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Review Article

Next Generation Probiotics for Butyrate Production in the Gut

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Abstract

Health beneficial effects of Short Chain Fatty Acids (SCFAs) produced in the gut, especially butyrate, and their decreased abundance in several gut-related disorders makes them a potent approach for disease management and prevention. New insights into the microbiome have enlightened the path for new probiotic formulations based on the combined actions (cross feeding interactions) of different human gut commensals to increase SCFAs concentrations in the colon. In this line, the Novobiome consortium was created in order to overcome technical and regulatory challenges to bring these next-generation probiotics (NGPs) to the market to complete the suite of probiotic products for supporting a healthy gut microbiome.

Keywords: *SCFAs; Next-Generation Probiotics; Butyrate; Cross-Feeding; Faecalibacterium prausnitzii; Akkermansia muciniphila; Novobiome; Probiotic, Microbiota*

SCFAs as essential metabolites for human health

SCFAs are the main products of saccharolytic fermentation of non-digestible carbohydrates in the large intestine by intestinal gut microbes [1]. Acetate, propionate, and butyrate are the most abundant SCFAs in the human body, and play a fundamental role in (gut) health. Those compounds are very important for the maintenance of the intestinal barrier integrity to prevent the translocation of pathogenic bacteria and/or bacterial compounds with pro-inflammatory potential (as for example, lipopolysaccharides) across the gut. Of those three metabolites, butyrate is very important because it consists on the primary source of energy for the colonocytes. It exerts a key regulatory role in maintaining the epithelial integrity, by regulating the tight junction proteins expression [2] and has helped to restore the gut barrier function in disease models [3]. SCFAs can also improve some of the immune defensive functions of the intestinal epithelium by increasing the expression of antimicrobial peptides by the gut epithelium cells [4].

As mention above, most butyrate produced by fiber fermentation is utilized by colon epithelial cells as energy source but, via the portal vein, SCFA can also reach the liver where acetate and propionate are metabolized and partly oxidized or used as substrate in gluconeogenesis and lipogenesis [5]. As a result, a small proportion of microbiota-derived SCFA enters the peripheral circulation and can exert a systemic effect acting as ligands for receptors expressed mainly in intestinal, adipose, skeletal muscle, liver and pancreatic tissues, affecting their functioning and metabolism. These bacterial metabolites trigger the production of metabolic hormones involved in insulin tolerance, which regulates blood sugar levels, and leptin, that is involved in the regulation of the energy balance in the body [6,7].

In addition, SCFAs can modulate inflammation and affect the immune system by regulating the differentiation, recruitment, and activation of immune cells [8]. Also, SCFAs (mainly butyrate) participate in the regulation of cell proliferation and differentiation by the inhibition of histone deacetylase activity and the promotion of histone acetylation, contributing to gut homeostasis [9].

SCFAs abundance has been found to be reduced in several diseases, such as among others IBD [9], atopic allergy [10] or obesity [11], and has been related to a dysbiosis of the gut microbiota composition, with a reduction of the microbial populations that are able to produce these compounds.

Including SCFAs into the diet to increase their abundance in the gut has no effect, because they are mainly absorbed in the upper gastrointestinal tract and do not reach the necessary concentrations in the colon [12]. For this reason, new strategies to increase SCFAs concentration in the targeted areas of the gut where their beneficial effects are exerted, are still necessary. Gut microbiota modulation through diet (non-digestible fiber consumption) and consumption of living bacteria (probiotics) and a combination thereof seem good strategies to achieve higher SCFAs concentrations in the colon. In the following sections, this concept is translated into novel ideas about probiotics strategies that will be further reviewed.

Strategies to increase the butyrate production in the gut: Next-Generation probiotics (NGPs)

At present, the most used definition for probiotics is that they are "live microorganisms that when administered in adequate amounts, confer a health benefit on the host" [13]. Since the probiotic concept was introduced by Ellie Metchnikoff in 1908, mainly *Lactobacillus* and *Bifidobacterium* genera have been used with this purpose (also known as classical probiotics), finding wide availability of them in the market [14].

With the expansion of cutting-edge next generation sequencing methods and available bioinformatics tools in human microbiome research, we now understand the composition and function of the human gut microbiome and its microbiota much better than 15 years ago. Consequently, a few distinct members of the human gut microbiota, referred as keystone species, have received particular attention for their key role in gut homeostasis, for example because of their special metabolic properties. In addition, several studies have associated a low abundance of these species with a negative impact on the remaining microorganisms and host's health [15,16] and consequently, some of these keystone species can be considered as candidate NGPs, for instance the ones that owe their relevance to their role as producers of essential metabolites such as SCFAs.

Most of the butyrate producers in the human colon belong to the Firmicutes phylum, in particular, to the family of *Lachnospiraceae* and *Ruminococcaceae* [17], which are highly oxygen-sensitive, strictly anaerobic, saccharolytic bacteria. In fact, the two most dominant bacterial species in the human colon, *Faecalibacterium prausnitzii* (*Ruminococcaceae)* and *Eubacterium rectale* (*Lachnospiraceae*), are both known butyrate producers [18]. Other important butyrate-producing bacterial species in the human colon are the *Lachnospiraceae Anaerobutyricum hallii*, *Roseburia* spp. [such as *R. faecis, R. inulinivorans, R. intestinalis*, and *R. hominis*], and *Anaerostipes* spp. (such as A. *caccae, A. butyraticus* and *A. hadrus*) [19]. Some of these species, such as *F. prausnitzii*, preferentially colonize close to the gut mucus layer whereas other species such as *A. caccae* are mainly present in the lumen of the colon [20].

In the last decade, several studies have shown that bifidobacteria can interact with other colon bacteria such as butyrate-producing bacteria by cross-feeding interactions. Particularly, *Bifidobacterium* species are known to produce acetate and lactate, which are crucial compounds for the gut health and promote gut epithelial integrity [21] through a cross-feeding relationship with SCFAs-producing keystone species. *Bifidobacterium* species, which belong to the group of classical probiotics, have important functions within the human colon [22,23]. Decreased abundances of these species in the colon have been associated with several disorders as antibiotic-associated diarrhea, IBS, IBD, obesity, allergies, and regressive autism [19]. Moreover, several works have been published demonstrating the cooperation between *Bifidobacterium infantis* and the butyrate producer *A. caccae* [24], establishing *B. infantis* as a carbohydrate first degrader

and *A. caccae* as a second degrader that utilizes intermediate metabolites (acetate), produced by *B. infantis*, for butyrate production and maintenance of the gut barrier integrity. In a similar way, *Bifidobacterium adolescentis* also establishes a cross-feeding relationship with butyrate producers that belong to the Firmicutes phyla as *A. caccae* and *A. hallii* [25].

New cross-feeding interactions have also been elucidated between *Akkermansia muciniphila,* a mucin degrader bacteria with a great potential as NGP [26,27], and *A. caccae*. The mucin degradation by *A. muciniphila* supports the growth of *A. caccae* and its concomitant butyrate production by increasing the availability of mucin sugars [28]. *A. muciniphila* is able to produce acetate and propionate and survive, on the contrary to the most part of butyrate producers that are strict anaerobic bacteria, in presence of nanomolar concentrations of oxygen [29]. This potential NGP can also exert beneficial effects independently of SCFAs production. For example, Cani and colleagues showed that this bacteria produces structural component protein in the outer membrane that has an important immunomodulatory action in the host [30].

F. prausnitzii is another potential NGP, which has been found to be decreased in some gut inflammatory diseases as Crohn's disease [31]. *F. prausnitzii* also collaborates in lessening the inflammation state in disease by other mechanisms apart from the butyrate production, as for example, stimulating the production of anti-inflammatory cytokines [e.g. IL-10] by host immune cells [32] and producing a microbial anti-inflammatory molecule [MAM] that showed anti-inflammatory properties in an induced colitis model [33]. As *F. prausnitzii* growth is stimulated by acetate [34], this commensal bacteria has the ability to establish interesting cross-feeding relationships with acetate-producers like *Bifidobacterium adolescentis* and *A. muciniphila* [35,36]. Also, cross-feeding interactions have been elucidated between *F. prausnitzii* and *Bacteroides thethaiotaomicron* [37], a symbiont that encodes an enormous repertoire of carbohydrate degrading enzymes [38] and has the ability to switch between diet- and host-derived carbohydrates, what makes it very adaptable to changes in the gut environment, where there is also a high competition for nutrients. In this line, *F. prausnitzii* can metabolize acetate produced by *B. thetaiotaomicron* to produce butyrate. What is more, it has been recently reported that *B. thethaiotaomicron* also stimulates degradation of quercetin and butyrate production by *Eubacterium ramulus* via cross-feeding of molecules released from bacterial starch fermentation [39].

Main challenges to bring NGPs to the market

For the reasons mentioned above, NGPs are expected to have a great impact on human health. But due to their novelty and intrinsic characteristics, there are several challenges during the development and introduction of NGPs to the market. The technical and regulatory challenges will be discussed here briefly.

Technical challenges

NGPs are considered to be extremely oxygen sensitive [EOS] microorganisms and they often have complex and yet unknown nutritional requirements. For these NGPs to be commercially attractive, first their production media need to be optimized based on the nutritional needs of each NGP; temperature, pH, water activity have to be adjusted accordingly as well amongst other parameters [40]. Moreover, any animal derived nutritional components need to be emitted and/ or be replaced in order to ensure the safety of the end users and in the same time preserving the properties of the final product [41,42]. The oxygen exposure needs to be limited throughout the whole production process including formulation and freeze drying therefore specified cryoprotectants and antioxidants or encapsulation need to be incorporated [41,43,44]. Consequently, their stability during the whole production process is hard to be achieved. In addition, the product final formulation, packaging and storage conditions might affect the viability of the bacteria and therefore the stability of the product which could impact its properties. Most NGPs candidates are human derived and they are expected to exert their impact in their natural niche. To do so they have to be delivered alive there. Thereby, viability after consumption needs to be considered as well. In order to reach the colon, bacteria will have to survive to a sufficient degree their passage through the gastrointestinal tract [oxygen, stomach pH, bile acids, enzymatic activity, small intestinal pH etc]. Hence, the selection of cell protectants and carriers is crucial for the efficacy of the product [45]. For example, for aerotolerant probiotic strains yogurts have been used as vehicles. Due to the intrinsic characteristics of NGPs, described above, other types of vehicles need to be considered e.g. microencapsulation [46].

Since these bacteria are endogenous, they are expected to colonize their respective niche and to interact with the intestinal epithelial cells [IEC]. Due to the oxygen limitations that the bacteria require and the oxygen-requiring human cells is really hard to study the host-microbe interactions. Consequently, model systems that would reflect the complexity of the host microbe interactions and will allow optimal growth of both bacteria and host cells, need to be developed [47-49].

Regulatory challenges

The legal possibilities to refer to microorganisms as 'probiotics', and to make health claims on them, vary across the world. In the EU, the European Food Safety Authority [EFSA] is the Risk Assessor who evaluates, when appropriate and required, the safety of foods and ingredients, as well as the scientific substantiation of health claims. The European Commission is the Risk Manager who, mandated by the European Parliament, authorizes or rejects e.g. novel foods and health claims. EFSA has developed the Qualified Presumption of Safety [QPS], and has allocated such Presumption to a [periodically updated] list of microorganism species. QPS has no legal status; it is an internal EFSA tool to simplify, where possible, their safety evaluation of microorganisms. The 'classical probiotics' discussed above belong to QPS species.

In the United States, a 'New Dietary Ingredient' [NDI] needs to be notified to the Food and Drug Administration, and this process takes substantial effort and time. An NDI that can be Generally Recognized as Safe [GRAS] can be exempt from the obligation to notify as NDI. A conclusion of GRAS is valid when reached and documented by reputable experts in the field, and when it can be assumed that other experts in the field would come to the same conclusion. Such dossier and conclusion can be sent to the FDA, who may respond with a letter stating that they have no questions, and it will be included in FDA's public GRAS Inventory. Alternatively, the Food Business Operator can simply keep such dossier on file, and show it to the FDA if requested.

Traditionally, safety of probiotics is mainly based on history of safe use. Due to the novelty of NGP strains and the lack of their presence in food known to EU before 1997, [50,51] they are expected to be regulated as Novel Food and/or Live Biotherapeutics (LBPs) from the authorities [52]. FDA's definition for a LBP is: "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" [52].

According to EFSA's recommendations for Novel Food applications [50,51], in order to introduce NGPs to the market a full safety assessment needs to be performed. A taxonomic classification to the species level must be provided accompanied by whole genome sequencing analysis focused on the detection of genes encoding toxins and virulence factors, genes that are conferring resistance to antibiotics with special focus on their potential for horizontal gene transfer and other potentially adverse metabolic features (e.g. D-lactate). The genotypic characterization must be accompanied by phenotypic characterization of the potential antimicrobial resistances (intrinsic or acquired) [53,54]. Based on the taxonomic classification and genomic information of the microorganism, other potentially adverse phenotypic features should be assessed (e.g. potential toxin production, hemolytic activity, infectivity, adverse immune effects, etc.) [50,51]. In some cases, in vitro tests might be sufficient to prove the safety of the bacterial strain in question. In other cases, testing in animal models and/or a small clinical study with healthy volunteers might be required as well. Finally, the stability of the final product and the number of viable cells in the final product must be provided as well. FDA also provided recommendations regarding Investigational New Drug Application (IND) for early clinical trials with LBPs in the United States (U.S.), marketed not only as drugs but as foods (e.g. conventional foods and dietary supplements) as well (Early Clinical Trials with Live Biotherapeutic Products: Chemistry, Manufacturing, and Control Information: Guidance for Industry [55]. This guidance focuses on the chemistry, manufacturing, and control (CMC) information. The IND application should contain amongst other information the following: i) a description of the LBP's drug substance, including its physical, chemical, or biological characteristics, ii) characterization of the drug substance (e.g. antibiotic resistance profiling and potential for horizontal gene transfer), iii) name and address of the manufacturer(s), iv) the method of Manufacture and v) drug Substance Specifications [55].

The Novobiome project: towards the NGPs for human health

Because NGPs are endogenous to the host they are expected to colonize their natural niche, to exert their beneficial effects in the site where they are needed and therefore to improve the quality of life of the consumers. As mentioned earlier, since there is no history of safe use of bacteria belonging to NGPs, is highly likely that LBPs will have to be tested first in healthy volunteers to look for adverse effects and ensure their safety for their intended use.

In order to overcome all the above mentioned challenges, we have formed the Novobiome consortium consisting of experts from industry and academic institutions. The goal is to select, develop, and produce NGPs. The consortium is focusing on the following aspects:

1. Isolation, genotypic and phenotypic characterization, meeting the regulatory requirements for complete characterization of strains;
2. Culturing and scale-up, in an economically feasible industrial scalability;
3. Establishment of safety and functionality, meeting the regulatory requirements for extensive safety profiles;
4. Delivery to the site of action, meeting the need for delivering effective strains to target sites and
5. Enabling multi-strain formulations of NGPs, to achieve an increased beneficial effect.

Since the safety and the efficacy of probiotics is strain dependent rather that species or genus dependent [56,57] the consortium will be focusing on vast isolations of human derived bacteria in order to identify strains that elicit effects and are safe, scalable and stable at the same time. By combining our expertises in the Novobiome platform we create ideal circumstance to tackle all the developmental challenges in accordance with the regulatory requirements.

Concluding Remarks

NGPs are a promising approaching to increase the concentration of SCFAs (butyrate) in the gut for the management and prevention of gut-related disorders. To this end, several technical and challenges need to be overcome, but with the cooperation between research institutions, industry and regulatory agencies, the development and market implementation of those novel probiotics are a step closer to being a reality.

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Competing Interests

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