiers S. Different human
1042 gut models reveal the distinct fermentation patterns of arabinoxylan versus inulin. J Agric Food
1043 Chem 2013;61:9819–27. https://doi.org/10.1021/jf4021784.
1044 Vass P, Pantea E, Domokos A, Hirsch E, Domján J, Németh Á, et al. Electrospun Solid
1045 Formulation of Anaerobic Gut Microbiome Bacteria. AAPS PharmSciTech 2020;21:214.
1046 https://doi.org/10.1208/s12249-020-01769-y.
1047 de Vos WM, Seegers JFML. Method for culturing and preserving *Eubacterium hallii* US Patent
1048 2019/0151377 A1, 2017.
1049 Wang Z, Zhao Y. Gut microbiota derived metabolites in cardiovascular health and disease.
1050 Protein Cell 2018;9:416–31. https://doi.org/10.1007/s13238-018-0549-0.
1051 Wassenaar TM, Zimmermann K. Lipopolysaccharides in food, food supplements, and
1052 probiotics: should we be worried? Eur J Microbiol Immunol 2018;8:63–9.
1053 https://doi.org/10.1556/1886.2018.00017.
1054 Wu W, Lv L, Shi D, Ye J, Fang D, Guo F, et al. Protective effect of *Akkermansia muciniphila*
1055 against immune-mediated liver injury in a mouse model. Front Microbiol 2017;8.
1056 https://doi.org/10.3389/fmicb.2017.01804.
1057 Yang F, DeLuca JAA, Menon R, Garcia-Vilarato E, Callaway E, Landrock KK, et al. Effect of diet
1058 and intestinal AhR expression on fecal microbiome and metabolomic profiles. Microb Cell Fact
1059 2020;19:1–18. https://doi.org/10.1186/s12934-020-01463-5.
1060 Zhang T, Li Q, Cheng L, Buch H, Zhang F. *Akkermansia muciniphila* is a promising probiotic.
1061 Microb Biotechnol 2019;12:1109–25. https://doi.org/10.1111/1751-7915.13410.
1062 Zheng DW, Li RQ, An JX, Xie TQ, Han ZY, Xu R, et al. Prebiotics-Encapsulated Probiotic Spores
1063 Regulate Gut Microbiota and Suppress Colon Cancer. Adv Mater 2020;32:2004529.
1064 https://doi.org/10.1002/adma.202004529.
1065 Zheng H, Liang H, Wang Y, Miao M, Shi T, Yang F, et al. Altered Gut Microbiota Composition
1066 Associated with Eczema in Infants. PLoS One 2016;11:e0166026.
1067 https://doi.org/10.1371/journal.pone.0166026.
1068 Zielińska D, Kolozyn-Krajewska D, Laranjo M. Food-Origin Lactic Acid Bacteria May Exhibit
1069 Probiotic Properties: Review. Biomed Res Int 2018;2018.

https://doi.org/10.1155/2018/5063185.

1072 Table 1. List of proposed NGP and their characteristics.

| Species (strain) | Gram | Metabolism | Classification[(1)] | Prevalence in human gut | Firstly Identified (Source – Location [Year]) | Relevant references |
|---|---|---|---|---|---|---|
| *Akkermansia muciniphila* | Negative | Anaerobic | P: Verrucomicrobia<br>C: Verrucomicrobiae<br>O: Verrucomicrobiales<br>F: Akkermansiaceae | 3% to 5% | Human feces – The Netherlands [2004] | (Derrien et al., 2008, 2004; Zhang et al., 2019) |
| *Faecalibacterium prausnitzii* | Positive | Strict anaerobic | P: Firmicutes<br>C: Clostridia<br>O: Eubacteriales<br>F: Oscillospiraceae | 3% to 5% | Human pus – Germany [1922] | (Duncan et al., 2002; Martín et al., 2017; Sokol et al., 2008) |
| *Anaerobutyricum hallii* | Positive | Strict anaerobic | P: Firmicutes<br>C: Clostridia<br>O: Eubacteriales<br>F: Lachnospiraceae | 2% to 3% | Human feces – Virginia [1974] | (Bunesova et al., 2018; Holdeman and Moore, 1974; Shetty et al., 2018) |
| *Prevotella copri* | Negative | Strict anaerobic | P: Bacteroidetes<br>C: Bacteroidia<br>O: Bacteroidales<br>F: Prevotellaceae | Up to 80% | Human feces – Japan [2007] | (Hayashi et al., 2007; Ley, 2016) |
| *Butyricicoccus pullicaecorum* | Positive | Anaerobic | P: Firmicutes<br>C: Clostridia<br>O: Eubacteriales<br>F: Clostridiaceae | n.a. | Chicken caecal content – Belgium [2008] | (Boesmans et al., 2018; Eeckhaut et al., 2008; Geirnaert et al., 2014) |
| *Bacteroides* spp. | Negative | Strict anaerobic | P: Bacteroidetes<br>C: Bacteroidia<br>O: Bacteroidales<br>F: Bacteroidaceae | 25% | Human gut – France [1898]<br>(First: *B. fragilis*) | (Dahiya et al., 2019; Tan et al., 2019) |
| *Roseburia* spp. | Positive | Strict anaerobic | P: Firmicutes<br>C: Clostridia<br>O: Eubacteriales<br>F: Lachnospiraceae | 3 to 15% | Mouse cecal mucosae – Illinois [1983]<br>(First: *R. cecicola*) | (Hillman et al., 2020; Tamanai-Shacoori et al., 2017) |
| *Clostridium butyricum* (MIYAIRI 588) | Positive | Strict anaerobic | P: Firmicutes<br>C: Clostridia<br>O: Eubacteriales | n.a. | Soil – Japan [1960s] | (Guo et al., 2020; Isa et al., 2016) |

| Species (strain) | Gram | Metabolism | Classification[(1)] | Prevalence in human gut | Firstly Identified (Source – Location [Year]) | Relevant references |
|---|---|---|---|---|---|---|
| | | | F: Clostridiaceae | | | |

[(1)] Taxonomic classification: P = phylum; C = class; O = order; F = family; n.a. = not available

1076 Table 2. Next-generation probiotics delivery systems

| Microorganism | Technique/method | Matrix | Main achievements | References |
|---|---|---|---|---|
| *Akkermansia muciniphila* MucT (CIP 107961T) | Water-in-oil-in-water (W/O/W) double emulsion | Sunflower oil, sodium Caseinate and polyglycerol polyricinoleate (PGPR) as emulsifier | 6.6% survival of encapsulated bacteria compared to 0.4% of controlled group after 2 h exposure to simulated gastric juice. | van der Ark et al. 2017 |
| *Akkermansia muciniphila* DSM22959 | Extrusion | Xanthan and gellan gum | 0.57 log CFU.$g^{-1}$ reduction over 1 month storage at storage at 4 °C under anaerobic conditions.<br>Improved survival when subjected to simulated gastric conditions. | Marcial-Coba et al. 2018 |
| *Akkermansia muciniphila* 139 | Spray drying | Succinate-grafted alginate Epigallocatechin-3-gallate | Improved viability up to 12 days at 4°C in anaerobic PBS buffer.<br>Improved survival when subjected to simulated gastric conditions. | Chang et al. 2020 |
| *Butyricicoccus pullicaecorum* 25-3T | Lyophilized culture used to fill hydroxypropyl methylcellulose (HPMC) capsules.<br>HPMC capsules sealed and coated with a pH-resistant coating of enteric polymer cellulose acetate phthalate (CAP) and plasticizer diethyl phthalate | Lyophilized formulation contained horse serum supplemented with trehalose and cysteine-HCl.<br>HPMC, CAP, diethyl phthalate | 67 % average survival ($6.7x10^7$ CFU) after 8 months storage at 4°C. | Boesmans et al. 2018 |
| *Clostridium butyricum* | Electrospinning of a bacterial cell/spores suspension under anaerobic conditions | Hydroxypropyl-beta-cyclodextrin (HP-β-CD) | Viability of sporulated bacteria in the fibers maintained during 1 year at room temperature and aerobic conditions.<br>No significant decrease in bacterial viability after milling and tableting the fibers. | Vass et al. 2020 |

| Microorganism | Technique/method | Matrix | Main achievements | References |
|---|---|---|---|---|
| *Clostridium butyricum* ATCC 19398 | Self-assembly of adamantane-modified spores and β-cyclodextrin-grafted dextran | β-cyclodextrin, adamantine (AD) and dextran | Dextran encapsulated probiotic spores specifically enrich in colon cancers after oral administration.<br>Additionally, encapsulated spores regulate gut microbiota, augment the abundance of SCFA-producing bacteria. | Zheng et al 2020 |
| *Faecalibacterium prausnitzii* CNCM I-4573 | Tableting by direct compression of previously lyophilized cells | Microcrystalline cellulose (MCC), HPMC, hydroxypropyl methylcellulose phthalate (HPMCP) | Survival above $10^8$ CFU/tablet after 28 days at 25 °C, anaerobic condition, 11% relative humidity. | Allouche et al. 2018 |
| *Faecalibacterium prausnitzii* CNCM I-4573 | 1. Extrusion-ionic gelation of fresh cells under anaerobic conditions and subsequent lyophilization | Amidated low-methoxyl pectin | Around 2 log units decrease after 14 days at 25 °C, anaerobic condition, 11% relative humidity.<br>3-5 log units decrease when exposed to stomach proximal and jejunum buffers for 30 min. | Raise et al.2020 |
| | 2. Lipid based-suspension introduced in gelatin capsules and let to solidify under anaerobic conditions | Gelucire®, gelatin capsules | Around 4 log decrease after 14 days at 25 °C, in anaerobic condition and with 11% relative humidity.<br>2 log units decrease when exposed to stomach proximal and jejunum buffers for 30 min. | |

1077