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Review Article

Next generation probiotics in disease amelioration

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ABSTRACT

Studies on the role of gut commensal bacteria in health development have rapidly attracted much more attention beyond the classical pathogens over the last decade. Many important reports have highlighted the changes in the gut microbiota (dysbiosis) are closely related to development of intra- and extra-intestinal, chronic inflammation related diseases such as colitis, obesity/metabolic syndromes, diabetes mellitus, liver diseases, cardiovascular diseases and also cancer and neurodegenerative diseases. To circumvent these difficulties, the strategy of modulating the structure of the gut microbiota has been under intensive study and shed more light on amelioration of these inflammation related diseases. While traditional probiotics generally show marginal ameliorative effects, emerging next generation probiotics start to reveal as new preventive and therapeutic tools. Recent studies have unraveled many potential next generation probiotics (NGP). These include *Prevotella copri* and *Christensenella minuta* that control insulin resistance, *Parabacteroides goldsteinii*, *Akkermansia muciniphila* and *Bacteroides thetaiotaomicron* that reverse obesity and insulin resistance, *Faecalibacterium prausnitzii* that protects mice against intestinal diseases, and *Bacteroides fragilis* that reduces inflammation and shows anticancer effect. New agents will soon be revealed for targeted therapy on specific inflammation related diseases. The important roles of next generation probiotics and gut microbiota normobiosis on the maintenance of intestinal integrity and homeostasis are emphasized.

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1. Introduction

Due to rapid development of advanced genetic sequencing tools and the bioinformatics platforms in the past 15 years, scientists are able to characterize the composition and function of microbiota and microbiomes from the intestine. Fast gathering of these big data strongly indicates that gut bacteria play important roles in maintaining the intestinal homeostasis and modulating host metabolism. During the process of microbiota study, some of the gut bacteria will soon be expected to emerge as potential sources of novel disease therapeutics [1]. The molecular mechanisms of interaction between the gut commensals and the host cells, and how commensals maintain optimal intestinal integrity and immunity are intensively studied [2]. Under the situation of gut microbiota dysbiosis, chronic inflammation related diseases such as metabolic syndromes, cardiovascular disorders, inflammatory bowel diseases, neuropsychiatric diseases, asthma and cancers …. etc. are developed [3]. As the microbiota studies proceed, single bacterial strains are screened and isolated, aiming to characterize their relationship with the amelioration of inflammation-related diseases. Among these, some of them are expected to emerge as next generation probiotics (NGP).

2. Interaction between microbiota and host intestinal immunity

Located along the border of the lumen in gastrointestinal tract, intestinal epithelial cells (IEC) play a most important role as a barrier bridging the key messages sent from gut microbiota to the immune and other cells residing in the lamina propia [2,3]. The host innate immunity system in IEC bears pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and NOD-like receptors (NLRs), essential elements recognizing the microbes' pathogen-associated molecular patterns (PAMPs) from microorganisms [4]. Important bacterial PAMP ligands comprise lipopolysaccharides (LPS), peptidoglycans, lipoteichoic acid, flagellin and muramyldipeptide(MDP). On top, there are also danger-associated molecular patterns (DAMP) signals derived from destroyed host tissues that can be sensed by PRRs [5]. On the other hand, intestinal immune systems are required to continuously monitor the structure and abundance of the microbiota [6]. Thus dynamic interactions exist between the intestinal cells and the vast diversity of microbiota microbes [7]. Under normal situations (normobiosis), the barrier function from IEC is efficiently maintained due to multiple mechanisms including production of optimal amount of tight-junction proteins, optimal thickness and sugar composition of the mucus layer, production of antimicrobial proteins/peptides, and production of a certain amount of immunoglobulin A (IgA) [7].

Under the situation of microbiota dysbiosis, overgrowth of some microbiota bacteria such as those belonging to the phylum Proteobacteria may occur and results in overproduction of the PAMPS, leading to enhanced inflammations and loss of immune tolerance. This is followed by abrogation of intestinal integrity, leading to leaky gut phenomena and occurrence of chronic inflammations-related diseases [8]. Taking the LPS from the PAMP as an example, under high-fat-diet life style, due to the compromised intestinal integrity, LPS translocates across the IEC into the blood. Increased blood LPS concentration initiates systemic low-grade inflammation via its interaction with the surface receptors (i.e. TLR-4 and CD14) of systematic innate immune and cellular systems [9]. These results lead to development of metabolic endotoxemia, overweight and subsequently obesity-related syndromes including insulin resistance, glucose intolerance, dyslipidaemia, and hepatic steatosis [10]. Besides LPS, many other PAMPs such as peptidoglycans or flagellin also have been shown to show similarly causal effects [11].

3. Microbiota metabolites affect host metabolism

Besides classical interactions between bacterial PAMPs and the host immune systems, metabolites derived from gut microbiota also involve interactive activities affecting the systematic host cellular metabolism and physiology [12]. Many bacterial metabolites that include folate, indoles, secondary bile acids, trimethylamine-N-oxide (TMAO), serotonin, gamma amino butyric acid, and also short chain fatty acids (SCFA, acetate, propionate and butyrate) have been characterized [13]. Under different concentrations, these bacterial metabolites play important roles in regulating host physiological phenotypes. In contrast, disease development may occur under unbalanced situation [13]. The functional molecular mechanisms of these metabolites can be via binding to specific host membranes or nuclear receptors [14]. For example, SCFAs trigger the secretion of intestinal peptides [glucagon-like peptide-1 or peptide YY (PYY)] involved in glucose metabolism or food intake via G-protein-coupled receptors such as GPR-41 and GPR-43 [15]. Besides, the butyrate works as an essential energy source, allowing colonic cells to proliferate and maintain healthy gut barrier function [16]. On top of this, butyrate also activates β-oxidation and oxidative phosphorylation in the mitochondria in colonic cells to consume oxygen (maintain anaerobic condition) in the intestinal lumen and protect the host from the expansion of pathobionts such as those belonging to facultative anaerobic Enterobacteriaceae [17,18]. Therefore, gut microbes may work as a endocrine organ regulating the activities of different distal organs through close interactions with intestinal cells [19].

4. Why NGP are essential?

To achieve the goal of amelioration of diseases related to leaky gut syndromes, the administration of probiotics is one of the optimal approaches. Main aims of administration of probiotics may comprise enhancing the integrity of intestinal epithelial layer, increasing in optimal IgA production, modulation of homeostatic bile acids production and secretion, and increase in production of antimicrobial peptides [20]). In general, traditional widely used probiotics such as *Bifidobacterium spp*., *Lactobacillus spp*. and many others were selected either randomly or through the gathering living experiences. While most of them show biological safety and some of them may show ameliorative effectiveness, however, the general effects and functions on amelioration of diseases are statistically marginal. On the other hand, the administration of traditional probiotics does not aim against specific diseases. Based on these situations, identification and characterization of novel and disease-specific NGP are urgently needed [21]. Besides safety concern, characteristics of the NGP will have to include comprehensive understanding of their targeted diseases, and the bacterial genetic features and physiological traits, including the growth dynamics, antibiotics sensitivity pattern. Moreover, the underlying molecular ameliorative mechanisms have to be clarified. To achieve this goal, screening and isolating the NGP through the cutting edge NGS (next generation sequencing) and bioinformatics technique platforms, followed by rigid functional validation of the new probiotics have to be performed. Strategically, these approaches are very different from those frequently used in isolation of traditional probiotics. Briefly, sectional (or even longitudinal) studies and more comprehensive bioinformatics analyses of the microbiota composition, metagenomics, and the host responses such as metabolites/metabolome produced (metabolomics) will have to be performed for selection of effective probiotics. Based on analyses results of the many multi-omics big data groups, potential probiotics or the consortium can then be highlighted and selected. These are followed by functional validation of the selected probiotics candidates, including assays of *in vitro* cell lines, *ex vivo* animal models, *in vivo* animals and even to human clinical trials. Furthermore, to get a more practical and applicable results, one may have to improve the quality of samples to be analysed, from the easily accessible faecal materials to those from local mucosa loci. Of note, standardized processing steps on samples harvesting, optimal storage conditions and detailed sequencing and bioinformatics analyses will have to be designed and performed strictly. Finally, the many big data results obtained from large scale analyses of blood/serum, tissues, urine, and fecal samples, under different environmental situations such as different nutrition and drug treatments … etc. will have to be integrated together to form a whole-picture oriented biochemical outputs. These results can then show more practical interaction situation between the microbiota and the host [22].

4.1. *How to isolate next generation probiotics?*

In order to achieve the goals of identifying next generation probiotics, the first stage is to unravel whether there are significant microbiota bacteria-host correlations between the study groups (such as the healthy, the disease and the experimental groups). Such associations can be derived either from animals or clinical studies. This is followed by isolation of potential probiotics and/or characterization of the related metabolites derived for subsequent functional validation studies. So far many microbiota bacteria still can not be cultured *in vitro*. However, as the technologies of culturomics are making rapid progress [23], targeted bacteria with significance can be specifically isolated and cultured by all means. Experimental design can subsequently be arranged in cell lines study to characterize whether there are any ameliorative effects originated from the bacterial isolate(s) at the cellular and molecular levels. The effects of selected probiotics on animal models of either SPF (specific pathogens free) or GF (germ free) mice will then be used for *in vivo* efficacy evaluation. These studies are basically performed for proof of concept. After completion of these studies, the functions, safety and molecular mechanisms of the probiotic(s) in animals will be addressed, followed by clinical human studies. Currently the United States FDA (food and drug administration) has started to initiate a program of LBP (live biotherapeutic products) to specifically regulate the application, clinical trial and commercialization of emerging new probiotics [24]. It is expected that in near future the NGP will start to come to reality in health bio-industry.

5. Candidates for next generation probiotics

Through analyses using the next generation sequencing and bioinformatics platforms, many potential NGP are currently under intensive development (Table 1). Some of them are selectively described below.

5.1. Bifidobacterium spp.

Bifidobacterium spp. are Gram-positive, non-spore-forming, nonmotile, and polymorphic rod bacteria that belong to the family Bifidobacteriaceae of the Actinobacteria class and phylum [25]. They are generally reconed as traditional probiotics. Bifidobacterium comprises >1% of the total microbiota, and are numbered at peak by the age of 3–4 months in infancy, followed by decreasing over time in adults [26,27].

Besides the many traditional beneficial effects reported, the efficacy of *Bifidobacterium spp*. on the outcomes of anticancer therapies is the subject of extensive ongoing research. Substantially reduced tumor growth rates and improved responses to anti-PD-L1 therapy were observed in mice [28]. Significantly, a probiotic cocktail consisting of *B. breve* and *B. longum* potentiates the antitumour efficacy of anti-PD-L1 immunotherapy [28]. The increased efficacy was, at least in part, mediated by the activation of DCs, leading to enhanced CD8+ T cell priming and accumulation within the tumor microenvironment (TME) [28]. On top of these, *Bifidobacterium spp*. as probiotics also gained much attention on cancer development and therapy against colitis and CRC [29]. In a rat model of azoxymethane (AOM)/DSS-induced CRC, a synbiotic (prebiotic and probiotic) comprising resistant starch and *Bifidobacterium animalis subsp. lactis* (*B. lactis*) showed

Table 1 – The NGP under intensive development.

Next generation probiotics	Main characterizations	Functions and mechanisms	Potential weakness	references
Bifidobacterium spp.	Some Bifidobacterium species strains may enhance the efficacy of Immune Checkpoint Inhibitors cancer therapy.	Enhance DC and $CD8^+$T cells functions.	The anti-cancer effects may be strain specific.	[23, 26, 27]
Prevotella copri	Ameliorate prediabetes syndromes.	Production of succinate, a TCA cycle intermediate.	Production of branch chain amino acids (BCAA) that may cause insulin resistance.	[33, 34]
Akkermansia muciniphila	Anti-obsogenicity and metabolic syndromes.	An outer membrane protein Amuc_1100 is reported to be responsible.	Positive association with Parkinson disease and multiple sclerosis.	[36–41]
Bacteroides fragilis	Anti-inflammations. Also may enhance efficacy of immune check point inhibitors cancer therapy.	A capsular polysaccharide PSA may enrich CD4+FoxP3 T cells after plasmacytoid DC cells presentation.	Enterotoxin containing *B. fragilis* is closely related to colorectal cancer development.	[42, 45–47]
Christensenella minuta	Anti-obsogenicity. Highly heritable in a lean host phenotype.	Unknown.	Not applicable.	[48, 49]
Faecalibacterium prausnitzii	Anti-inflammation. May ameliorate IBD and CRC.	Butyrate production.	Not applicable.	[53–55]
Parabacteroides goldsteinii	Anti-obsogenicity. Ameliorates prediabetes syndromes and liver inflammations.	Enhanced production of Treg and IL-10.	Not applicable.	[10, 56]

ameliorative effects on CRC development [30]. Furthermore, in patients with resected CRC, administration of a combination of inulin enriched with oligofructose, *B. lactis*, and *Lactobacillus rhamnosus* improved immune function [31]. In another study, CD8+ T cell memory responses against *B. longum* are positively associated with disease-free survival after tumor resection in patients with hepatocellular carcinoma [32].

Interestingly, *Bifidobacterium spp.* may also act as a vehicle for transporting anticancer genes to a target tumor [33]. *Bifidobacterium spp.* are shown to enter the bloodstream and can selectively accumulate in tumor mass due to the hypoxic condition in tumor. Take the *B. adolescentis* as an example, the underlying anti-tumor mechanism may lie in that *B. adolescentis* transformed with an antiangiogenic gene encoding endostatin can lead to bacterial proliferation within the tumor mass [34]. However, this does not occur in nonmalignant tissues. What's more, intratumoral expression of the endostatin can also inhibit tumor growth [34]. Further studies aiming at better understanding the mechanism of this association are warranted.

5.2. *Prevotella copri*

As a potential next generation probiotic, the *P. copri* belonging to the phylum Bacteroidetes was found to improve aberrant glucose tolerance syndromes and enhance hepatic glycogen storage in animals via the production of succinate, a tricarboxylic acid (TCA) cycle intermediate fermented from uptake of dietary fibers, that is responsible for glucose homeostasis through modulating intestinal gluconeogenesis [35].

Though *P. copri* shows beneficial effects on amelioration of prediabetic syndromes, however, recent study also showed that *P. copri* and *Bacteroides vulgatus* exacerbate glucose tolerance and enhance insulin resistance which occur before the development of ischaemic cardiovascular disease and type 2 diabetes. The underlying reason is related to synthesis of branch chain fatty acids (BCAA) [36]. In this study from 277 non-diabetic Danish individuals, the serum levels of BCAAs were observed to increase in insulin-resistant, prediabetes individuals. This phenomenon was further shown to be closely related to increased abundance of *P. copri* and *B. vulgatus* in a mouse study model [36]. Generally, whether *P. copri* works as a beneficial or deleterious bacterium requires further investigation. These phenomena strongly indicated that a careful analysis and demonstration of causality are essential before making functional conclusions of some microbes.

5.3. *Akkermansia muciniphila*

A. muciniphila belonging to the phylum Verrucomicrobia is another potential key probiotic candidate. This bacterium occupies up to 5% of the total microbiota bacteria and utilizes mucin as a nutrient for proliferation [37]. Numerous studies have shown that the composition of the gut microbiota differs between obese/T2D individuals and those of lean/non-diabetic ones. Currently it is widely accepted that administration of prebiotics such as the inulin-type fructans increases the abundance of *A. muciniphila*, leading to improvement of metabolic disorders and obesity [38]. The underlying mechanism by which *A. muciniphila* works is subsequently identified

to be through an immunomodulatory protein 'Amuc_1100' located in the bacterial outer membrane [39]. Other studies show *A. muciniphila* also modulates the endocannabinoid (eCB) system [40]. This is an important regulatory system in the aspect of targeting obesity, type 2 diabetes and inflammation. The eCB system is reported to involve the control of glucose and energy metabolism [40].

A. muciniphila may also involve anticancer immunotherapy such as anti-PD-1 treatment [41]. Besides clinical correlation, using the strategy of transferring of the faeces microbiota from responders or non-responders to germ free mice showed that *A. muciniphila* improved the compromised efficacy of anti-PD-1 blockade in contrast to the mice receiving the microbiota from non-responders [41]. How and why *A. muciniphila* shows such effects remain uncharacterized.

Even though *A. muciniphila* shows many beneficial effects, however, some other studies in animals reported a reverse situation on an increased abundance of *A. muciniphila* in the HFD mice [42] and the diseases multiple sclerosis (MS) and Parkinson's disease [43]. More studies on ameliorative effects of *A. muciniphila* are necessary.

5.4. *Bacteroides fragilis*

B. fragilis is another emerging probiotic [44]. As a prominent species of the genus Bacteroides in the phylum Bacteroidetes, *B. fragilis* is a Gram-negative absolute anaerobic commensal bacteria and occupies about 1% of the intestinal microbiota in humans [45]. Previous studies already showed that enterotoxin-containing *B. fragilis* strains (ETBF) significantly involve the abdominal infections and cause clinical abscesses arising from fecal spillage or bacteremia [46]. Although ETBF significantly contributes to intra-abdominal abscess formation, soft tissue infections, and bacteremia when present outside the gut, interestingly, *B. fragilis* strains that do not contain the enterotoxin gene show many beneficial effects [47].

After decades of studies using *B. fragilis* as a model organism, the research results from Dennis Kasper's group in Harvard University have highlighted the *B. fragilis* polysaccharide A (PSA) as an archetypical bacterial capsular polysaccharide example directing microbiota and host interactions [47]. PSA is rather unique due to its possessing zwitterionic motifs which enrich a specific subset of anti-inflammatory memory CD4+FoxP3 T cells after direct interactions of antigen presenting cells (APC) such as plasmacytoid dendritic Cells [48]. This can result in a systematic amelioration of inflammation related diseases such as abscess, neuro-inflammations and cancers [49].

5.5. *Christensenella minuta*

C. minuta also shows potential probiotic effects against obesity and associated metabolic disorders [50]. *C. minuta* is a Gram-positive, nonspore-forming, anaerobic probiotic candidate that shows the effects of reducing obesity and related syndromes [50]. Using the microbiota of more than 1000 fecal samples obtained from the UK twin pairs population, the family Christensenellaceae was found to form a co-occurrence network with other heritable bacteria [51]. The abundance of Christensenellaceae was found to enrich in individuals of low body mass index (BMI) [51]. Further study indicated that an obesity-associated microbiome can be ameliorated by *C. minuta* which amends the body weight gain and alters the microbiome pattern of recipient mice [50].

5.6. *Faecalibacterium prausnitzii*

F. prausnitzii is a Gram-positive bacterium belonging to the family Ruminococcaceae (the Clostridia class and the Firmicutes phylum). It is the only known species of the Faecalibacterium genus, and occupies about 5% of the gut microbiota in healthy adult humans [52]. *F. prausnitzii* ferments glucose, and produces SCFAs such as butyrate, formic acid, and D-lactate [53]. Due to production of butyrate, the intestinal homeostasis and integrity, and thus health are maintained [54].

Increasing evidences indicate that bacteria of the *Faecalibacterium* genus can influence the efficacy of immune checkpoint blockade (ICB) therapy [55]. Furthermore, there is positively correlation between the abundance of *Faecalibacterium spp.* and longer progression-free survival (PFS) durations. Importantly, strong evidences showed positive correlation between the abundance of *Faecalibacterium spp.* in the gut and CD8+ T cell infiltration within the tumor environment (TME), in addition to the frequency of effector CD4+ and CD8+ T cells in the periphery [56]. Further confirmation of the ability of *F. prausnitzii* to modulate responses to ICB therapy is warranted [57].

On top, an enrichment of this bacterium in baseline stool samples from patients with metastatic melanoma who respond to anti-CTLA-4 antibody therapy in comparison to the nonresponders was observed [57]. At the same time, the responders also showed a higher incidence of immune-related colitis [57]. This enrichment was positively correlated with expression of inducible T cell costimulator (ICOS) on the surface of circulating effector CD4+ T cells and negatively correlated with the numbers of regulatory T cells and the levels of pro-inflammatory proteins, such as IL-6, IL-8, and soluble IL-2 receptor-α (IL-2Rα), in the blood at baseline [57]. In brief, these findings highlight the tremendous potential of this bacterium as both a therapeutic target and a prognostic marker in patients with cancer.

5.7. *Parabacteroides goldsteinii*

Novel anti-obesity measures that are safe, effective and widely available are needed to combat the growing obesity epidemic. The bacterium *P. goldsteinii* also shows its potential as a novel probiotic for anti-obesity [10,58]. We recently reported that a water extract of the medicinal mushroom *Hirsutella sinensis* reduces obesity, inflammation and insulin resistance in high-fat diet (HFD)-fed mice. A high molecular weight polysaccharide fraction (>300 kDa) isolated from the water extract not only lowers body weight by 50% but it also reduces intestinal permeability, metabolic endotoxemia, inflammation and insulin resistance. Horizontal fecal transfer combined with antibiotic-induced depletion of specific gut bacteria shows that the effects of *H. sinensis* polysaccharides are dependent on neomycin-sensitive bacteria. Gut microbiota analysis reveals that the Gram-negative bacterium *P.*

goldsteinii is highly reduced in the microbiota of HFD-fed mice, while this bacterium is enriched in polysaccharide-treated mice. Notably, oral administration of live, but not heat-killed *P. goldsteinii* bacteria to HFD-fed mice considerably reduces weight gain and obesity-associated metabolic disorders. These results indicate that the anti-obesity, anti-inflammatory and insulin-sensitizing effects of mushroom polysaccharides are mediated by the gut microbiota and involve the newly identified probiotic *P. goldsteinii*. Mushroom polysaccharides and *P. goldsteinii* may thus be used respectively as prebiotics and probiotics for the treatment of obesity and related metabolic complications [10,56].

6. Immune checkpoints blockade (ICB) therapy and other potential candidates

A few other bacterial species also showed promising effects on promotion of the effectiveness of anticancer immunotherapies. These bacteria include *Eubacterium limosum*, *Enterococcus hirae*, *Enterococcus faecium*, *Collinsella aerofaciens*, and *Burkholderia cepacia*.

E. limosum belongs to the Class Clostridia, the order Clostridiales, and the phylum Firmicutes, and is an anaerobic, non-spore-forming, Gram-positive rod that is particularly prevalent in aged humans [59]. This bacterium shows effects on ameliorating DSS-induced colitis in mice, probably due to production of SCFAs to present anti-inflammation effects [59]. On top, the intestinal abundance of *E. limosum* was found to be related to a reduced risk of disease relapse and progression after allo-hematopoietic stem cell transplantation (HSCT) treatment in patients with haematological malignancies [60]. The underlying mechanisms of *E. limosum* to show the favorable outcome after allo-HSCT remains to be further characterized.

E. hirae belongs to Enterococcaceae and are grouped under the class of Bacilli and the phylum Firmicutes. It was found to be more abundant in faeces from responder patients with various carcinomas to PD-1—PD-L1 blockade than in samples from non-responders [61]. The potential amelioratived mechanism may be that *E. hirae* 13144 administration enhances the memory responses of T cells [62]. This was also closely related to progression-free survival (PFS) in patients with non-small cancer lung carcinoma (NSCLC) or ovarian cancer treated with immunogenic chemotherapy [61]. *E. hirae* was reported to translocate from the intestinal lumen to mesenteric lymph nodes, especially in the context of cyclophosphamide-based chemotherapy [62]. This may result in enhancement of T cells activity. However, *E. hirae* is also reported to cause clinical infections in humans [63]. Thus the roles of *E. hirae* in human health may be dual swords. One has to be careful in dealing with this bacterium.

In the aspect of clinical melanoma ICB study, the abundance of three bacterial species, *B. longum*, *C. aerofaciens*, and *E. faecium*, were found to increase in faeces from stool samples of responders to anti-PD-1 antibody therapy than those from non-responders [64]. Importantly, *E. faecium* was also found to be significantly increased in gut microbiota of responder patients with various carcinomas to PD-1 ICB therapy [65]. Even so, there is need of more independent cohort study to validate the positive association of *C. aerofaciens* with the outcomes of ICB. What's more, *E. faecium* is also frequently reported to be associated with nosocomial outbreaks and infections [66]. Again, one has to be cautious in management of this bacterium.

B. cepacia belongs to the Family Burkholderiaceae, the class betaproteobacteria, and the phylum Proteobacteria. It is a ubiquitous Gram-negative opportunistic pathogens often associated with nosocomial infections in immunocompromised patients [67]. Even so, in the aspect of anticancer immunotherapy, *B. cepacia* alone or in combination with B. fragilis can potentiate the efficacy and tolerability of CTLA-4 blockade in antibiotic-treated mice via the stimulation of TH1 cell immune responses [68].

7. Conclusion and perspectives

Although there are many studies, more works are needed to go beyond the identified association between healthy and disease state gut microbiota. Multiomics and longitudinal studies are needed to finally clarify the functions of the emerging probiotics.

As mentioned in the text, several faecal bacteria particularly enriched in clinically healthy individuals, in contrast to the abundances in various diseases groups — are associated with clinical responses to ICB (*A. muciniphila*, *F. prausnitzii*, *Bifidobacterium spp.*, and *B. fragilis*). The underlying reason may lie in that the abundance of these health-associated bacteria might reflect the presence of a well-equilibrated intestinal microflora, leading to a homeostatic host—microbiota ecosystem associated with good health. The other possibility is that reduced number of these NGP candidates may be directly responsible for the aberrant 'immune set point'. On the other hand, whether a single bacterial strain is enough to achieve such ameliorative effects, or a consortia are needed to achieve the effects of live bacterial biotherapeutics remain to be further addressed. On the other hand, is addition of prebiotics essential to significantly enhance probiotics effects? Future studies must discriminate between these possibilities using preclinical models and/or clinical trials.

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