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Citation for final published version:

O'Toole, Paul W., Marchesi, Julian Roberto and Hill, Colin 2017. Next-generation probiotics: the spectrum from probiotics to live biotherapeutics. *Nature Microbiology* 2 (5) , 17057. 10.1038/nmicrobiol.2017.57 file

Publishers page: <http://dx.doi.org/10.1038/nmicrobiol.2017.57>
<<http://dx.doi.org/10.1038/nmicrobiol.2017.57>>

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1 **Next Generation Probiotics; transitioning from probiotics to Live**
2 **Biotherapeutics**

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13

14 **Abstract**

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

26

27 Introduction

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

46 With the development of better culturing methodologies, more affordable genome and
47 metagenome sequencing and more powerful tools to edit and modify bacterial genomes, we are now
48 on the cusp of a new era in probiotic research, one which allows us to develop bespoke probiotics that
49 address specific consumer needs and issues. The knowledge of the composition and function of the
50 human gut microbiome, also accelerated by massively parallel sequencing, has dramatically extended
51 the range of organisms with potential health benefits, although many of these are still at the very early
52 stage of mechanistic investigation (Table 1). These organisms are sometimes referred to as “Next
53 Generation Probiotics” but may also be termed “Live Biotherapeutic Products” (LBPs³) in the context
54 of a new regulatory framework in the USA (see below). Both academic and industry scientists are
55 faced by a set of challenges which partly mirror those faced in recent decades by those engaged in
56 probiotic research, but which have additional distinguishing issues that may facilitate or complicate
57 their commercial development. There are many other candidate therapeutic organisms in various
58 phases of development in the burgeoning microbiome-based biopharma sector but Table 1 entries
59 are restricted to selected examples that have been published, and preferably tested in humans.

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

62

63 **What is a Next Generation Probiotic?**

64 Next Generation Probiotics (NGPs) obviously conform to the normal definition of a probiotic, but in
65 this discussion we are primarily referring to those microbes which have not been used to date as
66 agents to promote health, and which are more likely to be delivered under a drug regulatory
67 framework (Fig. 2). NGPs also fit well within the US Food and Drug Administration definition of Live
68 Biotherapeutic Product: “a biological product that: 1) contains live organisms, such as bacteria; 2) is
69 applicable to the prevention, treatment, or cure of a disease or condition of human beings; and 3) is
70 not a vaccine.”

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

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

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

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

97 **Examples of current NGP candidates**

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

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

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

126 *Bacteroides xyloxylosum* DSM 23964 has also been considered an NGP. It was isolated from human
127 faeces, and does not encode the *Bacteroides fragilis* enterotoxin or produce PSA⁹. It has been shown
128 to be tolerated in Phase I trials⁹, and in a later study in humans the same team showed that the heat
129 inactivated preparation of this organism was able to increase the levels of Thomsen-Friedenreich (TF α)
130 specific IgM antibodies in a manner which was dose-dependent and time constrained¹⁰. The authors
131 speculated that an increase in these antibodies would promote a more robust response to cancer and
132 thus ameliorate the host's own cancer immune surveillance system¹⁰. However, by heat inactivating
133 the organism they are effectively contravening what is one of the defining characteristics of probiotics;
134 that it must be a living organism. Furthermore, the desired outcome, to prevent cancer, is a difficult
135 one to prove, as it will require large cohorts prospectively studied over 20-30 years to assess efficacy.
136 Other *Bacteroides* spp. have also been considered as potential NGPs; *Bacteroides dorei* D8, has been
137 shown to convert cholesterol to coprostanol *in vitro*, and may be considered as a probiotic in the
138 context of the cholesterol-CVD axis; *B. acidifaciens* has been shown to increase IgA in gnotobiotic mice
139 mono-associated with the bacterium¹¹ and a strain of *B. ovatus*, when fed to mice, increased levels of
140 anti-TF α IgM and IgG antibodies.

141 The other common genus found in the colon, *Clostridium*, has not yet been explored to the same
142 extent as the *Bacteroides* species complex. One strain, *Clostridium butyricum* MIYAIRI 588 (CBM 588;
143 also referred to as *C. butyricum* FERM BP-2789), has been studied for over 50 years, mainly in Asia.
144 From the limited number of publications it appears that this organism has been used to treat
145 *Clostridium difficile* infections¹², *Helicobacter pylori* infections¹³, cholesterol levels^{14,15} and cancer¹⁶.

146 One of the most abundant species to be found in the large intestine is *Faecalibacterium prausnitzii*,
147 which has been reported to be depleted in individuals with inflammatory bowel disease¹⁷. Therefore,
148 it seems reasonable that if there was a causal link between disease status and the absence of this
149 organism, then by simply feeding it to the individual its health promoting features should be restored
150 and thus it may be considered an NGP. However, there is no evidence, either published or deposited
151 at ClinicalTrials.gov, for this organism's efficacy as a probiotic to be able to reverse the symptoms of
152 IBD when fed to humans. In animal models, evidence is available and feeding animals with *F.*
153 *prausnitzii* does lead to or associate with induction of anti-inflammatory cytokines¹⁸ or reduction of
154 pro-inflammatory cytokines¹⁹ in induced models of colitis/IBD.

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

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

173

174

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

176

177

Organism	Type	Disease Target	Level of Evidence	Study type	Ref
<i>Bacteroides xylanisolvens</i> DSM 23694	Natural (human)	Cancer	Medium: safety in humans has been established, while levels of T α specific-IgM have been shown to be elevated in humans.	In human	10
<i>B. ovatus</i> D-6	Natural (human)	Cancer	Low to medium: increases levels of murine T α specific-IgM and IgG.	Pre clinical in mice	28
<i>B. ovatus</i> 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
<i>B. ovatus</i> V975	GMO expressing Human transforming growth factor- β 1 (TGF- β 1)	Intestinal inflammation	Medium: Shows abrogation of symptoms of DSS induced in murine colitis model.	Pre clinical in mice	26
<i>B. dorei</i> D8	Natural (human)	Heart disease	Low, depletion of cholesterol in vitro	Pre clinical <i>in vitro</i>	29
<i>B. fragilis</i> ZY-312	Natural (human)	Clearance of infectious agents	Low: data only in vitro.	Pre clinical <i>in vitro</i>	4
<i>B. acidifaciens</i> 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
<i>Clostridium butyricum</i> 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
<i>Faecalibacterium prausnitzii</i>	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 <i>in vitro</i>	18,43,44
<i>L. lactis</i> ::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
<i>L. lactis</i> :: 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

178

179

180

181 **Issues facing the development and marketing of NGPs and LBPs**

182

183 *Current EFSA and FDA positions on probiotics and LBPs*

184 The existing regulatory positions for probiotics are not consistent across all jurisdictions, and so we
185 will briefly summarise the current situation in the United States and the European Union. When
186 considering regulatory positions on probiotics, it is important to recognize that probiotics can be
187 utilized in a variety of different product types. Probiotics can be delivered in the form of conventional
188 foods, infant formula, pet foods, dietary supplements, drugs, cosmetics and even medical devices¹.
189 The regulatory requirements and types of allowable claims for each of these products differ. Most
190 probiotics today are components of either foods or dietary supplements.

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

199 In the United States, regulatory authorities do not use the term ‘probiotic’. Even though precisely
200 defined¹, they instead use the term live microbial ingredients, when referring to ingredients in foods
201 or dietary supplements, or live biotherapeutic agents when referring to use as a drug. With regard to
202 claims in the United States, claims that a product can diagnose, cure, mitigate, treat, or prevent
203 disease are only allowed on drugs. Health benefit claims for foods or dietary supplements are of two
204 types. The first type, an approved Health Claim, has not been used for probiotics. This claim relates to
205 the ability of the food or supplement to reduce the risk of disease. This claim must be approved by
206 the FDA or an authoritative body (such as the Institute of Medicine). The second type of claim is the
207 structure/function claim. Such claims relate the probiotic to the normal structure and function of the
208 healthy human body. Recently, in the context of infant formula, the FDA expressed the opinion in a
209 draft guidance that such claims are acceptable on dietary supplements, but that such claims on foods
210 must relate to the taste, aroma or nutritive function of the food⁴⁶.

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

213 topic⁴⁷. This draft contains the statement: “Bacteria that have never been consumed as food are
214 unlikely to be dietary ingredients.” In short, any probiotics on the market prior to the adoption of the
215 dietary supplement regulations (Dietary Supplement Health and Education Act of 1994) in October 15,
216 1994 can be grandfathered in as a dietary supplement ingredient. However, the FDA does not provide
217 a direct path to a dietary supplement for any novel probiotics. If an NGP is first marketed in food, it is
218 considered a dietary ingredient, and then has a path to become a dietary supplement. This is a
219 cumbersome, indirect pathway that will likely result in any microorganisms being developed instead
220 as LBPs.

221 As stated earlier, the FDA Center for Biologic Evaluation and Research (CBER) defined a live
222 biotherapeutic product (LBP) as ‘*a biological product that: 1) contains live organisms, such as bacteria;*
223 *2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and 3)*
224 *is not a vaccine*’⁴⁸. This would appear to be a very useful category which could be exploited for novel
225 microbes ‘mined’ from the microbiota. CBER requires a very detailed characterisation of any
226 microorganisms in this category, similar to that required for vaccines. LBPs would have to be produced
227 to Good Manufacturing Practice (GMP) standards. CBER also allows for the development of
228 recombinant LBPs, composed of microorganisms that have been genetically modified through the
229 purposeful addition, deletion, or modification of genetic material. The path for conducting human
230 research on LBPs is clear, though we know of no examples that have completed it yet. The
231 Investigational New Drug (IND) process must be followed. Over past years, the FDA had considered
232 essentially all probiotic research to be drug research. Under the auspices of the International Scientific
233 Association for Probiotics and Prebiotics (ISAPP), several researchers challenged FDA on this position,
234 demonstrating the negative impact it has had on the conduct of human research on probiotics in the
235 United States as well as pointing out that such research on foods or dietary supplements is legal under
236 U.S. law⁴⁹. Recently, the FDA relaxed their position, seemingly to provide a path for human research
237 on probiotic foods or dietary supplements without needing an Investigational New Drug (IND)
238 approval⁵⁰.

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

246

247 *What do proponents of LBPs need to demonstrate?*

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

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

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

265

266 **Production challenges and scale-up**

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

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

289

290 **Conclusions and Action Required**

291 The term probiotic is not a taxonomic one, but refers to functionality. Nothing in the definition of
292 the term limits the species, genus or even Kingdom from which probiotics can be selected, nor does
293 it dictate whether they must be naive strains or whether they can have been subjected to any form
294 of genetic manipulation. Why do we therefore feel the need to use the term 'Next Generation
295 Probiotics'? We believe that it is highly likely that in the near future the enormous amount of
296 research on the beneficial impact of the microbiome on human health will lead to the discovery and
297 development of novel microorganisms derived from our microbial symbionts. In many cases these
298 may belong to unusual and formerly 'uncharacterised' microorganisms with unusual properties, or
299 perhaps may even be microorganisms formerly thought of as pathogens or pathobionts. These
300 developments will present significant challenges for scientific research, for industrial exploitation
301 and for regulatory agencies. For the moment the term NGP can serve as a useful descriptor for
302 these 'non-traditional' microbes. Other human commensals developed and approved through a
303 pharmaceutical route for curing disease or alleviating symptoms will likely retain the LBP moniker.
304 The success of faecal microbiota transplantation (FMT) for curing diarrhoea associated with
305 recurrent *Clostridium difficile* infection⁵² has provided a conceptual framework for isolating
306 organisms or consortia that might improve diseases associated with gut microbiota alteration⁵³.
307 These could include GMMs, bacterial spores, or bacteriophages, that would also be more readily
308 developed as LBPs.

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

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

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

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

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

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

336

337 **Figure Legends**

338

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

340

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

343

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

345

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505 **Acknowledgements**

506 We thank the Panel members of the ISAPP 2016 meeting for stimulating discussions, and Mary Ellen
507 Sanders for reviewing the section on regulations. The opinions in this article are those of the authors
508 only, and do not represent a consensus of the ISAPP convened panel. We are grateful to three
509 anonymous reviewers for constructive criticism that helped refine our thinking.

510

511 **Disclosures**

512 PWOT and CH are funded in part by Science Foundation Ireland (APC/SFI/12/RC/2273) in the form of
513 a research centre which is/has recently been in receipt of research grants from the following
514 companies: Cremo, Mead Johnson Nutrition, Kerry, General Mills, GE Healthcare, Friesland Campina,
515 Sigmoid, Alimentary Health, Second Genome, Nutricia, Danone, Janssen, AbbVie, Suntory Morinaga
516 Milk Industry Ltd, Pfizer Consumer Health, Radisens, 4D Pharma, Crucell, Adare Pharma, Artugen
517 Therapeutics, Caelus. PWOT is a founder shareholder of Tucana Health Ltd. CH is a founder
518 shareholder in Artugen therapeutics. These relationships with industry have no bearing on the present
519 work and neither influenced nor constrained it. JRM has consulted and received payment from Cultech
520 Ltd, Takeda Pharmaceuticals and Unilever.

521