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Effects of genetic, processing, or product formulation changes on efficacy and safety of probiotics

Mary Ellen Sanders,[1] Todd R. Klaenhammer,[2] Arthur C. Ouwehand,[3] Bruno Pot,[4] Eric Johansen,[5] James T. Heimbach,[6] Maria L. Marco,[7] Julia Tennilä,[3] R. Paul Ross,[8] Charles Franz,[9] Nicolas Pagé,[10] R. David Pridmore,[11] Greg Leyer,[12] Seppo Salminen,[13] Duane Charbonneau,[14] Emma Call,[2] and Irene Lenoir-Wijnkoop[15]

[1]International Scientific Association for Probiotics and Prebiotics, Centennial, Colorado. [2]North Carolina State University, Raleigh, North Carolina. [3]DuPont Nutrition and Health, Kantvik, Finland. [4]Institut Pasteur de Lille, Lille, France. [5]Chr Hansen A/S, Hørsholm, Denmark. [6]JHEIMBACH LLC, Port Royal, Virgina. [7]University of California, Davis, California. [8]Alimentary Pharmabiotic Centre, Teagasc Food Research Centre, Cork, Ireland. [9]Max Rubner-Institut, Federal Institute for Nutrition and Food, Karlsruhe, Germany. [10]Nestlé, Konolfingen, Switzerland. [11]Rue du Centre 61b, St Sulpice, Switzerland. [12]UAS Laboratories LLC, Madison, Wisconsin. [13]Functional Foods Forum, University of Turku, 20014, Turku, Finland. [14]Procter & Gamble, Mason, Ohio. [15]Danone Research, Palaiseau, France

Address for correspondence: Mary Ellen Sanders, 7119 S. Glencoe Ct., Centennial CO 80122. mes@isapp.net

Commercial probiotic strains for food or supplement use can be altered in different ways for a variety of purposes. Production conditions for the strain or final product may be changed to address probiotic yield, functionality, or stability. Final food products may be modified to improve flavor and other sensory properties, provide new product formats, or respond to market opportunities. Such changes can alter the expression of physiological traits owing to the live nature of probiotics. In addition, genetic approaches may be used to improve strain attributes. This review explores whether genetic or phenotypic changes, by accident or design, might affect the efficacy or safety of commercial probiotics. We highlight key issues important to determining the need to re-confirm efficacy or safety after strain improvement, process optimization, or product formulation changes. Research pinpointing the mechanisms of action for probiotic function and the development of assays to measure them are greatly needed to better understand if such changes have a substantive impact on probiotic efficacy.

Keywords: probiotic; genetics; gene expression; regulatory; safety; efficacy

Introduction

A key scientific question facing the probiotic field today is how to determine when efficacy and safety studies need to be redone following strain improvement measures, changes in production, or final product reformulation. Growth conditions, growth substrates, cryoprotectants, food formulation, food processing conditions, and storage conditions may affect probiotic properties as scientists seek to optimize processes, viability, and function. Such modifications concomitantly may generate detectable differences in genes (mutations, genome rearrangements), gene expression patterns, or metabolic output. This raises the question of when such changes warrant a re-examination of efficacy or safety. Time, expense, and ethical considerations dictate that human studies are repeated only when there is a reasonable expectation that efficacy or safety have been compromised. This review explores the nature of genetic and phenotypic alterations induced by changing industrial processes or delivery format and will consider what type of testing—short of full safety assessments or new human efficacy trials—would enable reasonable and meaningful conclusions on efficacy and safety. We focus here on probiotics for food and supplement use, not for drug use. We exclude discussion of genetically modified microorganisms, as well as newly isolated strains, regardless of similarity to existing probiotic

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Table 1. Guiding principles and situational context

- The standard to be applied is reasonable certainty, not absolute assurance, of functional equivalence.
- Any changes should not compromise delivery of metabolically active, live probiotics.
- Any changes should not compromise any original probiotic properties determined to be important to functionality.
- Any regulatory requirements for additional safety or efficacy testing should have a reasonable potential to add to overall assurance of safety or efficacy. If that potential is very low, additional testing should not be imposed.
- The impact of consumer diet, genetics, and colonizing microbiota on probiotic function is a relevant context for evaluating the importance of changes to probiotics.
- In foods, there is precedent in nutrition science that food ingredients can be delivered in different formats, without negation of claim of health benefit.

strains. We include probiotic strains subjected to genetic improvement techniques short of introduction of foreign DNA. Techniques such as mutagenesis and screening or plasmid curing fall within this scope. The principles and context guiding the conclusions of this review are shown in Table 1. This paper provides useful perspective for researchers, industry scientists, and regulators grappling with the efficacy and safety implications of inevitable changes in handling of probiotics.

Genetic tools applied to probiotics

Whole-genome sequences for numerous probiotic strains have been made available since the first was published in 2003 (*Lactobacillus plantarum* WCFS1).[1] These sequences enable scientists to (1) understand the genetic content and thereby the potential for a strain to elicit probiotic properties; (2) search for genotypes that might disqualify the strain from commercial development due to safety concerns; and (3) define unique genetic characteristics that can specifically identify and differentiate one strain from others. This last use of genomic sequencing provides a reference identity for a strain subjected to efficacy and safety evaluations with a depth and specificity that can inform the extent and significance of genetic changes.

The combination of genome sequencing and development of genetic tools for probiotic cultures[2–4] has initiated a mechanistic understanding of how some genes and proteins affect probiotic functionality, including survival through the gastrointestinal tract, bile tolerance, association with mucus and the intestinal epithelium for probiotics targeting GI endpoints, and immunomodulatory properties. For example, *in vitro* and *in vivo* (mouse) models reveal that the cell surface properties of probiotic microbes and the display of those surface components can direct major immunological responses in the gastrointestinal tract, negating or abating inflammatory bowel conditions such as colitis and colonic polyp formation in mice.[5–8] Such detailed insights into mechanisms of action provide targets for directed strain improvement efforts as well as identify properties that may be assessed to confirm functionality.

Genetic changes in probiotics

Scale-up of probiotic cultures from a 10-ml seed culture ($\sim 10^{10}$CFU) to 10,000 liters ($\sim 10^{16}$ CFU) requires approximately 21 generations. In this scenario, rare mutations may occur and mutant strains are likely to be present in the population. It has been suggested that, in the absence of any selective pressure, mutations appear in a population at a rate of 0.0033 changes/genome/generation regardless of genome size.[9] Thus, the vast majority of cells in the population will be genetically identical. Homogeneity is promoted by the use of seed cultures from a single stock and minimal subculturing to reduce genetic drift in a given strain population. However, quality-control measures should be considered to preserve the original probiotic properties.[10] Furthermore, mechanisms have been identified in lactic acid bacteria (LAB) that potentially increase the frequency of mutations, including (1) plasmid curing, (2) hypermutability,[11] (3) the presence of prophages or prophage remnants and insertion sequences in the genome, and (4) CRISPR sequences (clustered regularly interspaced short palindromic repeats).[12] Hypermutability has been reported in *Oenococcus oeni*, owing to mutations in *mutR* and *mutS*, encoding proofreading enzymes that correct DNA

replication errors. Without the proofreading function, *Oenococcus* shows higher mutation rates across the genome and is thus one of the fastest evolving lactic acid bacteria. Such hypermutable strains would clearly accentuate genetic changes in a population of microbes during fermentation scale-up. What this implies in terms of functionality (loss or gain) is difficult to predict, but may be comparable to the intra-species variation, which is generally observed within LAB or *Oenococcus* in particular.[13]

Prophages may also promote genetic variability. Of note, during scale-up operations, it has been observed that 5–10% of lysogenic LAB can undergo spontaneous curing of resident prophages (Greg Leyer, unpublished observation). Notably, this loss of the prophage was not accompanied by detectable phenotypic or metabolic changes in the bacterium, even if the potential exists. Elimination of prophages may also be used to increase technological performance of the large-scale culturing process.[14] A comparative genome analysis of three dairy product isolates of *Lactobacillus rhamnosus* GG and the ATCC 53103 reference strain to the published genome sequence of *L. rhamnosus* GG[15] showed that in two of three isolates, major DNA segments were missing. The deleted genes included important genetic determinants encoding 34 genes in one isolate and 84 genes in another, including the genes encoding pilin subunits involved in adhesion to mucus and persistence of the strains in the human intestinal tract. The study further substantiated the relevance of assessing genome stability in probiotic strains and any potential impacts on outcomes to *in vitro* and *in vivo* efficacy studies.

Lastly, CRISPR elements are also common in LAB and probiotic cultures. Despite the palindromic sequence repeats in these elements, analysis of *Streptococcus thermophilus* cultures propagated over 4200 generations did not generate a single rearrangement, deletion, or base pair change in the CRISPR region (Greg Leyer, unpublished observation). CRISPR sequences can also provide important strain genotyping signatures that indicate strain identity and evolutionary history with closely related strains.[12] They may have an important functional role in microbiome ecosystem management and in shaping human microbial communities.[16,17]

These examples highlight the potential for genetic variation to occur in LAB and probiotics and suggest that such change can occur among stock cultures held in varying locations and within normal commercial processes. Such variation has the potential to affect gene expression. Whether this affects efficacy or safety of a probiotic will be addressed in the following sections. It should be noted that changes in a very small proportion of naturally occurring spontaneous mutations will have a negligible overall effect on efficacy or safety, but similar changes when applied to the master probiotic stock may require further investigation.

When considering genetic changes, it is the nature of the difference, not the magnitude, that carries the most weight. In the case that a directed genetic alteration has been made, it is important to determine what the differences are. A high-quality genome sequence of both strains is required. From this point, the genetic changes can be assessed for function. This should be done on a difference-by-difference basis by answering the questions below:

1. What are the exact differences between the strains? This may be answered by genome sequencing and transcriptional analysis.
2. What are the likely effects of the differences on the physiology of the cell? Silent mutations or changes in intergenic regions probably are not substantive. Mutations that affect the amino acid sequence of a protein, mutations that are likely to affect the expression of one or more genes (e.g., promoter mutations), or changes such as insertions, duplications, deletions, and stop codons have the potential to be substantive.
3. Can the predicted differences in physiology be confirmed by laboratory experiments and if so, what is their possible impact on probiotic function?
4. Are there reasonable mechanisms by which these genetic or physiological changes could affect the efficacy or safety of the strain?

If the answer to question 4 is *yes*, further experimentation is warranted and may include *in vivo* toxicity tests in the case of safety, and extensive laboratory testing and possibly some amount of *in vivo* testing in the case of efficacy, although no preclinical assay is currently known to fully predict the situation in the host.

Environmental challenges that affect probiotic recovery

The viability and functional expression of probiotics are influenced by a variety of external factors, including fermentation conditions, the method of concentration of the culture, the method of stabilizing the culture (freezing, freeze drying, spray drying), final product format, and storage conditions.[18] There are several endpoints that can serve as a measure of physiological changes, including recovery of the probiotic from the product; survival of the probiotic through gastrointestinal transit as reflected by fecal recovery; impact on host biomarkers, such as immune cells; or ultimate health endpoint. In the case of probiotic survival, methods of reconstitution, recovery and enumeration will also affect results.

Table 2 gives examples of responses and/or consequences of some probiotic bacteria to different growth, storage, or reconstitution conditions. These examples clearly show the impact of external processing and preparation treatments, at least with regard to numerical recovery of the probiotic. For the purposes of this paper, however, what remains to be determined is how, for a given dose of viable probiotic, altered conditions might change a strain's ability to influence a final health outcome.

Impact of product format on fecal recovery, host microbiota, biomarkers, and health endpoints

Fecal recovery

Although a variety of product formats have been evaluated, few studies have investigated the impact of different product formats in parallel (Table 3). Saxelin and co-workers[19] compared the fecal recovery of *L. rhamnosus* GG and *L. rhamnosus* LC705 in a probiotic combination, as consumed in capsules, yogurt, or cheese. No effect of product format on fecal counts was found for either strain; each was excreted in high amounts in feces ($> 10^8$ genome copies/g).[20,21] Similarly, entero-coated tablet and fermented milk were both good carriers of *L. rhamnosus* GG, according to a study of 44 healthy adults.[22] Both entero-coated tablets and fermented milk yielded higher fecal counts of the strain than freeze-dried powder studied earlier.[23] These results are in agreement with a comparative study by Goldin and co-workers,[24] that showed higher fecal recovery of *L. rhamnosus* GG when consumed in fermented whey as compared to frozen concentrated cells. Low-fat spread and milk-based fruit juice were also demonstrated to be good carriers of *L. rhamnosus* GG.[25,26] The consumption of low-fat spread resulted in fecal counts that were in line with the study of Saxelin and co-workers[19] using a similar molecular enumeration method, whereas the consumption of milk-based fruit juice resulted in 1 log lower fecal counts. It seems that *L. rhamnosus* GG is relatively persistent, whether consumed in capsules or food matrices, whereas dried-powder forms may likely yield lower fecal counts. This was noted also with *Lactobacillus plantarum* MF1298 in the study of Klingberg and Budde,[27] which demonstrated more frequent recovery of the strain when consumed in fermented sausage compared to freeze-dried powder. In contrast, fecal *Lactobacillus acidophilus* NCFM counts were significantly and similarly increased whether consumed in cheese or synbiotic powder.[28,29]

We identified two studies on children (0.5–6 years) that used milk or formula as a delivery format for *L. rhamnosus* GG.[20,21] Fecal recovery did not differ between the two formats.

Bifidobacterium animalis subsp. *lactis* BB-12 yielded the highest fecal counts and had the longest excretion time after the intervention when consumed in yogurt compared to capsules and cheese.[19] Fermented milk may contribute to adaptation to acidic conditions, thereby improving resistance to stomach acid during transit. The study of Savard and co-workers[30] supports this, as there was a significant increase of *B. animalis* subsp. *lactis* BB-12 consumed in yogurt after 4 weeks (up to 10^8 CFU/g), although capsules have been proved to be good carriers as well.[19,31] However, in two other studies, using culture-based enumeration methods, *B. animalis* subsp. *lactis* BB-12 or "*B. lactis*" (strain number not designated) consumed in yogurt at 10^{11} CFU/d or ice cream at 5×10^9 CFU/d, respectively, was detected in fewer than 80% of the subjects.[32,33] For some bifidobacteria strains, the delivery format does not seem to play an important role in fecal recovery. For example, in the case of *B. animalis* subsp. *lactis* DN-173010 (dose 10^{10}–10^{11} CFU/d), no difference was seen between fecal recovery of the strain delivered in fermented product compared to lyophilized form.[34] It remains to be clarified if this type of observation is strain dependent.

Table 2. Examples of changes in probiotic expression of stability in response to changed environmental conditions

Condition	Mechanism	Outcome	Reference
Pre-exposure to sublethal stress (heat, salt)	Increased expression of heat shock protein GroESL in *L. paracasei*	Increased survival during spray drying	86,87
Adaption *of L. plantarum* to ethanol exposure	Higher membrane fatty acid saturation	Increased heat resistance	88
Growth of *L. johnsonii* in media enriched in different C18 fatty acids	Differing levels of fatty acid saturation in cell membranes	Increased ability to withstand heat and acid stress	89
Reconstitution studies with dried *L. johnsonii* and *B. longum*	Unknown	Survival of *B. longum* increased up to 8-fold by adjusting the conditions used	90
L. rhamnosus GG, *L. casei* Shirota, *B. animalis* subsp. *lactis* BB-12	Acquisition of heat and freeze tolerance	Changes in probiotic properties, adhesion, and pathogen exclusion	91
Spray drying *L. paracasei* in the presence of gum acacia	Unknown	10-fold increase in survival during spray drying and 1000-fold during storage at 30 °C	92
Survival of *L. rhamnosus* in acid in the presence of metabolizable sugar	Proton exclusion via glycolysis	6 log enhanced survival at pH2 after 90 min	93

Other probiotic strains, such as *Propionibacterium freudenreichii* subsp. *shermanii* JS, have been recovered in similar amounts regardless of the delivery format.[19,35] Yogurt yielded the highest fecal quantity, followed by capsules, whereas cheese consumption resulted in the lowest, but still high ($>10^7$ genome copies/g feces) fecal counts.[35] When consumed in whey-based orange juice, strain JS was also recovered in high numbers.[35] In another study, the fecal counts of the JS strain were lower when consumed in capsules,[36] but this could be explained by the culturing method used.

On the basis of the studies available, it appears that the most commonly used formats, food matrices and capsules, are good carriers for most probiotic strains. However, fecal recovery of the strain is highly dependent on the dose and detection method used. Fecal levels of *L. rhamnosus* GG, *L. rhamnosus* LC705, and *L. acidophilus* NCFM did not appear to be influenced by the delivery format. *B. animalis* subsp. *lactis* BB-12, *P. freudenreichii* spp. *shermanii* JS, and *L. plantarum* MF1298 were affected by the delivery format, resulting in 1–2 $\log_{10}$ cycles difference between the highest and lowest detection levels.[19] Conclusions on delivery format effects, however, can be confounded by differences in dose and recovery methods used in different studies.

Host microbiota

Data are limited on the impact of delivery format of the probiotic on host microbiota. Consumption of *B. animalis* subsp. *lactis* BB-12 at a dose of 10^9–10^{10} CFU/day and inulin in yogurt increased *Bifidobacterium* levels in those with low initial *Bifidobacterium* levels and reduced clostridia counts in those with high initial clostridia levels. However, no changes were observed in the majority of subjects.[37] Similarly, no change in microbiota of healthy adults was observed when BB-12 was consumed in ice cream at 5×10^9 CFU/day.[33] *B. animalis* subsp. *lactis* HN019 consumed in ice cream at 10^9 CFU/day did not influence the composition of the microbiota of healthy adults.[33] However, consumption of the strain at similar doses in milk has been reported to lead to increases in bifidobacteria, lactobacilli, and enterococci and a reduction in enterobacteria.[38,39] Consumption of a combination of *L. rhamnosus* IMC 501 and *Lactobacillus paracasei* IMC 502

Table 3. Impact of delivery format on fecal recovery of different probiotic strains (the original authors' conclusions are reflected in results column)

Strain	Delivery format	Dose	Subjects in active group(s)	Results	Reference
L. rhamnosus GG	Capsules, yogurt, or cheese	5.2×10^9 (capsule), 4.7×10^9 (yogurt), 2.8×10^9 (cheese) cfu/d for 2 weeks	36 healthy adults	GG counts were high in feces (8.08 $\log_{10}$ genome copies/g); delivery format did not affect the fecal counts or the recovery time after ceasing the intervention.	19
	Low-fat spread	3.3×10^{10} to 5.6×10^{10} cfu/d for 3 weeks	16 healthy adults	Counts of GG significantly increased from the baseline (ND->7.3 $\log_{10}$ cells/g); also a significant increase in viable lactobacilli in feces.	25
	Fermented milk or tablet	2.1×10^9 or 1.2×10^{10} cfu/d (milk), $1–8 \times 10^9$ cfu/d (tablet) for 7 days	44 healthy adults	Clear dose response in milk group, no statistical differences between tablet groups, both good carriers.	22
	Milk-based fruit juice	6.5×10^9 cfu/d for 3 weeks	41 healthy adults	Increase in GG counts from 5.18 $\log_{10}$ to 7.24 $\log_{10}$ cfu/g.	26
	Frozen concentrate, fermented milk, or whey	4×10^{10} cfu/d (frozen concentrate) for 28 days, 3.6×10^{11} cfu/d (fermented milk) for 7 days, 1.6×10^{11} cfu/d (fermented whey) for 35 days)	76 healthy adults	GG counts increased to 6.3 $\log_{10}$ (day 28, concentrate), 7.7 $\log_{10}$ (day 35, whey), and 6.0 $\log_{10}$ cfu/g (day 7, milk).	24
	Freeze-dried powder	$1.5 \times 10^6 – 1.1 \times 10^{11}$ cfu/d for 7 days	~36 healthy adults	10^{10} and 10^{11} cfu/d yielded $10^5–10^6$ and $10^6–10^7$ cfu/g feces, respectively (strain isolated from all subjects).	23
	Formula/milk	3×10^9 cfu/100 ml (ad libitum)	21 infants (5–28 months) with rotavirus diarhhea	GG counts increased in active group; strain isolated from all subjects.	20
	Milk (Gefilus)	3 times a day with meals for 28 weeks (mean 10^8 cfu/d)	261 children (2–6 years)	GG counts increased from 6.7 to 7.5 $\log_{10}$ genome copies/g in active group.	21
L. acidophilus NCFM	cheese	10^9 cfu/d for 3 weeks	31 healthy elderly	Significant increase of NCFM in feces (from log 1.46 to log 4.35 cells/g); NCFM counts after 4-week wash-out were higher compared to run-in phase.	28
	synbiotic powder containing NCFM and lactitol	2×10^{10} cfu/d for 2 weeks	24 healthy elderly	NCFM counts significantly higher in NCFM group (5.63 $\log_{10}$ cfu/g) compared to placebo (3.67 $\log_{10}$ cfu/g).	29
L. rhamnosus LC705	capsules, yogurt, or cheese	7.4×10^9 (capsule), 3.3×10^9 (yogurt), 4.2×10^8 (cheese) cfu/d for 2 weeks	36 healthy adults	LC705 counts were high in feces (8.67 $\log_{10}$ genome copies/g); delivery format did not affect the fecal counts or the recovery time after ceasing the intervention.	19
	capsules	2×10^{10} cfu/d for 4 weeks	36 healthy men	Increased level of LC705, 75% of participants had detectable levels.	36
L. plantarum MF1298	Fermented sausage or freeze-dried powder	6×10^9 cfu/d (powder), 1.2×10^{10} cfu/d (sausage) for 18 days	17 healthy adults	When consumed in sausage, the number of MF1298 positive subjects increased from 4 (powder) to 10.	27
B. animalis subsp. *lactis* BB-12	capsules, yogurt, or cheese	1.8×10^9 (capsule), 1.4×10^{10} (yogurt), $4.2 \times 10^7–1.2 \times 10^6$ (cheese) cfu/d for 2 weeks	36 healthy adults	Yogurt yielded the highest BB-12 counts (9.89 $\log_{10}$ genome copies/g). BB-12 counts were lowest when consumed in cheese (also lowest daily dose). Longest excretion time in the yogurt group; unable to distinguish between dose effect or delivery vehicle effect as both were varied.	19

Continued

Table 3. *Continued*

Strain	Delivery format	Dose	Subjects in active group(s)	Results	Reference
	capsule	$10^{7–11}$ cfu/d for 3 weeks	57 healthy adults	BB-12 increased significantly with increasing dose (plate counts).	31
	yogurt	10^{9}–10^{10} cfu/d for 4 weeks	38 healthy adults	Significant increase in bifidobacteria and BB-12 (8.5 $\log_{10}$ cfu/g) counts.	30
	yogurt	10^{11} cfu/d for 10 days	14 healthy adults	BB-12 was detected in the feces of 79% of the study subjects (6.9 $\log_{10}$ cfu/g).	32
	Ice cream	5×10^{9} cfu/d for 28 days	30 healthy adults	Increased level of *B. lactis*; 60% of volunteers had detectable levels; no change in analyzed genera.	33
B. animalis subsp. *lactis* DN-173 010	fermented product or lyophilized form	6×10^{10}–2×10^{11} cfu/d for 1 week	12 healthy adults	Similar quantities ($\geq 10^{8}$ cfu/g) of DN-173010 detected in feces in both groups; average survival of 22% for the lyophilized form and 20% for the fermented product.	94
L. reuteri DSM17938	pudding	10^{9} cfu/d for 7 days	18 healthy adults	Alternate-day intake resulted in equivalent colonization of *L. reuteri* (10^{4}–10^{5} cfu/g feces) to daily intake; colonization declined rapidly when dosing stopped.	95
	low-fat spread	5.7×10^{9} to 1×10^{10} cfu/d for 3 weeks	13 healthy adults	Counts of DSM17938 significantly increased from baseline (ND->6.7 $\log_{10}$ cfu/g); also a significant increase in viable lactobacilli in feces.	25
Propionibacterium freudenreichii subsp. *shermanii* JS	capsules, yogurt, or cheese	4.2×10^{9} (capsule), 7.5×10^{9} (yogurt), 1.7×10^{9} (cheese) cfu/d for 2 weeks	36 healthy adults	Yogurt yielded the highest fecal quantity of JS (8.01 $\log_{10}$ genome copies/g), lowest when consumed in cheese. Longer excretion times in the capsule and yogurt groups. Unable to distinguish between dose effect or delivery vehicle effect, as both were varied.	19
	whey-based orange juice	6×10^{10} for 2 weeks	10 healthy adults	Recovered in high numbers (7.6 $\log_{10}$ cfu/g) during the intervention; not detected after 2-week follow-up period.	35
	capsules	2×10^{10} cfu/d for 4 weeks	36 healthy men	Increased level of JS; 61% of participants had detectable increase also in total *Propionibacterium*.	36

at 10^{9} CFU/day in the form of yogurt, ricotta, mozzarella, chocolate, chocolate mousse, ice cream, or powder was reported to increase fecal lactobacilli and bifidobacteria.[40,41] Overall, the most common change in microbiota composition observed in response to probiotic consumption is an increase in the genus to which the consumed probiotic belongs, typically *Bifidobacterium* or *Lactobacillus*.[42] This most likely reflects fecal recovery of the consumed strain, as discussed above, rather than a true shift in colonizing microbiota.

Biomarkers

Human intervention studies with probiotics have measured various biomarkers, most commonly immune biomarkers. Unfortunately, few studies have measured the same biomarker in comparable populations, making it difficult to make comparative conclusions.

B. animalis subsp. *lactis* HN019 increased natural killer (NK) cell activity when delivered in reconstituted fat-free milk at 5×10^{9} or 5×10^{10} CFU/day[43] or in ice cream at 10^{9} CFU/day,[33] when consumed by elderly or younger adults, respectively. *Lactobacillus casei* (strain not reported) improved NK cell activity when consumed in either fermented milk (4×10^{10} CFU/day)[44] or as powder (4×10^{10} CFU/day).[45] These studies provide limited support that the ability to affect NK cell activity is independent of the delivery format.

Clinical effects

Lactobacillus casei Shirota delivered in fermented milk (6.5×10^9 CFU/day)[46] or a lyophilized powder (10^{10} CFU three times daily) was tested for its ability to relieve constipation. Both studies concluded that constipation was relieved in otherwise healthy adults. Children receiving *L. rhamnosus* GG either as lyophilized powder or as fermented milk recovered from diarrhea equivalently.[47] Authors concluded that *L. rhamnosus* GG shortened the duration of the diarrhea compared to the placebo by 1 day, regardless of the delivery format. These limited data suggest that the delivery format did not affect clinical effects.

Conclusions on delivery format

Here, we have limited our discussion to orally consumed probiotics that have been tested in a substantial variety of delivery formats: capsules,[48] sachets with powder,[45] fermented milks,[44] non-fermented milk,[43] cheese,[49] ice cream,[33] infant formulas,[50] chocolate,[51] juices,[52] spreads,[25] and cereal bars.[53] Although the use of different product formats in the same study enables direct comparison of effects, it also makes blinding impossible. It may therefore be necessary to study different product formats in separate studies with their own, appropriate placebo. This is the case with most published studies; it is rare that different formats are evaluated in parallel.[19]

Although various delivery formats have been tested with many probiotic strains, often different biomarkers or health outcomes have been measured. Furthermore, different doses, strain combinations, and other confounding components (e.g., prebiotics) may have been included. Finally, different study populations (e.g., healthy and patient populations or adults and infants) have been used in studies, precluding direct comparisons of outcomes.

Few studies allow comparison of probiotic performance in different delivery systems. Instead, we are left with a mosaic of data points that are suggestive but not conclusive. Fecal recovery of probiotics may be influenced by the delivery format, but not in all cases. The available data suggest that when making substantive changes in delivery formats (for example, from lyophilized to fermented milk, or from fermented milk to dried cereal), functional assays (such as those indicated in Table 4), in addition to survival in the new product format, should be performed to the extent possible. In some cases, the matrix can interfere with *in vitro* assays, making a direct comparison impossible.

Host effects on probiotic function

As we consider the impact that genetic and environmental changes may have on probiotic function *in vivo*, it is important to also consider that the intestinal environment into which the probiotic is introduced might affect function. The opportunity for probiotics to interact with the host is provided during transit through the human digestive tract, which takes between 24 and 60 hours.[54] This duration of residence is sufficient for these bacteria to adapt for growth and survival at various sites along the intestine. Thus diet, genetics, and resident

Table 4. ***In vitro*** **or animal model assays useful for developing a "performance map" of functionality**

- If mechanism is known, test for the relevant marker of functionality *in vitro* or in a relevant animal model. Examples: lactase activity, bacteriocin production, type and rate of acid production, SCFA production, competitive exclusion, improved barrier function, reduced translocation risk, anti-inflammatory profile, H_2O_2 production
- Growth curves
- Acid resistance, preferably stomach acid
- Bile resistance
- *In vitro* immune profiling: effects of bacteria on different epithelial and immune cell types or on immune molecules (e.g., production of defensins, mucus production, Paneth cells, cytokines, chemokines)
- Fecal recovery for probiotics targeting intestinal function; recovery from oral, stomach, or vaginal sites may be more relevant to probiotics targeting these sites.

NOTE: Such tests provide insight into equivalence of probiotics when comparing pre- and post-changes. Note that food matrix may preclude testing certain products in certain assays. This list of assays is not comprehensive, nor applicable to all probiotic applications. For example, tests such as bile resistance or fecal recovery would not be applicable to a probiotic for oral health. Standardized methods for such tests are currently often not available.

microbiota of the consumer all potentially affect probiotic function.

In vivo responses by probiotics likely include adaptation to the low pH and gastric enzymes, bile salts in the small intestine, and the many indigenous bacterial inhabitants of the colon.[55] This is supported by a number of studies concluding that probiotic *Lactobacillus* are transcriptionally active in the intestine and increase expression of certain genes that confer adaptations to the gut.[55] Site-specific expression of gut-inducible genes[56,57] and their value to adaptation of the probiotic to the intestinal environment[58–60] have been documented in a few studies.

Less well understood are the effects of host-specific factors on probiotic function. Diet, genetics, age, health, and geography are correlated with the composition and functional characteristics of the indigenous intestinal microbiome.[61] It therefore follows that strains of probiotics might also be influenced by such factors *in vivo* in a manner that alters their capacity to influence host health.

An example where host health-associated factors may affect probiotic functionality is their use by individuals with active symptoms of Crohn's disease who harbor genetic polymorphisms that may directly or indirectly affect probiotic efficacy. In these patients, polymorphisms of NOD2 genes on chromosome 16 have been described.[62–64] Macho Fernandez *et al.*[8] showed that a muramidyl-tri-peptide residue was responsible for the anti-inflammatory capacity of strain *Lactobacillus salivarius* Ls33 in mice. This effect depended on local IL-10 production and was abolished in Nod2-deficient mice, suggesting that patients with a NOD2 deficiency may not be susceptible to treatment with these types of anti-inflammatory probiotics.

Other influencing factors from the host may be more temporary in nature. Figure 1 shows a series of probiotic strains evaluated for their potential to induce the cytokine Interleukin 10 (IL-10) in human peripheral blood mononuclear cells (PMBCs) derived from four different healthy donors. The image shows that individual donors react differently to these bacteria, some having a high IL-10 induction capacity, while others induce much lower levels of the same cytokine. Moreover a single donor tested at different time points will display considerable variation, on the basis of the actual status of the immune system (not shown). Factors such as summer/winter variations, presence of viral or bacterial infection, and other variables might alter an individual's immune response. It would be interesting to conduct studies on the impact of specific probiotics on subjects with different patterns of microbiota composition and activity.

Inclusion of specific nutrients such as prebiotic oligosaccharides into the diet might encourage growth or activity of probiotics in the intestine.[65] For example, TNBS-mediated colitis was significantly reduced in mice administered *L. plantarum* WCFS1 and fed a diet with high quantities of sucrose and animal fat, as compared to mice provided the same strain but fed a low-fat diet enriched in plant polysaccharides.[66] These effects were likely the result of diet-induced changes in *L. plantarum* gene expression and persistence *in vivo*.[66] These findings were supported by prior genome-wide transcriptome studies on this strain in mono-associated mice[67] and human subjects.[68] Notably, the composition of the indigenous intestinal microbiota was also altered by the mouse diet, and significant reductions in indigenous *Lactobacillus* populations observed in mice consuming the high-sucrose, high-fat diet might have resulted in an vacant ecological niche that supported *L. plantarum* WCFS1.[66] Therefore, diet and other factors specific to the physiological status of the consumer might therefore result in significant variations in probiotic functionality and correlate with the occurrence of non-responders in probiotics studies.[69] Although difficult to quantitate comparatively, a consumer's overall diet, health status, and colonizing microbiota may be more important for probiotic function than production and delivery format conditions.

Regulatory perspectives

Our discussion has considered the potential for genetic or phenotypic changes in probiotics due to environmental factors from a scientific perspective. What remains is to consider the regulatory implications. Regulatory documents suggest that efficacy studies should be conducted on the same food format that will be marketed.[70,71] The Food and Drug Administration (FDA) issued a guidance document on the need for new dietary ingredient notifications—a document addressing dietary supplement ingredient safety—in July 2011.[72] This document, which was met with strong criticism from the International Scientific Association for

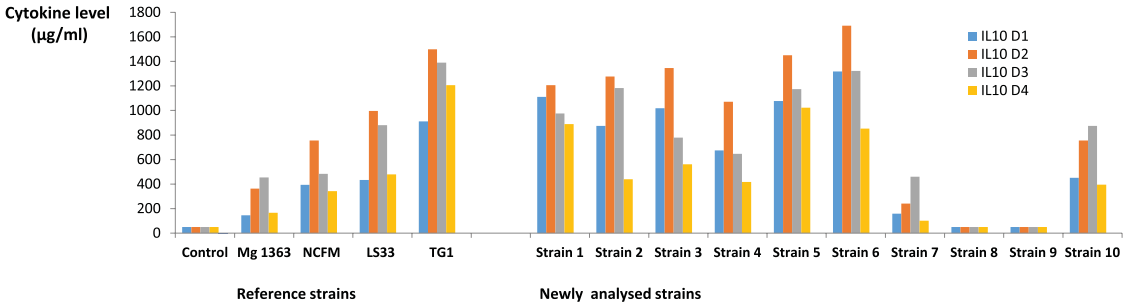

Figure 1. Differential IL10 response in peripheral blood mononuclear cells (PMBCs) derived from four different healthy human donors (IL10D1-IL10D5) to different strains (four control strains and 10 test strains) (Pot, *et al.* unpublished data). Control strains: *Lactococcus lactis* MG 1363; *Lactobacillus acidophilus* NCFM; *Lactobacillus salivarius* Ls33; *Escherichia coli* TG1 (non-pathogenic strain); control = buffer. Tested strains include strains from both *Lactobacillus* and *Bifidobacterium* species.

Probiotics and Prebiotics (ISAPP) and industry, states that changes in fermentation conditions, for example, could result in an identity change for a substance, triggering the need for a new dietary ingredient notification. However, it is unclear how regulatory authorities will determine what changes are substantive. In the absence of a structure for such decisions, it is incumbent on the scientific community to propose them. This discussion is reduced to a question of maintenance of substantial equivalence between the old probiotic and the new one. Some background on substantial equivalence follows.

Substantial equivalence

The term "substantial equivalence" entered the regulatory vocabulary two decades ago in a statement by the Organization for Economic Cooperation and Development (OECD) regarding principles for the safety evaluation of the products of biotechnology.[73] The concept of substantial equivalence was developed in recognition that toxicological approaches were (and are) inadequate to determine the safety of a complex food. For example, the only real evidence that rice is safe is that it has been consumed in large quantities by a large number of people over a long period of time with no apparent adverse effects. Furthermore, the fundamental idea has been implicit for many years in the expectation that new cultivars developed through traditional crossbreeding will be safe and are substantially equivalent with regard to their safety profiles to parent cultivars.

The application of the concept of substantial equivalence was further developed in a joint Food and Agriculture Organization (FAO) and World Health Organization (WHO) consultation,[74] which stated that, "substantial equivalence embodies the concept that if a new food or food component is found to be substantially equivalent to an existing food or food component, it can be treated in the same manner with respect to safety (i.e., the food or food component can be concluded to be as safe as the conventional food or food component). Account should be taken of any processing that the food or food component may undergo as well as the intended use and the intake by the population."

The FAO/WHO[74] further described substantial equivalence in the context of biotechnology as including "demonstration that the characteristics assessed for the genetically modified organism, or the specific food product derived therefrom, are equivalent to the same characteristics of the conventional comparator ... within the natural variation for such characteristics" and "consideration of the molecular characterization of the genetically modified organism, its phenotypic characteristics, and the key nutrients and toxicants for the food source in question."

It has since been recognized that the substantial-equivalence paradigm has broader application in the area of food-safety assessment and regulation beyond issues raised by biotechnology. Safety of synthetic analogues of natural products, ingredients from novel sources that are substantially equivalent to those from traditional sources, and traditional ingredients processed in novel ways have been evaluated using the substantial-equivalence approach. Substantial-equivalence has rarely, however, been employed to address issues regarding the safety of probiotic bacteria. In the United States, the only probiotic for which the FDA has accepted a safety

evaluation based largely on substantial equivalence is *Lactobacillus reuteri* strain DSM 17938, which was shown to be substantially equivalent to its parent strain, *L. reuteri* ATCC 55730, differing only in the curing of two plasmids carrying antibiotic resistance genes, and so the safety evaluation could rely heavily on research conducted with the parent strain.[75] Subsequent human studies have shown equivalent efficacy as well. For example, the parent *L. reuteri* strain (ATCC 55730) was shown to be effective in reducing colic symptoms.[76] Subsequently, the daughter strain (DSM 17938), cured of the antibiotic resistance plasmids, was shown to have equivalent effects in a second study.[77] Similarly, gastric emptying in infants was accelerated both by *L. reuteri* ATCC55730[78] and the daughter strain *L. reuteri* DSM17938.[79]

Nevertheless, the substantial equivalence paradigm offers an approach to addressing the safety of probiotics with known safety profiles for which fermentation conditions, growth substrates, food matrices, or cryoprotectants have changed, or that have experienced genetic drift. One difficulty is in defining the characteristics that must be evaluated to determine that the new strain is indeed substantially equivalent to the old.

First, we must identify what characteristics might or might not be susceptible to change under the conditions being assessed. For example, if a gene encoding an immunomodulatory protein is not present in the genome, no change in fermentation or growth conditions can change that, while if such a gene is present but apparently unexpressed, it may be necessary to demonstrate that the change in production conditions has not affected its expression.

Second, having identified what genotypic or phenotypic differences can exist, we must evaluate whether there is a reasonable basis to suspect that the difference may have a significant impact on safety or efficacy. If these conditions can be fulfilled, it might be suggested that together they establish that the safety or efficacy of the new strain is reasonably expected to be the same as that of the old strain and a new assessment of safety or efficacy is not necessary.

Decision frameworks

The discussion to this point has established that (1) genetic changes, by accident or design, can occur in probiotic populations, (2) phenotypic changes can occur with environmental manipulations, and (3) the varied diet, health status, and inter-individual differences in the genome and microbiome can affect probiotic expression. This section seeks a framework for considering when such changes result in a probiotic that functions in a substantially different way than the one tested in the efficacy or safety studies. A guiding principle is that repeating human studies should only be required when there is reasonable expectation that the previous body of information no longer applies to the probiotic in question. Such requirements should not be imposed if the potential to add to the overall understanding of safety or efficacy is very low. We propose the following decision trees when evaluating the impact that production or delivery format changes have on probiotic efficacy or safety.

Efficacy concerns with changes to production conditions. When imposing changes to probiotic production, including growth (such as media formulation, source of ingredients, or temperature of growth), concentration (such as membrane filtration or centrifugation) or preservation (such as cryoprotectants, freeze drying, or spray drying), we propose that the key measures of equivalency for the probiotic resulting from the new process are (1) equivalent or improved survival of the probiotic in the final product, and (2) equivalent results from phenotypic performance mapping (Table 4). A performance map comprises an array of functional and physiological tests of biological activity but does not entail full clinical endpoint evaluations. This list of assays suggested in Table 4 is not comprehensive, nor applicable to all probiotic applications. For example, tests such as bile resistance or fecal recovery would not be applicable to a probiotic for oral health. Standardized methods for the indicated functional tests are not necessarily available; the important issue is to use the same method for comparison before and after production changes were instituted. The need to perform additional human studies to test for equivalency should be made on a case-by-case basis, on the basis of the outcomes of the selected panel of *in vitro* functional tests. The most extensive mapping assay may require recovery of the live probiotic from the site of action, if it is known (e.g., intestinal tract, vaginal tract, or oral cavity). Assessing fecal samples is an accepted proxy for the intestine, since most compartments of the intestinal tract are too difficult to sample or would require too invasive a procedure. Although failure to recover a probiotic

from the feces does not prove that the probiotic is not harbored in upstream intestinal sites, the relevant issue in this context is comparison of fecal recovery before and after production changes have been instituted. Detection of the probiotic from oral or vaginal swab samples is suitable for those respective sites. It is worth noting that viability at the site of action may not be essential for probiotic activity *in vivo*, as bioactive molecules derived from the probiotic may mediate functionality.

Not all changes to the production process should warrant the need for assessment of functional and physiological tests of biological equivalency. Changing the supplier of an ingredient used in the growth medium might be considered an insubstantial change. Changes in cultivation protocols (e.g., change in nitrogen source), harvesting procedures (e.g., growth phase at time of harvest, or use of cell shock procedures) or drying protocols (e.g., freeze dry to spray dry) may be more substantial, considering the different stresses imposed by these methods, but further studies are needed to determine if such changes are substantial. Figure 2 shows a general decision framework to determine if efficacy evaluations need to be conducted when production changes have been instituted. However, there are currently insufficient comparative studies investigating the impact of specific probiotic processing modification on the efficacy to allow for rational identification of substantial changes. Improved knowledge on what constitutes a substantial change and on the type of functional tests required to assess the impact of the change are needed. For such tests, it would also be necessary to define the threshold at which the results will be considered comparable or not.

Efficacy concerns with changes to delivery format. When imposing changes to the format in which the probiotic is delivered, the need to institute new efficacy evaluations should be guided by the following principle: it must be recognized that the impact of host-dependent factors such as varied diet, genetics, and microbiota are likely substantial and may dwarf the impact of small changes due to different delivery formats. With this in mind, a change in delivery format could be considered to be substantial when there is a change in product category, such as a probiotic tested in a fermented milk product being delivered as a dried capsule. Indeed, substantial alterations in delivery format that result in substantial changes in water activity are known to have particular effects on survival during product storage. In contrast, changes such as flavors, percent fat, nutritive sweeteners being substituted with artificial sweeteners, or introduction of new inert carriers are likely not substantive. Probiotic viability must be evaluated, and if any substantive difference in survival is noted, equivalent dose delivery must be adjusted. To establish confidence that a new delivery format won't impose a large shift in functionality, a performance map—as described above and in Table 4— is suggested. The new product format can be compared to the former one in functional assays. For such tests, it would also be necessary to define the threshold at which the results will be considered comparable or not.

Note that different food formats may preclude the conduct of some assays. For example, whereas probiotic cells in a dried format may make a suitable test substance, the same probiotic in a yogurt matrix may not. Figure 2 shows the decision framework for changes in delivery format on efficacy.

Efficacy concerns with spontaneous genetic changes. Inadvertent changes during production are rare and will be present in only a small fraction of the total population in a particular batch. Furthermore, reversion to master seed cultures will minimize the importance of any spontaneous genetic changes. If spontaneous mutants are being selected during a strain-improvement research effort, however, identification of the nature of the mutation through genomic sequencing can be conducted in a short time period and for a reasonable cost and is recommended. A number of categories of genetic changes may be observed.

The uptake of heterologous DNA, such as plasmids or insertion sequences. These types of events have the potential to change the functionality of a strain considerably. In such cases, the changed strain should be abandoned and revert to the original stock culture.

The loss of extra-chromosomal DNA. As argued above, simple tests may confirm if functionality is influenced or not.

Chromosomal rearrangements. Since the DNA content will not change during these events, it is sufficient to confirm functionality of the strain through performance mapping (Table 4).

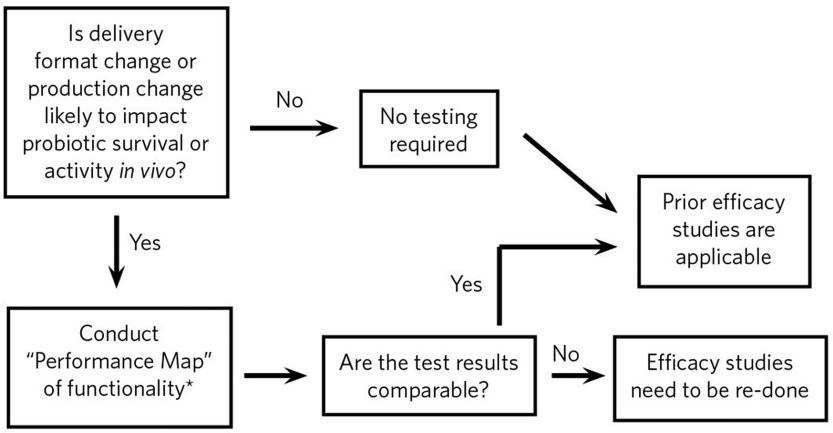

Figure 2. **Impact of delivery format or production changes on probiotic efficacy. Scheme assumes that alteration of delivery format does not reduce probiotic survival in final product. See Table 4 for details on performance mapping.**

Point mutations. Single nucleotide polymorphisms (SNP) are inevitable in any growing culture. The number of normal SNPs within a population will largely depend on the type of microorganisms (slow grower versus fast grower), the growth environment (presence of antibiotics, oxidants, or other substances) and the nature of the bacteria itself (such as presence and efficacy of restriction/modification systems or CRISPR systems). The impact of any SNP will depend on the gene it has affected and the nature of the mutation (such as silent, missense, or frameshift).

Knowledge of the mechanisms for probiotic and metabolic activity will largely assist in the decision concerning whether specific changes have an impact on probiotic functionality. There is a pressing need for more studies in this area.

In general, it is advisable to minimize subculturing and work from a large stock of seed cultures of the probiotic production strain and to preserve freeze-dried back-up cultures in recognized culture collections, which will serve as reference materials in case substantial genetic modifications are suspected. However, the fraction of cells with genetic changes will be very small compared to the mass of cells in the product, unless it provides a significant selective advantage under those growth conditions.

Safety. Specific guidance on appropriate safety assessments for probiotic foods or supplements from regulatory bodies does not exist. When considering the impact that spontaneous genetic changes, production, or delivery format changes would have on probiotic safety, the guiding principle is that any imposed safety assessments should have a reasonable potential to add to the overall understanding of safety. If that potential is very low, additional testing would impose costs and delays on development and use of a probiotic strain without any commensurate public health or safety benefit. (Note that the assessment pathway for safety decisions is different for GMO strains altered through directed genetic engineering processes.) It should also be noted that genetic shift occurs commonly in bacterial populations, without apparent consequences for safety.

The first step in evaluating the impact of any of these changes on safety is to determine if a higher dose is being delivered. Since a safety evaluation is conducted assuming a specific maximum dose delivered, changes that result in delivery of higher doses trigger the need to consider if the higher dose reflects any safety concern. Assuming delivered dose has not been increased, we consider that it is very unlikely that a microorganism judged to be safe for a particular use would become unsafe through non-GMO genetic changes, production changes, or delivery format changes. A basic framework for assessing the safety of existing commercial probiotic strains that have undergone changes to production conditions or delivery format or spontaneous mutation (inadvertent or to improve performance) is shown in Figure 3.

This starting point for this framework is a strain that has been determined to be safe for the intended use, which in our opinion should include full genomic sequencing, including plasmids, and

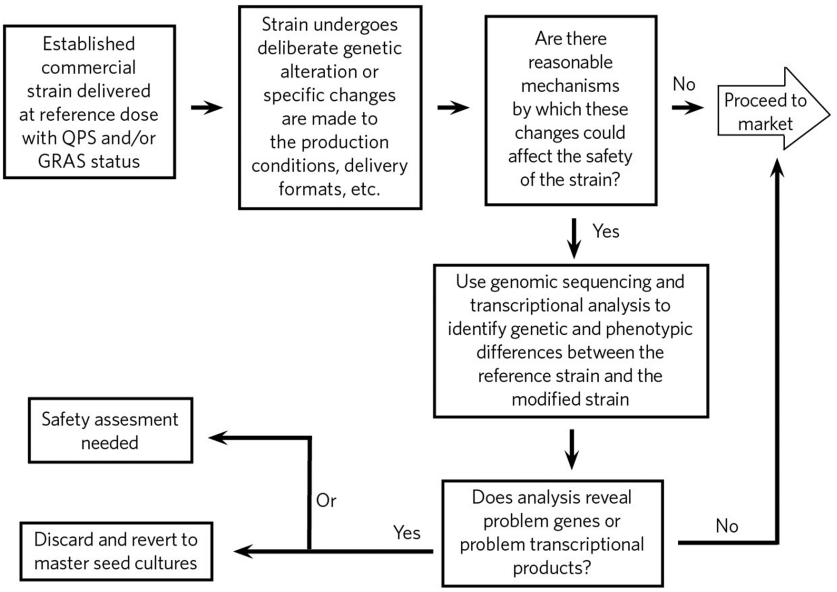

Figure 3. **Safety decision tree for changes to existing commercial strains. In the case that inadvertent strain modifications such as spontaneous mutations are detected, the appropriate action is to discard and revert to master seed cultures. Where addressing genes of concern, this decision tree is informed by Bennedsen *et al.*[80] and Hill.[83] This framework presumes that a full genetic sequence is known for the established commercial strain. Overall approach to safety relies on the European QPS approach (European Food Safety Authority Qualified Presumption of Safety[96]) and is similar to the U.S. FDA Generally Recognized as Safe[97] process.**

annotation consistent with state-of-the-art technology. The time and cost for genomic sequencing and annotation has fallen to the point that this is a reasonable expectation for safety assessments of probiotics. Knowing the complete genetic landscape of probiotic microbes provides the ability to screen for questionable genes and phenotypes, most notably virulence factors and antibiotic resistance determinants, which most often drive regulatory decisions on the commercial acceptability of the strain. A number of databases and programs are now available to scan genomes for known virulence factors and antibiotic resistance determinants *in silico*.[80–82] On virulence determinants, Hill[83] offered the perspective that microbes occupying similar habitats will evolve niche factors that promote survival and activity in those environments. Intestinal pathogens and commensal microbes, both occupying and competing in the gastrointestinal tract would be expected to develop similar survival strategies and niche factors (e.g., mucin-binding proteins, pili, bile tolerance). The key difference is that commensals and probiotics are not toxigenic or infectious. With the exploding interest in using gut microbes as probiotics, the distinction between virulence factors and niche factors must be considered carefully by scientists and regulators when assessing safety.

Research needs

Clearly, gaps exist in fundamental knowledge needed to fully inform the decision frameworks suggested here. The following research would greatly facilitate further progress in resolving the issues addressed in this review.

Research pinpointing the mechanisms of action for probiotic function is greatly needed. Decisions on the impact changes may have on efficacy are greatly informed by assays that give insight into physiological processes that contribute to health effects of the probiotic. Additionally, once mechanisms are understood, the site of action of the probiotics may be determined, perhaps enabling measurement of viability and activity at the site of action.

Validated, standardized, meaningful functional assays (e.g., those identified in Table 4) are also important to develop. Currently, we have assays that provide a window into probiotic physiology, but assays that are causally linked to properties that lead to probiotic efficacy would be a significant improvement. Reference materials for functional

and genetic assays are needed. When conducting the performance map described above, suitable positive controls, negative controls, and internal standards render the assays more useful and more translatable among different laboratories. Thresholds must be defined that allow interpretation of results from these assays and enable a conclusion on equivalency. The assays may not be perfect, but when conducted in a controlled manner, according to standardized protocols, and comparably between before and after, such assays would be of great value.

Validated, meaningful safety assessments are needed. Assays developed primarily to test toxicological effects are not the most suitable for probiotics.

In specific cases, the ability to measure probiotic function at the site of action would be an improvement over measuring viability at consumption and at excretion. A database with molecules linked to specific mechanisms[8,84,85] would also be useful.

Conclusions

The purpose of this review is to provide a framework developed by scientists in the probiotic field for considering when genetic, production, or delivery format changes to commercial probiotic strains trigger the need for reassessing efficacy or safety. Guidance is provided to assist these assessments for a rational, science-based approach to framing regulatory approaches. This discussion focuses on food/dietary supplement uses (not drug uses), with the general population as consumers. Conclusions do not apply to genetically modified microorganisms. In general, the principles used do not provide absolute certainty, but provide an approach that imposes the need for reassessments only when there is a reasonable possibility that they are needed and will contribute important information on safety and/or efficacy. Clearly, additional testing may be necessary under certain circumstances.

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Conflicts of interest

The authors declare no conflicts of interest.

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