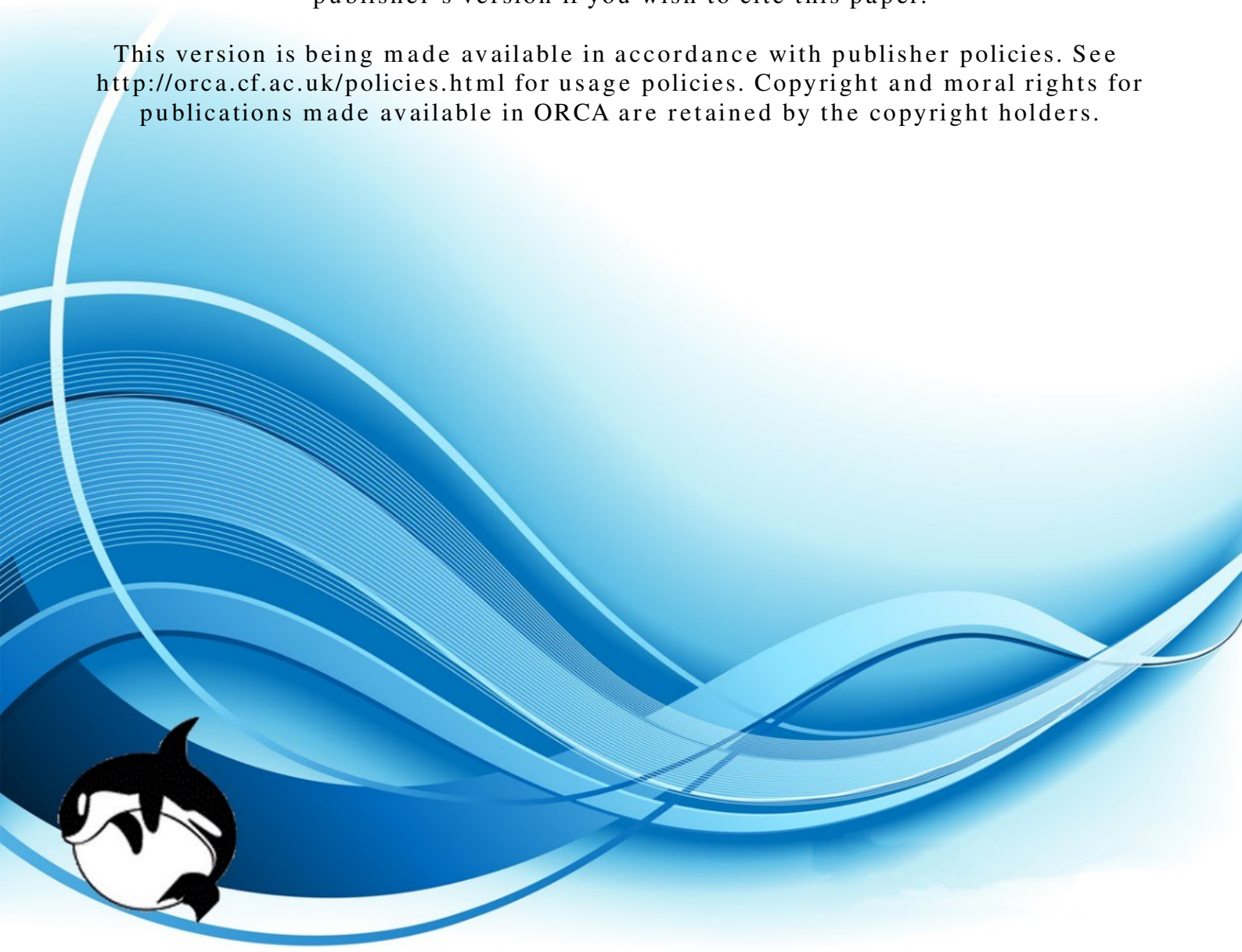

Next Generation Probiotics; transitioning from probiotics to Live Biotherapeutics

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Abstract

The leading probiotics currently available to consumers are generally drawn from a narrow range of organisms. Knowledge of the gut microbiota and its constituent actors is changing this paradigm, particularly given the phylogenetic range and relatively unknown characteristics of the organisms under investigation as novel therapeutics. For this reason, and because their development is likely to be more amenable to a pharmaceutical than a food delivery route, these organisms are often operationally referred to as Next Generation Probiotics, a concept which overlaps with the newly emerging concept of Live Biotherapeutic Products. The latter is a class of organisms developed exclusively for pharmaceutical application. In this perspective we discuss what lessons have been learned from working with traditional probiotics, explore the kinds of organisms likely to be used as novel microbial therapeutics, discuss the regulatory framework required, and propose how scientists may meet this challenge.

Introduction

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host"[1]. Probiotics have a centuries-long history of safe use (Fig. 1) but have only been recognised as being of economic value during the 20th century. The global probiotics market is projected to reach a turn-over value of USD$46.55 billion by 2020 (http://www.marketsandmarkets.com/PressReleases/probiotics.asp), and is dominated by food companies, nutritional supplement companies, and dedicated probiotic production companies. The probiotic organisms that feature in these products have been mainly sourced from the gut or from traditional fermented foods such as pickles, yoghurts, and kefir grains. Thus the majority of the probiotics sold and used both in probiotic research and commercial probiotic development are from a limited list of genera, which mainly include *Lactobacillus* spp. and *Bifidobacterium* spp. The more commonly exploited strains/species among the lactobacilli and bifidobacteria have been accepted as having Generally Regarded as Safe (GRAS) status in the United States (http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices) or have been granted Qualified Presumption of Safety status by the European Food Safety Authority (EFSA)[2]. Other probiotics currently available in the marketplace include *Saccharomyces*, *Bacillus* spp., *Escherichia coli*, enterococci and *Weissella* spp. We consider it likely that these organisms will continue to be developed and regulated under the current mechanisms for probiotics rather than the novel pathways discussed below.

With the development of better culturing methodologies, more affordable genome and metagenome sequencing and more powerful tools to edit and modify bacterial genomes, we are now on the cusp of a new era in probiotic research, one which allows us to develop bespoke probiotics that address specific consumer needs and issues. The knowledge of the composition and function of the human gut microbiome, also accelerated by massively parallel sequencing, has dramatically extended the range of organisms with potential health benefits, although many of these are still at the very early stage of mechanistic investigation (Table 1). These organisms are sometimes referred to as "Next Generation Probiotics" but may also be termed "Live Biotherapeutic Products" (LBPs[3]) in the context of a new regulatory framework in the USA (see below). Both academic and industry scientists are faced by a set of challenges which partly mirror those faced in recent decades by those engaged in probiotic research, but which have additional distinguishing issues that may facilitate or complicate their commercial development. There are many other candidate therapeutic organisms in various phases of development in the burgeoning microbiome-based biopharma sector but Table 1 entries are restricted to selected examples that have been published, and preferably tested in humans.

Expanding this parsimonious list will require completion of pre-clinical safety trials, and safety and efficacy trials in humans.

What is a Next Generation Probiotic?

Next Generation Probiotics (NGPs) obviously conform to the normal definition of a probiotic, but in this discussion we are primarily referring to those microbes which have not been used to date as agents to promote health, and which are more likely to be delivered under a drug regulatory framework (Fig. 2). NGPs also fit well within the US Food and Drug Administration definition of Live Biotherapeutic Product: "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."

(http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/General/UCM292704.pdf).

Given that the term LBP is now a formally recognised concept, at least in the USA, one may reasonably question if a term such as NGP is necessary at all. We suggest that at this juncture that classifying certain microbes as NGPs can serve a useful purpose, in that the term emphasises that they differ from traditional probiotics in how they are likely to be viewed by regulators, and recognises the likelihood that NGPs will also include genetically modified microorganisms (GMMs). Probiotics have been largely included in food delivery vehicles or as supplements, marketed and regulated as foods or functional foods, and are clearly positioned in consumer perception a long way from the controversial issue of GMMs or Genetically Modified Food. Since the likely route to market for LBPs and NGPs will follow a path marked by studies of preclinical mode of action, safety, pharmacokinetics, pharmacodynamics, phase 1-3 trials, accompanied by passing appropriately timed regulatory approval hurdles (see below), it seems that referring to these organisms as simply "probiotics" will generate confusion rather than clarity, to scientists and consumers alike.

It is also worth considering if both terms NGP and LPB are different and necessary. The differences are mainly but not exclusively operational ones; NGPs tend to be investigated by laboratories previously engaged in probiotic and microbiome research and often have a development trajectory based on the probiotic experience in the laboratory; LBPs tend to be investigated by start-up biotechnology companies or pharmaceutical companies with the expressed intention of seeking approval for pharmaceutical marketing. GM probiotics arguably span both label domains, with there being a reasonable case that calling them LBPs rather than NGPs is less likely to erode consumer confidence

that probiotics are simple unmodified organisms. We suggest that NGP is a reasonable attempt to mark the transition from traditional microbes with long histories of safe use, to untried microbes with no such historical acceptance. In time, we believe that the term NGP will disappear and its members will either merge with current probiotics or will take a pharmaceutical route to market, in which case they would be developed as LBPs.

Examples of current NGP candidates

A scan of the primary literature for the period of 2000-2016 using the term "probiotic*" reveals 16,064 articles, 9,811 of which contain the word *Lactobacillus* and 3,463 *Bifidobacterium*, either in the title or abstract. The majority of papers that mentioned non-canonical probiotic genera, for example *Clostridium* or *Bacteroides*, did so in the context of these genera being pathogenic strains to be modulated by the consumption of the probiotic, rather than as actual probiotics. Furthermore, any conflations of the term with other genera such as *Faecalibacterium* or *Akkermansia* were very rare. Where non lactobacilli or bifidobacterial probiotics were mentioned, it is evident that there are two strategies being employed to develop them as NGPs. As with current probiotics, one strategy involves associating the presence or absence of a specific strain with a health phenotype and exploring whether the chosen strain, when administered in sufficient quantities, can recapitulate the health phenotype. The second strategy is to adopt a well-characterised probiotic strain and use them as delivery vehicles for a specific molecule, again choosing the molecule to be delivered based on either a strong association or some mechanistic insight which shows that addition of the molecule would abrogate the disease phenotype and thus promote health.

The two most abundant families in the colon are *Bacteroidales* and *Clostridiales*. The former are being explored as potentially novel second-generation probiotics. For example, Deng and colleagues [4] isolated *B. fragilis* strain ZY-312 from the faeces of a healthy breastfed infant and proceeded to show that the organism possessed potentially health promoting phenotypes when incubated with colonocytes and macrophages. These phenotypes include the promotion of the production of microbicidal molecules and phagocytic functions in macrophages. However, these functions appear to be strain dependent; for example *B. fragilis* has been reported to make fragilysin [5,6] which has been implicated as a risk factor for developing colorectal cancer [7], which would not be a desirable trait in a next-generation probiotic. The bacterial polysaccharide, PSA, which was reported in 2005 [8] is another probiotic feature of *B. fragilis*. PSA is part of a larger family of zwitterionic polysaccharides (ZPS) and has been reported to play an immunomodulatory role, and depending on the type of polysaccharide, this can be either immunoregulatory or pro-inflammatory. These results show that it is important to

identify the strain being used because its health promoting features will be closely aligned to its evolutionary history, a feature which is also true for traditional probiotics.

Bacteroides xylanisolvens DSM 23964 has also been considered an NGP. It was isolated from human faeces, and does not encode the *Bacteroides fragilis* enterotoxin or produce PSA [9]. It has been shown to be tolerated in Phase I trials [9], and in a later study in humans the same team showed that the heat inactivated preparation of this organism was able to increase the levels of Thomsen-Friedenreich (TFα) specific IgM antibodies in a manner which was dose-dependent and time constrained [10]. The authors speculated that an increase in these antibodies would promote a more robust response to cancer and thus ameliorate the host's own cancer immune surveillance system [10]. However, by heat inactivating the organism they are effectively contravening what is one of the defining characteristics of probiotics; that it must be a living organism. Furthermore, the desired outcome, to prevent cancer, is a difficult one to prove, as it will require large cohorts prospectively studied over 20-30 years to assess efficacy. Other *Bacteroides* spp. have also been considered as potential NGPs; *Bacteroides dorei* D8, has been shown to convert cholesterol to coprostanol *in vitro*, and may be considered as a probiotic in the context of the cholesterol-CVD axis; *B. acidifaciens* has been shown to increase IgA in gnotobiotic mice mono-associated with the bacterium [11]and a strain of *B. ovatus*, when fed to mice, increased levels of anti-TFα IgM and IgG antibodies.

The other common genus found in the colon, *Clostridium*, has not yet been explored to the same extent as the *Bacteroides* species complex. One strain, *Clostridium butyricum* MIYAIRI 588 (CBM 588; also referred to as *C. butyricum* FERM BP-2789), has been studied for over 50 years, mainly in Asia. From the limited number of publications it appears that this organism has been used to treat *Clostridium difficile* infections [12], *Helicobacter pylori* infections [13], cholesterol levels [14,15] and cancer [16].

One of the most abundant species to be found in the large intestine is *Faecalibacterium prausnitzii*, which has been reported to be depleted in individuals with inflammatory bowel disease [17]. Therefore, it seems reasonable that if there was a causal link between disease status and the absence of this organism, then by simply feeding it to the individual its health promoting features should be restored and thus it may be considered an NGP. However, there is no evidence, either published or deposited at ClinicalTrials.gov, for this organism's efficacy as a probiotic to be able to reverse the symptoms of IBD when fed to humans. In animal models, evidence is available and feeding animals with *F. prausnitzii* does lead to or associate with induction of anti-inflammatory cytokines [18]or reduction of pro-inflammatory cytokines [19]in induced models of colitis/IBD.

An alternative route to developing some NGPs is to take GRAS organisms or commensals and use them as a delivery vehicle for a bioactive molecule. In this approach the bacterial "vehicle" is known not to

produce any virulence factors and will be tolerated by the host and if chosen carefully, may not even colonise the host. Two groups have used *Lactococcus lactis* strains (not normally considered to be probiotics) as their vehicle for delivering a range of anti-inflammatory molecules. *L. lactis* was engineered to deliver the serine protease inhibitor, elafin, and shown that in an animal model of colitis administration of the GMO reduced elastolytic activity and inflammation [20]. Another laboratory engineered *L. lactis* to deliver several different human molecules, most notably IL-10 [21] for controlling allergen sensitivity and Trefoil Factor 1 [22] to treat oral mucositis, with other examples being covered in more detail elsewhere [23]. While these approaches used a GRAS food-derived bacterium as their delivery vehicle, the common colonic bacterium *Bacteroides ovatus* has been employed as a host to express and produce either murine IL-2 [24], keratinocyte growth factor-2 (KGF-2) [25] or TGF-β1 [26], all under the control of a xylan inducible promoter, which was re-purposed from its original task of driving expression of the *B. ovatus* xylanase gene [27]. In one animal trial, TGF-β1-producing *B. ovatus* was administered to mice with DSS-colitis, and induced production of the TGF-β1 *in situ*, by inclusion of xylan in the drinking water. The authors concluded that this GMO was able to significantly improve the clinical scores and accelerate healing, and stated that the results "are comparable and most cases superior to that achieved by conventional steroid therapy" [27].

175 **Table 1. Selected examples of Next Generation Probiotics**

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Organism	Type	Disease Target	Level of Evidence	Study type	Ref
Bacteroides xylanisolvens DSM 23694	Natural (human)	Cancer	Medium: safety in humans has been established, while levels of TFα specific-IgM have been shown to be elevated in humans.	In human	[10]
B. ovatus D-6	Natural (human)	Cancer	Low to medium: increases levels of murine TFα specific-IgM and IgG.	Pre clinical in mice	[28]
B. ovatus V975	GMO (originally from human gut samples) expressing Human keratinocyte growth factor-2 (KGF-2)	Intestinal Inflammation	Medium: Shows abrogation of symptoms of DSS induced in murine colitis model.	Pre clinical in mice	[25]
B. ovatus V975	GMO expressing Human transforming growth factor-β1 (TGF-β1)	Intestinal inflammation	Medium: Shows abrogation of symptoms of DSS induced in murine colitis model.	Pre clinical in mice	[26]
B. dorei D8	Natural (human)	Heart disease	Low, depletion of cholesterol in vitro	Pre clinical *in vitro*	[29]
B. fragilis ZY-312	Natural (human)	Clearance of infectious agents	Low: data only in vitro.	Pre clinical *in vitro*	[4]
B. acidifaciens JCM 10556(T)	Natural (mouse)	Clearance of infectious agents	Low-medium: Increases IgA levels in the large intestine of gnotobiotic mice.	Pre clinical in mice	[11]
Clostridium butyricum MIYAIRI 588	Natural (human)	Multiple targets including cancer, inflammation and infectious agents	Low-Medium: Evidence gathered for claims in human and animals trials	In human	[12-16,30-42]
Faecalibacterium prausnitzii	Natural (human)	Mainly IBD, but also asthma, eczema and Type II diabetes	Low to Medium: Mainly focused animal models of colitis and in associative studies	Pre clinical in mice and *in vitro*	[18,43,44]
L. lactis::elafin	GMO (Host isolated from food)	Mainly inflammatory disease such as IBD	Medium: Good evidence from animal models of IBD	Pre clinical in mice	[20]
L. lactis:: Trefoil Factor 1 or IL-10	GMO (Host isolated from food)	Allergen sensitivity and autoimmune diseases – Type I Diabetes	Medium: Mainly animal based efficacy.	In humans Phase I trial	[23]

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Issues facing the development and marketing of NGPs and LBPs

Current EFSA and FDA positions on probiotics and LBPs

The existing regulatory positions for probiotics are not consistent across all jurisdictions, and so we will briefly summarise the current situation in the United States and the European Union. When considering regulatory positions on probiotics, it is important to recognize that probiotics can be utilized in a variety of different product types. Probiotics can be delivered in the form of conventional foods, infant formula, pet foods, dietary supplements, drugs, cosmetics and even medical devices[1]. The regulatory requirements and types of allowable claims for each of these products differ. Most probiotics today are components of either foods or dietary supplements.

In the European Union the responsible regulatory agency is the European Food Safety Authority (EFSA). The EFSA Panel on Dietetic Products, Nutrition and Allergies has evaluated over 400 probiotic applications, but has not reached a positive opinion on any health claims. Indeed, even the use of the term 'probiotic' has been effectively outlawed by an amendment which regulates the use of 'generic descriptors'[45]. It is not clear whether any NGPs would be subjected to any additional regulatory scrutiny, but any genetically modified microbes would also have to be approved by the EFSA Panel on Genetically Modified Organisms, while the authorisation of any microbe as a drug would have to be authorised by the European Medicines Agency.

In the United States, regulatory authorities do not use the term 'probiotic'. Even though precisely defined[1], they instead use the term live microbial ingredients, when referring to ingredients in foods or dietary supplements, or live biotherapeutic agents when referring to use as a drug. With regard to claims in the United States, claims that a product can diagnose, cure, mitigate, treat, or prevent disease are only allowed on drugs. Health benefit claims for foods or dietary supplements are of two types. The first type, an approved Health Claim, has not been used for probiotics. This claim relates to the ability of the food or supplement to reduce the risk of disease. This claim must be approved by the FDA or an authoritative body (such as the Institute of Medicine). The second type of claim is the structure/function claim. Such claims relate the probiotic to the normal structure and function of the healthy human body. Recently, in the context of infant formula, the FDA expressed the opinion in a draft guidance that such claims are acceptable on dietary supplements, but that such claims on foods must relate to the taste, aroma or nutritive function of the food[46].

Importantly to the context of development of NGPs, the FDA position on what constitutes a 'new dietary ingredient' must be considered. In August 2016, the FDA published a draft guidance on this

topic[47]. This draft contains the statement: "Bacteria that have never been consumed as food are unlikely to be dietary ingredients." In short, any probiotics on the market prior to the adoption of the dietary supplement regulations (Dietary Supplement Health and Education Act of 1994) in October 15, 1994 can be grandfathered in as a dietary supplement ingredient. However, the FDA does not provide a direct path to a dietary supplement for any novel probiotics. If an NGP is first marketed in food, it is considered a dietary ingredient, and then has a path to become a dietary supplement. This is a cumbersome, indirect pathway that will likely result in any microorganisms being developed instead as LBPs.

As stated earlier, the FDA Center for Biologic Evaluation and Research (CBER) defined a live biotherapeutic product (LBP) as '*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*'[48]. This would appear to be a very useful category which could be exploited for novel microbes 'mined' from the microbiota. CBER requires a very detailed characterisation of any microorganisms in this category, similar to that required for vaccines. LBPs would have to be produced to Good Manufacturing Practice (GMP) standards. CBER also allows for the development of recombinant LBPs, composed of microorganisms that have been genetically modified through the purposeful addition, deletion, or modification of genetic material. The path for conducting human research on LBPs is clear, though we know of no examples that have completed it yet. The Investigational New Drug (IND) process must be followed. Over past years, the FDA had considered essentially all probiotic research to be drug research. Under the auspices of the International Scientific Association for Probiotics and Prebiotics (ISAPP), several researchers challenged FDA on this position, demonstrating the negative impact it has had on the conduct of human research on probiotics in the United States as well as pointing out that such research on foods or dietary supplements is legal under U.S. law[49]. Recently, the FDA relaxed their position, seemingly to provide a path for human research on probiotic foods or dietary supplements without needing an Investigational New Drug (IND) approval[50].

While EFSA is the competent authority for legislating and oversight with regard to probiotics, The European Directorate for the Quality of Medicines (EDQM) enables the development, implementation and monitoring of the application of quality standards for safe medicines and their use (https://www.edqm.eu/en/EDQM-mission-values-604.html). The EDQM appointed a Live Biotherapeutic Products Working Party in 2014, to develop a monograph for Live Biotherapeutic Products (LBPs). The purpose of this monograph will be to harmonise quality standards for LBPs as biological medicinal products and it is expected to be enacted shortly.

What do proponents of LBPs need to demonstrate?

According to FDA regulations all LBP applications must include a 'description of the drug substance', to include the biological name and strain designations; the original source of cells from which the drug substance was derived; the culture/passage history of the strains; a description of the clinical health of the donor; a summary of the phenotype and genotype of the product strains; and documentation and summary of modifications, if any, to the LBP, e.g., intentional introduction of foreign genes or mutations, along with details of the genetic construction. These demands should be possible for most LBPs isolated from the microbiome, although providing a complete description of the precise culture/passage history of the strains may be challenging for strains isolated a number of years ago.

Complete 'characterisation' of an LBP must also be provided. This comprehensive list includes, *inter alia*, methods for detection and identification, antibiotic resistance, methods used and a justification for any genetic manipulation, and any support for a mechanism of action. The manufacturer must also provide a complete and comprehensive description of the manufacturing method and infrastructure, the materials used in the manufacturing process, and details of any other products produced in the same facility.

LBPs will be subjected to the normal IND requirements as would any other drug substance. Initial studies in humans will be concerned with safety, and so are likely to involve healthy volunteers to look for adverse events (see below).

Production challenges and scale-up

Many of the commercially successful probiotics that currently dominate the marketplace were selected in large part based on their technological robustness, by which is meant that they withstand the process of growth, enrichment, freeze-drying or product incorporation, and retain viability during product shelf-life. The *Bifidobacterium* and *Lactobacillus* species that form the mainstay of the commercial supply are anaerobic or microaerophilic organisms, but are much less sensitive to atmospheric oxygen than the strict anaerobes such as *Faecalibacterium prausnitzii*, *Akkermansia muciniphila* and others that are currently being explored as NGPs. Bacterial fermentation is, by definition, an anaerobic process, but nevertheless current production lines were not developed to allow harvesting viable bacterial cells with the complete exclusion of oxygen throughout. Even for the initial product development stage of supporting trials, fermentation of pilot cultures up to 100 litres is required to prepare inocula for large-scale fermentation in thousand-litre volumes. As a further

challenge, the whole process must be performed under GMP conditions that are regulated and inspected at national level in EU member states. Following fermentation, the microbial cell biomass requires (typically) to be free-dried, again under strictly anaerobic conditions, followed by microbial quality control steps (microbial purity, viable cell counts). If being encapsulated, the freeze-dried material must be milled into an homogenous powder that is tested for galenic properties (powder characterization, disintegration, dissolution properties). Finally, the powder must be encapsulated in the absence of oxygen but also with very low water content, with or without excipients or other agents, typically based on pilot data from intestinal transit studies used to determine how to optimize viability. This chain of technological stages presents a significant challenge to the large number of start-up companies aiming to develop novel therapeutics based on anaerobic gut commensals (reviewed in ref.[51])

Conclusions and Action Required

The term probiotic is not a taxonomic one, but refers to functionality. Nothing in the definition of the term limits the species, genus or even Kingdom from which probiotics can be selected, nor does it dictate whether they must be naive strains or whether they can have been subjected to any form of genetic manipulation. Why do we therefore feel the need to use the term 'Next Generation Probiotics'? We believe that it is highly likely that in the near future the enormous amount of research on the beneficial impact of the microbiome on human health will lead to the discovery and development of novel microorganisms derived from our microbial symbionts. In many cases these may belong to unusual and formerly 'uncharacterised' microorganisms with unusual properties, or perhaps may even be microorganisms formerly thought of as pathogens or pathobionts. These developments will present significant challenges for scientific research, for industrial exploitation and for regulatory agencies. For the moment the term NGP can serve as a useful descriptor for these 'non-traditional' microbes. Other human commensals developed and approved through a pharmaceutical route for curing disease or alleviating symptoms will likely retain the LBP moniker. The success of faecal microbiota transplantation (FMT) for curing diarrhoea associated with recurrent *Clostridium difficile* infection [52] has provided a conceptual framework for isolating organisms or consortia that might improve diseases associated with gut microbiota alteration [53]. These could include GMMs, bacterial spores, or bacteriophages, that would also be more readily developed as LBPs.

A suggested development pathway for these products is summarized graphically in Fig. 3. The most challenging initial task is to identify a candidate LBP. Hypothesis-based approaches to this

include identifying organisms whose relative abundance levels are depleted in subjects with a condition associated with an altered microbiome; organisms that are associated with successful FMT treatment of a particular condition; organisms already known to modulate the microbiome composition or function; organisms known to influence a particular host pathway or phenotype relevant to a particular disease. Alternatively, one may screen a bank of strains for a desired *in vitro* or *in vivo* activity.

The next phase is to characterize the LBP, initially by genome sequencing to screen for transmissible antibiotic resistance genes, and presumptive virulence factors such as toxins. Unless already performed during candidate LBP screening, trials in enzyme assays, cell models, animal models or *ex vivo* models are required to confirm phenotype related to the desired LBP effect. Depending on strain identity and any safety information for that species or closely related species, safety and toxicity in animal models may require additional focus.

The production phase should have already been scoped out so that pilot scale, defined medium, conditions have been established for rapid GMP scale-up. Establishment of an effective formulation for delivery will include confirmation of LBP survival and bioavailability upon ingestion. GMP product approval will be required so that production of batches for human trials may commence.

Finally, a typical series of pharmaceutical clinical trials will be implemented. Phase 1 will, for many LBPs, be a *First in Human* trial and will establish safety, and examine dosage ranges. Phase 2 will revolve around the primary endpoint expected for the LBP, in small group sizes. Phase 3 will examine efficacy, side effects, and relative benefits in larger group.

Accompanying all of these milestones will be achieving deliverables relevant to seeking regulatory approval by CBER, EDQM or relevant competent authority. These agencies should (continue to) engage with relevant stakeholders, especially as legislation is being developed, so that all parties have a clear understanding of precisely what documentation is required for approval of LBPs for commercial sale.

Figure Legends

Figure 1. Time-line of selected milestones in the history of probiotics and next-generation probiotics.

Figure 2. Schematic diagram summarizing some differences in the history and route to market of probiotics, next-generation probiotics, and Live Biotherapeutic Products.

Figure 3. Graphical summary of the pathway to regulatory approval for Live Biotherapeutic products.

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