

Validating bifidobacterial species and subspecies identity in commercial probiotic products

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BACKGROUND: The ingestion of probiotics to attempt to improve health is increasingly common; however, quality control of some commercial products can be limited. Clinical practice is shifting toward the routine use of probiotics to aid in prevention of necrotizing enterocolitis in premature infants, and probiotic administration to term infants is increasingly common to treat colic and/or prevent atopic disease. Since bifidobacteria dominate the feces of healthy breast-fed infants, they are often included in infant-targeted probiotics.

METHODS: We evaluated 16 probiotic products to determine how well their label claims describe the species of detectable bifidobacteria in the product. Recently developed DNA-based methods were used as a primary means of identification, and were confirmed using culture-based techniques.

RESULTS: We found that the contents of many bifidobacterial probiotic products differ from the ingredient list, sometimes at a subspecies level. Only 1 of the 16 probiotics perfectly matched its bifidobacterial label claims in all samples tested, and both pill-to-pill and lot-to-lot variation were observed.

CONCLUSION: Given the known differences between various bifidobacterial species and subspecies in metabolic capacity and colonization abilities, the prevalence of misidentified bifidobacteria in these products is cause for concern for those involved in clinical trials and consumers of probiotic products.

Probiotics are dietary supplements containing nonpathogenic microbes that provide a health benefit to the host. A broad array of probiotics containing many different bacterial strains are commercially available, many of which were selected based on factors related to ease of production rather than identified mechanisms of protection. Well-designed studies of commercially available probiotics with established composition and purity are essential to establish safety and clinical efficacy, particularly in high-risk patients such as neonates and the immunocompromised. A thorough description of the components of the product is also crucial to understand the mechanism by which administration of a probiotic leads to desirable health outcomes. Factors to consider when

establishing the efficacy of probiotic administration include the accurate identification and labeling of strains used, the viability of organisms administered, and consistency in product formulation over the time course of the study. Unfortunately, many commercial probiotics have been shown to fail at one or more of these criteria (1–8).

One area in which probiotics may have a strong benefit is in guiding the development of the microbial community of the gastrointestinal tract of the neonate. The development of the microbiota early in life has been shown to influence risk for susceptibility to infection and development of allergies and atopic disease (9,10). Perhaps the most compelling case for the use of probiotics is in the premature infant, where multiple randomized clinical trials have demonstrated a decreased risk of necrotizing enterocolitis in infants receiving probiotics (11,12). The hospital environment has been shown to be a likely source of inoculum for premature infants (13,14), and the gut microbiota of premature infants in the neonatal intensive care unit was shown resemble that of neonatal intensive care unit fomites (14). In order to combat this phenomenon, probiotics are increasingly administered to introduce alternative nonpathogenic species to colonize the gastrointestinal tract and occupy niches potentially open to pathogens. Rare cases of infection from probiotic organisms or contaminants in premature infants underscore the importance of providing probiotic products with established composition and purity (15,16).

Bifidobacterium-containing products are often used in a neonatal intensive care unit setting due to their status as generally recognized as safe microorganisms, their ubiquity in the gut of healthy breast-fed infants, and their observed health effects (17,18). Infants with bifidobacteria-dominated gastrointestinal tracts have improved responses to some vaccines, higher resistance to colonization by some pathogens, and better gut barrier function (19–22). Bifidobacteria aid the proper development of the infant's acquired and innate immune systems, enhancing surveillance while reducing inflammation (22–25). Comparison studies between strains or species of bifidobacteria are limited. In a recent study, *Bifidobacterium*

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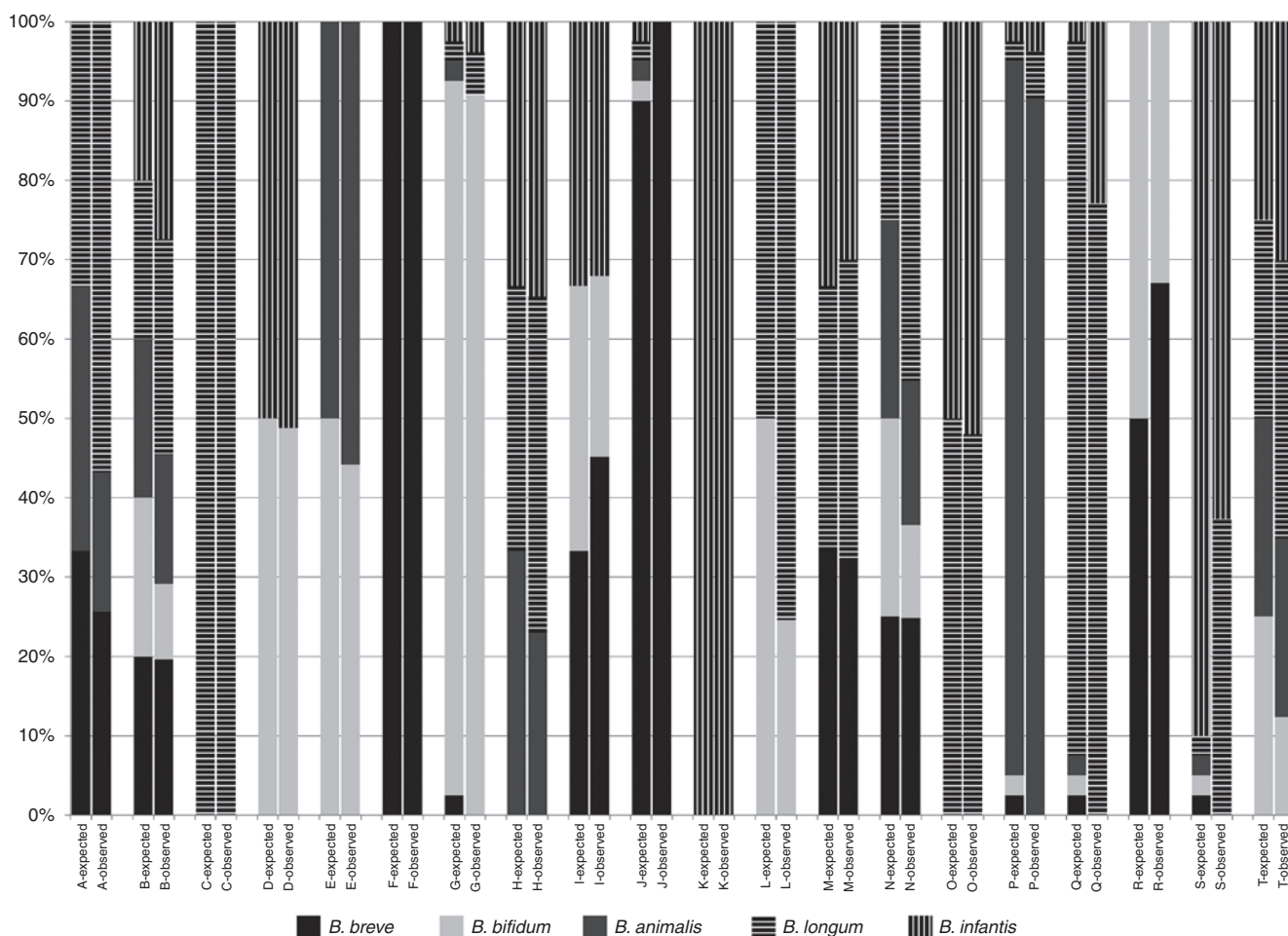


Figure 1. Mock community composition and measurement. The expected values (as defined by the ratios of input DNA initially measured by A_{260}) and observed values for each of the 20 different mock communities assayed are shown here.

longum subsp. *infantis* was found to be a better colonizer of the premature gut than *Bifidobacterium animalis* subsp. *lactis*, especially in the presence of human milk (26). This advantage is likely due to the capacity of *B. longum* subsp. *infantis* to consume a wide spectrum of human milk oligosaccharides as a direct result of the extensive array of human milk oligosaccharide binding, transport and degrading enzymes encoded in its genome but not found in many species of *Bifidobacterium* including *B. animalis* subsp. *lactis* (27–30). *B. longum* subsp. *infantis* colonization is associated with improved responses to some vaccines; in addition *B. longum* subsp. *infantis* appears to decrease intestinal epithelia permeability, and to have anti-inflammatory effects in the premature intestine (20,22,31).

B. longum has two subspecies found in humans that historically have been challenging to distinguish, *B. longum* subsp. *longum* and *B. longum* subsp. *infantis*. Previous studies indicate *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* possess different suites of glycolytic enzymes (27,29,32). While closely related, the two subspecies are not distinguishable using common 16S rRNA gene sequencing methods (27,29,32,33). One commonly used probiotic product whose label lists two subspecies, *B. longum* subsp. *longum* and *B. longum* subsp. *infantis*, as ingredients recently reclassified the included strains as

B. animalis subsp. *lactis*. This unfortunately has led to confusion, as there is a history of published results using this product listing the strains as *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* (34). The risk of species and subspecies misidentification is high, especially given the recently refined definition of these two *B. longum* subspecies further confirmed through genome sequencing (27,32,35). Motivated by the potentially unfortunate consequences of species and subspecies confusion in clinical trials, we surveyed several *Bifidobacterium*-containing probiotic products to evaluate their label claims with regard to these two *B. longum* subspecies and to other bifidobacterial species. To facilitate this effort, we developed a reliable and inexpensive polymerase chain reaction (PCR)-based method for rapid identification of *B. longum* subsp. *infantis* at the subspecies level (20,36). To verify that these DNA-based methods give data that accurately reflect input DNA, we also validated our methods against a series of artificial mock communities.

RESULTS

Method Validation Using Mock Community

We have previously developed bifidobacterial terminal restriction length polymorphism (Bif-TRFLP) analysis to readily

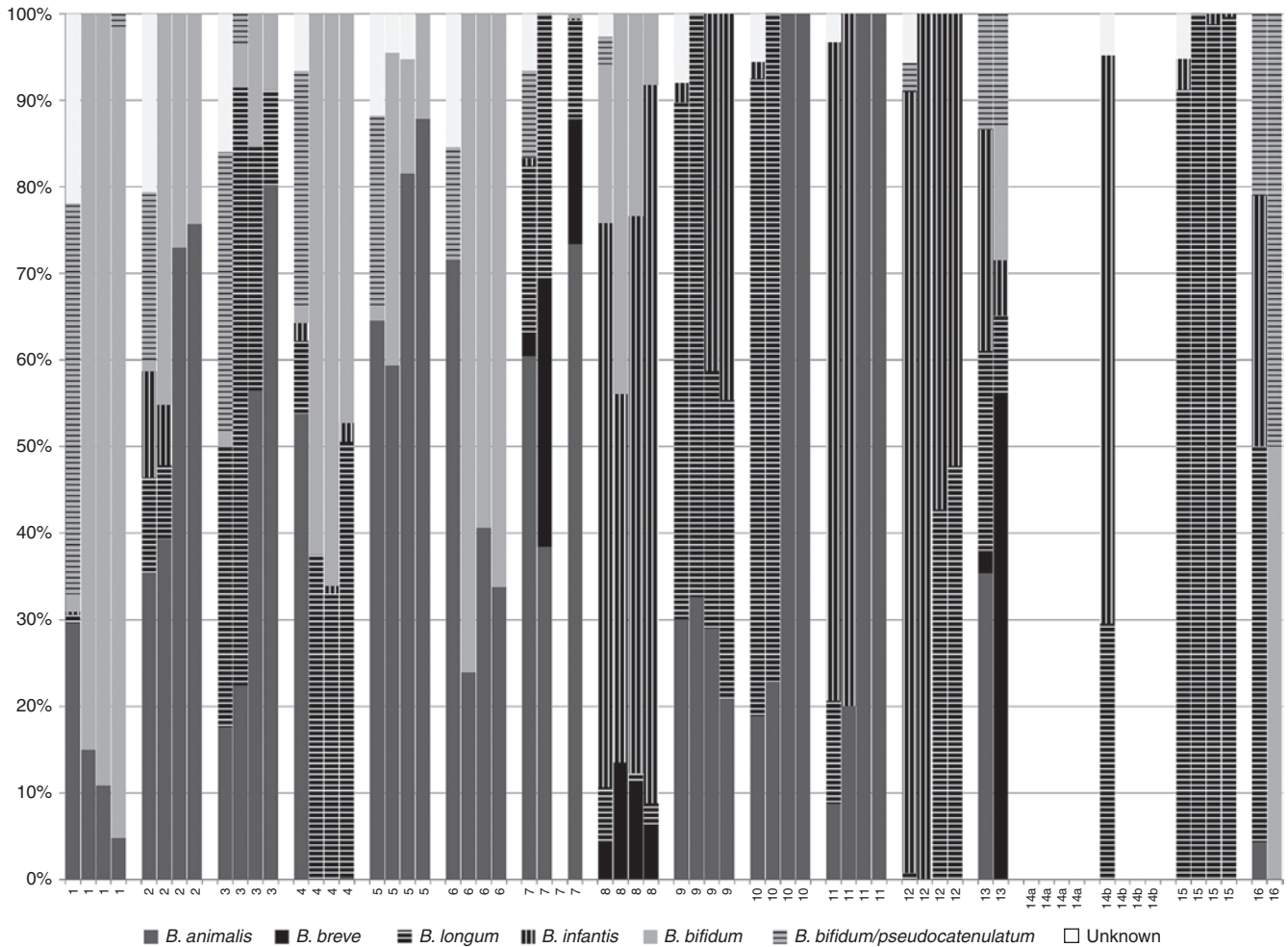


Figure 2. Bifidobacterial composition of probiotic products by polymerase chain reaction–based methods. Each product was assayed four times, shown here in order grouped by product, lot 1 pill 1, lot 1 pill 2, lot 2 pill 1, lot 2 pill 2. Blank plot area not between two different products indicates no amplicon was detected.

differentiate common bifidobacterial species and *Bifidobacterium longum/infantis* ratio analysis (BLIR) to differentiate *B. longum* subspecies (20,33). In order to evaluate commercial probiotic products, we first examined if the Bif-TRFLP/BLIR correctly differentiates a number of mock communities containing different combinations of bifidobacterial strain DNA. The Bif-TRFLP/BLIR results generally reflected the known mock communities of input DNA from common bifidobacteria probiotic species (Figure 1). All five tested species were observable and distinguishable from each other, and there was no specific bias against any species or subspecies. Communities containing two, three, four, or five different strains in approximately equal levels were identified as containing the correct mix of strains. However, when multiple strains were present each in levels under 5% of total DNA, some minority strains were not observed in the output data, a known limitation of the TRFLP-based technique (33). Specifically, no *B. animalis* and *B. breve* were observed in mixture G, mixture J appeared as containing only *B. breve* despite all five species being added, mixture P was missing *B. breve* and *B. bifidum*, and mixtures Q and S were missing *B. breve*, *B. bifidum*, and *B. animalis*.

Bif-TRFLP/BLIR of Commercial Probiotics

After successful validation of the identification methods, they were used to describe the bifidobacterial contents of the selected probiotic products. Species observed in these probiotics include *B. longum* (both subspecies), *B. bifidum*, *B. breve*, and *B. animalis* subsp. *lactis*. Ambiguous peaks that match both *B. bifidum* and *B. pseudocatenulatum* were observed, but were assumed to be *B. bifidum* due to the probiotic product context. The measured contents of the probiotic products were often consistent across both pills and lots (Figure 2). The analysis showed pill-to-pill variation (within a lot) in probiotic 16, and lot-to-lot variation in probiotics 2, 10, and 11. Multiple probiotic products either contained unlisted species and/or did not contain the species their label claimed (Table 1). Only one probiotic (#5) perfectly matched its label claims in all four samples assayed, with no missing or additional bifidobacterial species. Amplification of bifidobacterial DNA was obtained from every sample except for probiotic 7 lot 2 pill 1, all probiotic 14a pills, and all probiotic pill 14b pills except lot 1 pill 1. It is unknown whether this represents a lack of bifidobacteria in the samples or potential PCR inhibitors that coeluted with the extracted

Table 1. Label claims vs. observed polymerase chain reaction–based results

Sample	<i>B. longum</i> subsp. <i>longum</i>												<i>B. breve</i>						<i>B. animalis</i>						<i>B. bifidum</i>					
	<i>B. longum</i> subsp. <i>infantis</i>						<i>B. longum</i> subsp. <i>longum</i>						First lot		Second lot		First lot		Second lot		First lot		Second lot		First lot		Second lot			
	Label	First lot pill	Second lot pill	First lot pill	Second lot pill	Label	First lot pill	Second lot pill	First lot pill	Second lot pill	Label	First lot pill	Second lot pill	Label	first pill	second pill	Label	first pill	second pill	Label	first pill	second pill	Label	first pill	second pill	Label	first pill	second pill		
1	~									X																				
2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
3	X					X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
4	~					X	X	X	X	X																				
5																														
6	X					X				X																				
7	~					X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
8	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
9	X	~				X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
10	X	~				X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
11	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
12	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
13	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
14a	X					X				X																				
14b	X					X				X																				
15	X	~				X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		
16	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		

Species mentioned as present on each label are marked with an X in the label column, along with the species detected in each sample in its column. ~ = present only in trace amounts. N/A = not applicable, no sample tested.

DNA. Attempting to reduce the concentration of potential PCR inhibitors by a 1:50 dilution of the genomic DNA from these failed samples, did not result in positive amplification (data not shown).

Bifidobacterial Isolation

For purposes of content comparison, bifidobacterial isolates were obtained and identified (by MALDI Biotyper and BLIR for *B. longum* group isolates) from each of the probiotic products. Not all listed species were isolated from the probiotics (**Supplementary Table S1** online) likely a consequence of differing viability after storage, as well as differences in culturability. *B. animalis* subsp. *lactis* was the most commonly-isolated organism, and was frequently the only species isolated from the products. Indeed, *B. animalis* subsp. *lactis* was the only species not listed on the product labels that was found by isolation. In three out of four cases where Bif-TRFLP found *B. animalis* subsp. *lactis* where it was not listed on the label, the species was also successfully isolated (Probiotics 4, 6, and 11, but not 13).

Genome Sequencing to Confirm Results of BLIR Analysis

The BLIR method is based on unique genetic loci found in the genomes of multiple strains of each *B. longum* subspecies. Several potentially mislabeled products containing *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* were observed using this method. To further confirm the results of the BLIR method, the genomes of two isolates from Product 15, where the label claimed *B. longum* subsp. *infantis* and BLIR indicated the contents were *B. longum* subsp. *longum*, were sequenced for comparison. 2.5 million reads were obtained for each isolate and the average genome size was 2.239 Mb. This predicted genome size is noticeably smaller than that of *B. longum* subsp. *infantis* ATCC 15697 (2.832 Mb) (27). Reads from the Product 15 isolate did not map to key loci in the *B. longum* subsp. *infantis* ATCC15697 genome, including the subspecies-stereotypic HMO cluster described by Sela *et al.* (27,29). While average read coverage for this genome was 272-fold, the average read coverage for the 40 kb HMO cluster was zero (**Supplementary Figure S1** online). Conversely, the Product 15 sequence aligned well with *B. longum* subsp. *longum* DJO10A (37), indicating that the Product 15 isolates were *B. longum* subsp. *longum* strains rather than the advertised *B. longum* subsp. *infantis*, (although strain-level differences were observed, as indicated by small gaps in coverage in **Supplementary Figure S1a** online. This is consistent with the classification given by the BLIR method.

DISCUSSION

Consistent with previous studies, we present further evidence that the advertised content of many probiotic products containing bifidobacteria vary significantly from the actual content (1–8). Indeed, only 1 of the 16 products tested exactly matched the bifidobacterial species claims on the label in every sample tested. Some products were not internally consistent as both pill-to-pill and lot-to-lot variation were observed. We note

that many of these products also contained non-bifidobacterial species contents, which, for the purposes of this study, were not evaluated. These results suggest that quality control of probiotics is lacking. In order for clinical trials to provide meaningful data about the benefits of specific probiotic strains and enable clinicians to make informed decisions about prescribing or recommending probiotics, increased standards of strain identification are needed, particularly given the lack of regulatory oversight for certification of probiotics in the United States.

Debate continues regarding probiotic administration to premature infants, with several authors arguing for the routine prophylactic use of probiotics for the prevention of necrotizing enterocolitis (38,39). Which probiotic strain and whether a single strain or a combination product is superior are open questions requiring further trials. There is evidence supporting the use of *B. longum* subsp. *infantis* as a potential candidate species for infants, especially when provided in combination with breast milk (26,40). However, the current study shows that *B. longum* subsp. *infantis* is commonly misidentified in commercial probiotics, some of which are marketed towards infants. To this end, we present and validate potentially useful tools for evaluating probiotic contents, including a method to distinguish *B. longum* subsp. *infantis* from *B. longum* subsp. *longum*, a subspecies differentiation not possible on the basis of traditional 16S ribosomal RNA sequencing alone.

While the clear congruence between the known mock community input and resultant Bif-TRFLP/BLIR data was encouraging from a methodological standpoint, the results from the products tested was concerning. There were numerous mismatches between label claims and our results. Probiotic 11, for example, was advertised as containing *B. bifidum* and *B. longum* subsp. *infantis*, but Bif-TRFLP/BLIR indicated its contents were *B. animalis* subsp. *lactis* and *B. longum* subsp. *infantis*, with some *B. longum* subsp. *longum* present in one pill. The second lot of Probiotic 11 had the same stock keeping unit number as the first lot, was produced by the same manufacturer, and was even purchased from the same store. However, the product label from the second lot differed from the first lot. It now listed a *B. animalis* subsp. *lactis* strain (with the same trade name as the previous *B. bifidum*), no *B. longum* subsp. *infantis*, and a *Lactobacillus* strain. The Bif-TRFLP data from lot 2 of this product agrees with the new label claim, and the relabeling of the *B. bifidum*-named strain as *B. animalis* subsp. *lactis*. Such an unannounced change may or may not be noticed and included in the analysis of the results of a clinical trial using this product.

Probiotic 15 was consistently listed as containing only a *B. longum* subsp. *infantis* strain, while it clearly contained *B. longum* subsp. *longum* by Bif-TRFLP/BLIR. Genomic comparisons of two isolates from the same product lot showed it was missing large genomic sections present in the *B. longum* subsp. *infantis* type strain (ATCC 15697) including gene clusters dedicated to catabolism of human milk oligosaccharides and urea—genetic loci specific to the *B. longum* subsp. *infantis* subspecies (27,29). Conversely, the genome sequence of Product 15 aligned very closely with *B. longum* subsp. *longum*

DJO10A thus clearly showing membership within that subspecies. Products 3, 9, and 10 also had misidentified *B. longum* subspecies contents in some samples. These results demonstrate the difficulty of distinguishing between these two *B. longum* subspecies. BLIR analysis is an inexpensive tool for quality control screening by clinical labs or the probiotics industry to rapidly differentiate *B. longum* product subspecies that would otherwise only be possible via whole genome sequencing (27) or multilocus sequence typing (29).

The contents of probiotic 6 were especially different from its labeling, as it did not appear to contain any of the three label species (*B. longum* subsp. *longum*, *B. longum* subsp. *infantis*, and *B. breve*) and contained two non-label species (*B. bifidum* and *B. animalis*). However further investigation of the probiotic 6 label revealed a footnote indicating that the manufacturers reclassified both their *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* strains as *B. animalis* subsp. *lactis*, which is in agreement with the Bif-TRFLP/BLIR data developed in this study. However it is unclear if such subtle footnoted-label changes, *in lieu* of actual relabeling, will be fully understood and recognized by clinicians and consumers.

While our use of the Bif-TRFLP and BLIR techniques has revealed numerous inconsistencies of product labeling with product contents, there are limitations to the methods used in this study. It is possible for a mutation causing an rRNA gene restriction site to change a cognate Bif-TRFLP fragment length and cause an incorrect (or unknown) species identification. In addition, while our terminal restriction fragment database is extensive, it does not contain every known bifidobacterial strain. Moreover, in products containing a mixture of strains, one strain might be present but not detected due to it being below the limit of detection in a mixed community, a known limitation of the technique (33). The absence of any detectable bifidobacterial amplicon in some samples was concerning, however, the possibility remains that there may have been PCR inhibitors in the sample which resulted in a false negative.

In conclusion, the state of species and subspecies quality control for many bifidobacteria-containing probiotic products remains inconsistent. In our study, we found that probiotic products often do not correctly identify the bifidobacterial species they list on their labels. Probiotic products can shift their contents over time without warning, even while keeping the same stock keeping unit. Differentiating the two subspecies *B. longum* subsp. *longum* from *B. longum* subsp. *infantis* appeared to be of particular difficulty.

Clinicians face a number of challenges when administering probiotics to patients. While not addressed in our study, the issues of strain viability upon dosing and potential contamination by adventitious microorganisms (and even pathogens) are also concerns for those planning clinical trials. Despite the generally recognized as safe status of probiotic supplements, the strain identity issues discussed here must be taken into account when planning and conducting clinical trials to ensure the proper interpretation the data generated. Misidentified probiotics are not likely to pose a danger to patients, however they certainly cloud clinic trial interpretations and are

particularly problematic when the properties possessed by one strain are confused with another. Indeed, the use of appropriate tools (such as those described here) to distinguish between closely related strains such as *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* is needed to discriminate between these microorganisms with significantly different metabolic capacities that impact colonization behavior as well as host health outcomes (20,22). Close and continued monitoring of probiotic products is recommended, and consideration should be given to those probiotic products for which validated, good manufacturing practice production is known. Certainly federally funded clinical trials using commercial probiotics would benefit from increased strain validation solely to ensure meaningful, interpretable, data is generated.

METHODS

Construction of Mock Communities

Type strains of various *Bifidobacterium* species (*B. longum* subsp. *infantis* ATCC15697, *B. longum* subsp. *longum* DJO10A, *B. animalis* subsp. *lactis* ATCC27536, *B. breve* ATCC15700, and *B. bifidum* DSM20456) were grown in de Man Rogosa Sharpe (MRS) broth supplemented with 500 mg/l of L-cysteine-HCL, and DNA extraction was performed using the Epicentre MasterPure Gram Positive DNA Purification kit (Epicentre, Madison WI). DNA concentrations were determined by nanodrop spectrophotometry and adjusted to 40 ng/ μ l. DNA from the various bifidobacteria was then combined in different ratios, as noted, to construct 20 mock community DNA pools. The final volume of DNA was adjusted to 15 μ l and diluted to a final concentration of 20 ng/ μ l. One microliter of the resulting mix was used to perform Bif-TRFLP/BLIR as described below.

Probiotic Products and DNA Extraction

A search was performed both online and in local Davis, CA stores for probiotics listing bifidobacteria as an ingredient, and 16 such products were selected for evaluation. One product (#14) contained two formulations in a single package that we list as 14a and 14b. Two separate lots of each probiotic product were purchased approximately 2 years apart. Probiotics 13 and 16 had been discontinued at the time of purchase for the second lot. Genomic DNA was extracted from either two separate pills or two 100 mg aliquots of powder of each probiotic product using the Masterpure Gram Positive DNA Purification Kit (Epicentre) after dilution and vortexing in 9 ml of phosphate-buffered saline. Label claims of bifidobacterial content of each product were recorded.

Bifidobacterial Terminal Restriction Fragment Length

Polymorphism (Bif-TRFLP)

The method of Lewis et al. (33) was used to perform the Bif-TRFLP assay. Briefly, DNA from feces was amplified in triplicate by PCR using primers NBIF389 (5'-(HEX)-GCCTTCGGGTGTGAAAC) and NBIF1018 REV (GACCATGCACCACCTGTG) (Supplementary Table S1 online). DNA was purified using the Qiagen Qiaquick PCR purification kit (Qiagen, Valencia, CA) and then cut with restriction enzymes AluI and HaeIII. The resulting fragments were analyzed on an ABI 3100 Capillary Electrophoresis Genetic Analyzer at the UC Davis College of Biological Sciences Sequencing Facility and sizes were compared against the published database for species identification. The samples were analyzed with PeakScanner 2.0 software (Applied Biosystems, Carlsbad, CA). Negative controls containing DNA-free water instead of sample DNA were included with each run.

BLIR

A PCR-based assay, BLIR, was developed in order to determine which of the two human-associated subspecies of *B. longum* (*B. longum* subsp. *longum* and *B. longum* subsp. *infantis*) were present in each sample and to gain an estimate of their relative abundance to each other (20). In brief, three primers

(FWD_BL_BI (5-(HEX)-AAAACGTCCATCCATCACA), REV_BL (5-ACGACCAGGTTCCACTTGAT), and REV_BI (5-CGCCTCAG TTCTTTAATGT)) targeting a conserved portion of the genome (between Blon_0424 and Blon_0425) shared by both subspecies were designed using multiple genome sequences of each subspecies. FWD_BL_BI is complementary to a sequence in both subspecies while REV_BL and REV_BI are complementary to nearby sequences in only *B. longum* and *B. infantis*, respectively. FWD_BL_BI and REV_BL amplify a fragment of the *B. longum* genome 145 bp in length, while FWD_BL_BI and REV_BI amplify a fragment of the *B. infantis* genome 114 bp in length, allowing differentiation of the amplicons.

Genomic DNA from each probiotic was amplified by PCR using 0.5 μ l of 10 μ mol/l stock of each of the above primers, 12.5 μ l GoTaq Green Master Mix (Promega, Madison, WI), 1 μ l of 25 mmol/l MgCl₂, 1 μ l of template DNA, and 9 μ l of nuclease-free water. Cycling conditions were 95 °C for 2 min, 30 cycles of 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 30 s, followed by a 72 °C extension for 5 min. PCR products were purified from the mixture using the QIAquick PCR purification kit (Qiagen) and diluted 1:10 to avoid off-scale peak sizes. Capillary electrophoresis and analysis of the amplicon sizes proceeded as with the Bif-TRFLP procedure above. A positive control of known genomic DNA was included with each PCR run to ensure potential amplification of both *B. longum* subsp. *longum* and *B. longum* subsp. *infantis* products. The percentage of peak area corresponding to each subspecies was multiplied by the percentage of *B. longum* species peak area from the Bif-TRFLP data to arrive at final abundances of each subspecies. Negative controls containing DNA-free water instead of sample DNA were included with each run.

Bifidobacterial Isolations

To isolate bifidobacteria, 100 mg or one pill of each product was aseptically transferred to a sterile tube, diluted tenfold with sterile phosphate buffered saline (PBS) and homogenized by vortex. Serial dilutions were prepared in PBS and inoculated on modified BSIM agar (41). Modified BSIM agar was prepared by supplementing MRS media with 13 g/l agar, 500 mg/l of L-cysteine-HCL, 20 mg/l of nalidixic acid, 50 mg/ml mupirocin, 50 mg/ml kanamycin, 50 mg/ml polymixin B sulfate, 100 mg/ml Iodoacetate, 100 mg/ml 2,3,5-triphenyltetrazolium chloride. The plates were incubated for 48 h at 37 °C in an anaerobic chamber with an atmosphere containing approximately 5% carbon dioxide, 3% hydrogen, and the remainder nitrogen. Up to 10 resulting colonies from each sample with the appropriate colony appearance were streaked onto MRS-cysteine plates for purity for two passages. The resulting pure strains were grown in MRS broth supplemented with 0.05% L-cysteine and stored at -80 °C in 50% glycerol.

Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) Identification of Isolates

Glycerol stocks of each isolate were streaked on MRS plates and incubated at 37°C for 48 h in anaerobic chamber. A colony from each plate was added to 300 μ l nuclease free water in a 1.5 ml tube and homogenized by vortex. Next, 900 μ l of 98% ethanol was added to the tube, briefly vortexed, and centrifuged for 2 min at maximum speed. The supernatant was removed and the tubes were again centrifuged for 2 min. All liquid was decanted and the samples were left at room temperature to allow the ethanol to evaporate. Subsequently, 25 μ l formic acid was then added to each tube and homogenized by vortex, followed by the addition of 25 μ l acetonitrile. Samples were then centrifuged for 2 min and 1 μ l of extract was placed on a MALDI target plate, left to dry at room temperature, covered with a α -Cyano-4-hydroxycinnamic acid (HCCA) matrix and air dried. The MALDI target plate was then analyzed by a MALDI Biotyper (Bruker, Fremont, CA) and the best database match for each isolate was recorded. The MALDI Biotyper was not found to reliably differentiate between *B. longum* subspecies (data not shown); accordingly BLIR was used on DNA extracted from an overnight MRS broth culture of each *B. longum* group isolate using the Epicentre Masterpure Gram Positive DNA Purification Kit (Epicentre) to identify each *B. longum* group isolate at the subspecies level.

Genome Sequencing

Libraries of genomic DNA from each of two isolates of probiotic 15 identified by MALDI as members of the *B. longum* species were created using standard Illumina library prep and sequenced on an Illumina MiSeq by the UC Davis Genome Center DNA Technologies core, using 150 bp single read settings. Reads were mapped to the *B. longum* subsp. *infantis* ATCC 15697 and *B. longum* subsp. *longum* DJO10A ggenomes and visualized using CLC Bio software (Qiagen, Boston, MA) (27,29,32). An equal number of reads were mapped to both genomes. This Whole Genome Shotgun project has been deposited at DDBJ/EMBL/GenBank under the accessions LKSU00000000 and LKSV00000000. The versions described in this paper are versions LKSU01000000 and LKSV01000000.

Ethical Considerations

As no human or animal subjects were involved in this research, no ethical approval (e.g., Institutional Review Board) was sought.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/pr>

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